Regulation of chromatin modifier genes by microRNA vis-à-vis regulation of microRNA by DNA methylation and histone modifications in human cancer

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Regulation of chromatin modifier genes by microRNA vis-à-vis regulation of microRNA by DNA methylation and histone modifications in human cancer

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Under the supervision of

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November, 2016

Supervisors' Certificate

This is to certify that the work embodied in this thesis entitled "*Regulation of chromatin modifier genes by microRNA vis-à-vis regulation of microRNA by DNA methylation and histone modifications in human cancer*", by *Dipta Sengupta*, Roll Number 510LS102, is a record of original research carried out by him under my supervision and guidance in partial fulfilment of the requirements for the degree of *Doctor of Philosophy* in *Life Science*. Neither this thesis nor any part of it has been submitted for any degree or diploma to any institute or university in India or abroad.

Samir Kumar Patra
Supervisor

Dedicated to

My parents and brother

Declaration of Originality

I, Dipta Sengupta , Roll Number 510LS102 hereby declare that this thesis entitled *"Regulation of chromatin modifier genes by microRNA vis-à-vis regulation of microRNA by DNA methylation and histone modifications in human cancer"* represents my original work carried out as a doctoral student of NIT Rourkela and, to the best of my knowledge, it contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the thesis. Works of other authors cited in this thesis have been duly acknowledged under the section 'Bibliography' and kept in the reference as well. I have also submitted my original research records to the scrutiny committee for evaluation of my thesis.

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November, 2016 *Dipta Sengupta*

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Abstract

This thesis deals with the role of microRNA (miRNA) regulating other epigenetic modifiers like DNA methyltransferase 1 (DNMT1), and histone methyltransferase myeloid/lymphoid or mixed-lineage leukaemia (MLL1) also known as Histone-lysine N-methyltransferase 2A. It also divulges the reason for aberrant expression of miRNAs (miR-152, miR-148a, and miR-193a) in breast and prostate cancer.

Silencing of the miR-152 gene due to promoter DNA methylation alter the expression pattern of several other genes. E-cadherin (CDH1) forms the core of adherent junctions between surrounding epithelial cells, link with the actin cytoskeleton and affects cell signalling. CDH1 gene is downregulated by promoter DNA methylation during cancer progression. In this investigation, we attempt to elucidate the correlation of miR-152 and CDH1 function, as it is well known that the loss of CDH1 function is one of the primary reasons for cancer metastasis and aggressiveness of spreading. For the first time here it has been shown that loss of CDH1 expression is directly proportional to the loss of miR-152 function in breast cancer cells. mRNA and protein expression profile of DNMT1 implicate that miR-152 targets DNMT1 mRNA and inhibits its protein expression. Tracing the molecular marks on DNA and histone 3 for understanding the mechanism of gene regulation by ChIP analyses leads to a paradoxical result that shows DNA methylation adjacent to active histone marking (enrichment of H3K4me3) silence miR-152 gene.

This thesis also demonstrated that miR-148a remains downregulated in hormone-refractory prostate cancer compared to other healthy cells and its upregulation induce apoptosis in hormone-refractory and metastatic prostate cancer cells. Here for the first time, it was analyzed the role of miR-148a in the regulation of DNMT1 in prostate cancer cells. The ectopic expression of miR-148a shows a noticeable amount of programmed cell death and repression of cancer cell proliferation. It also revealed the silencing of miR-148a in prostate cancer cells was done by DNMT1. This finding gives a new avenue to targeting prostate cancer cells and proved the role of miR-148a as a therapeutic.

Moreover, other experiments also demonstrate the regulation of MLL1 by miR-193a. MiR-193a has been downregulated in prostate cancer by DNA methylation and help in MLL1 overexpression during prostate cancer progression. Most importantly it was found by inhibiting MLL1 it changes the global H3K4 methylation pattern increasing the mono-methylation and decreasing trimethylation at H3K4 positions. H3K4 trimethylation is an active gene mark present in various oncogenes during cancer progression. By inhibiting H3K4, tri-methylation cancer progression can be repressed. Ectopic expression of miR-193a results in cell death, inhibition of cellular migration, and anchorage-independent growth of cancer cells.

All together this thesis supports that miR-152, miR-148a, and miR-193a are regulated by DNA methylation, and they affect the expression of the various epigenetic modifiers. Hence these can be targeted for therapeutic intervention for breast and prostate cancer.

Keywords: miRNA, miR-152, miR-148a, miR-193a, DNA methylation, DNMT1, E-Cadherin, MLL1, Breast cancer, Prostate cancer, Apoptosis

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Abbreviations

APC : Adenomatous Polyposis Coli

BCL2 : B-cell lymphoma 2

BAX : BCL2-associated X protein

CGI : CpG Island

DAB : 3,3′-Diaminobenzidine tetrahydrochloride

DAPI : 4',6-Diamidino-2-Phenylindole, Di-acetate

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : Dimethyl Sulphoxide

ddNTP : Dideoxynucleotide triphosphate

DGCR8 : DiGeorge Syndrome critical Region 8

DNA : Deoxyribonucleic acid

DNMT1 : DNA Methyltransferase 1

DNMT3A : DNA Methyltransferase 3A

DNMT3B : DNA Methyltransferase 3B

EGFR : Epidermal Growth Factor Receptor

EHMT2/G9A : Euchromatic Histone-Lysine N-Methyltransferase 2

EMT : Epithelial to Mesenchymal Transition

EphB : Ephrin Type-B Receptor

ESC : Embryonic Stem Cell

ESRRB : Estrogen-Related Receptor B

EZH2 : Enhancer of Zeste Homolog 2

F-12 : Nutrient Mixture F-12 Ham (Kaighn's Modification) Medium

FACS : Fluorescence Activated Cell Sorter

FAK : Focal Adhesion Kinase

FBS : Fetal Bovine Serum

FFPE : Formalin-Fixed Paraffin Embedded

FITC : Fluorescein Isothiocyanate

GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase

GATA6 : GATA-Binding Factor 6

H3K14Ac : H3 Lysine14 Acetylation

H3K14Ac2 : H3 Lysine 14 Di-Acetylation

H3K27me3 : H3 Lysine27 Tri- Methylation

H3K36me1/2 : H3 Lysine36 Mono and Di-Methylation

H3K4me1 : H3 Lysine4 Mono-Methylation

H3K4me2 : H3 Lysine 4 Di-Methylation

H3K4me3 : H3 Lysine 4 Tri-Methylation

H3K4MT : H3 Lysine4 Methyl Transferase

H3K64me3 : H3 Lysine64 Trimethylation

H3K9Ac : H3 Lysine 9 Acetylation

H3K9Ac2 : H3 Lysine 9 Di-Acetylation

H3K9AcS10p : H3 Lysine9 Acetylation Serine10 Phosphorylation

H3K9me1 : H3 Lysine9 Mono-Methylation

H3K9me3 : H3K9 Trimethylation

H4K12Ac : H4 Lysine 12 Acetylation

HAT : Histone Acetyltransferase

HDAC : Histone Deacetylases

JAK/STAT3 : Janus Kinase/Signal Transducers And Activators of Transcription 3

KAT2A/6A : K (lysine) acetyltransferase 2A/6A

KDM : Lysine (K)-specific Demethylase

KLF4 :Kruppel-Like Factor 4

KMT :Lysine (K)-specific Methyltransferase

L-15 : Leibovitz's L-15 Medium

LIF : Leukemia Inhibitory Factor

LRH1 : Liver Receptor Homolog 1

LSD1 : Lysine Specific Demethylase1

MBD1 : Methyl-CpG Binding Domain 1

MBD3	: Methyl-Binding Domain 3
MCF-7	: Michigan Cancer Foundation-7
MEM	: Minimum Essential Medium
MLL1	: Mixed Lineage Leukemia 1
MLL4	: Mixed-Lineage Leukemia 4
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCCS	: National Centre for Cell Sciences
Paf1C	: RNA polymerase II-Associated Factor 1C
PBS	: Phosphate Buffered Saline
PBST	: Phosphate Buffered Saline with Tween-20
PC-3	: Prostate Cancer-3
PE	: Proximal Enhancer
PI	: Propidium Iodide
PI3K	: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
SP1	: Specificity Protein 1
STAT	: Signal Transducer and Activator of Transcription
STAT3	: Signal Transducer and Activator of Transcription 3
STK40	: Serine/Threonine Kinase 40
SWI/SNF	: SWItch/Sucrose Non-Fermentable
TBS	:Tris Buffered Saline

Notations

r	: correlation coefficient
h	: Hour
°C	: Degree Celsius
%	: Percentage
µM	: Micromolar
nm	: Nanometer
nt	: Nucleotide
nM	: Nano molar
mg	: Milligram
µg	: Microgram
bp	: base pairs
π	: Pi
Å	: Angstrom
Ps	: picosecond

Chapter 1
Introduction

1 Introduction

In the last decade the term 'epigenetics' attracted much attention among researchers and scientists to unravel the clues hidden in the gene expression without changing the sequence of DNA. Epigenetics is a molecular mechanism which governs the expression of genes and converts genetic information to phenotypic expression. Although each cell inherites same genetic code yet the expression of genes are regulated by their 'epigenetics landscape'; the term which was coined by Conrad Hal Waddington [1, 2]. In many cases, epigenetic traits remain preserved through mitosis or even meiosis without altering the primary DNA sequence. Epigenetic marks not only regulate gene expression in a cell but also carry the heritable traits. Recent discoveries have helped us to understand the role of epigenetic modification as a contributor in the development of different lethal diseases including cancer. The covalent modification in cytosine base of DNA and modifications of histones change the nucleosome dynamics which forms the driving factors for epigenetic modifications. MicroRNA (miRNA) expression is also regulated by these modifications, and it can also act as an epigenetic modifier. They together control cellular processes like DNA-protein interactions, cellular differentiation, embryogenesis, X-chromosome inactivation, genomic imprinting, and suppression of transposable element mobility. Methylation of cytosine base in the 5-carbone position of DNA is the most common phenomenon of gene regulation which was first discovered in calf thymus DNA in 1948 by Hotchkiss [3]. DNA methylation takes place in adenine and cytosine bases and recognized by the host restriction system to identify self and non-self DNA in prokaryotes. It also helps the DNA repair machineries to identify the mother strand during replication. But in eukaryotes, the methylation occurs only in cytosine residue and act as a repressive mark followed by transcriptional repression and silent chromatin formation [4, 5]. Eukaryotic DNA is organized into chromatin, which folds the genetic information that is essentially an array of nucleosomes or histone cores. The nucleosome is the fundamental and repeating unit of chromatin that is composed of repeated units of ~147 bp of DNA wrapped around histone octamers consisting of two copies of each histone H2A, H2B, H3, and H4, which provides flexibility to the DNA and keeps it in a compact form. In eukaryotes, the histone fold domain (HFD) [6] of each histone protein helps it to dimerize and help in pairs formation of H3 with H4 and H2A with H2B [7-10]. The linker histone (H1) binds to the linker DNA to stabilize the folding of the chromatin fiber and also seals the DNA turns at the nucleosome DNA entry/exit points [11]. miRNAs are a group of small non-coding RNAs (ncRNAs) of about 19–25

nucleotides (nt) in length that constitute an integral part of the post-transcriptional gene expression. miRNAs act as a binary switch for gene silencing by inhibiting translation and/or triggering degradation of their target mRNAs. The emerging role of miRNAs in different biological functions includes embryogenesis, developmental pattern formation, and apoptosis [12-14] . For example, brain development, including patterning, neurogenesis, neuronal differentiation, subtype specification, and neuronal activity is scripted via miRNAs. Furthermore, miRNAs are reported to be involved in many dreadful diseases, including cancer [15-19]**.**

In multicellular organisms, epigenetic marks are transmitted to offspring because it generates multiple phenotypes from the same genotype [20-22] . The importance of epigenetic mark was understood when the incorrect mark was observed in diseased cells. For an example, DNA hypomethylation in p16INK4a, p14ARF, and MGMT genes was found in the early stage of tumorigenesis [23, 24].

1.1 Epigenetic modulation and modulating machineries

For understanding the regulation of genes by epigenetic modification, they can be divided into three categories: DNA methylation, histone modification, and small RNA regulation. The combined effect of these factors is the regulatory system for gene expression.

1.1.1 DNA methylation

DNA methylation reaction is catalyzed by DNA methyltransferase enzymes known as DNMTs. The DNMT family mainly consist of three active enzymes DNMT1 (maintenance methyltransferase), DNMT3A and DNMT3B (*de-novo* methyltransferase). It methylated cytosine residue in the presence of cofactor SAM (S-Adenosyl methionine), which donates the $–CH_3$ group and gets converted to SAH (S-Adenosyl homocysteine) [24]. Methylation mostly occurs in CpG dinucleotide rich region of DNA. This cluster of CpG dinucleotides is defined as CpG islands which contain at least 50% GC contain in a region of 200 bases. Human gene promoters consist of 60% of CpG islands which are usually unmethylated in normal cells but become methylated in a tissue-specific manner during early development or in differentiated tissues [25]. CpG island methylation, in general, is related to gene silencing. It also plays a key role in the genomic imprint, X chromosome inactivation. Methyl-CpG-binding domain (MBD) proteins recognize methylated DNA region and facilitate the recruitment of histone modifiers and chromatin-remodeling complexes that establish repressive histone marks, such as H3K27me3,

H3K9me3, etc. [26]. Moreover, DNA methylation also prevents binding of DNA binding proteins at their target sites and inhibits transcription. In contrast, unmethylated DNA CpG island is associated with active gene transcription. Unmethylated CpG promotes Cfp1/ Setd1 mediated H3K4 trimethylation (H3K4me3) enrichment which turns on the transcription process. Apart from CpG island DNA methylation also occurs in the CpG shore (close proximity, near about ~2 kb, of CpG islands) and gene body. A recent study explains that in CpG island shores, methylation is tightly associated with transcriptional inactivation of different genes such as Caveolin1 (CAV1) [27]. DNA methylation also plays a key role in chromosomal integrity. A significant fraction of methylation is found in repetitive elements which prevent reactivation of endoparasitic sequences, causes chromosomal instability, translocation and gene disruption [24]. Methylation on non-CpG has been described in stem cells, and it was enriched in gene bodies which are directly correlated with gene expression and depleted in protein binding sites and enhancers [28]. The non-CpG methylation decreases during differentiation but again restored in induced pluripotent stem cells which suggest it plays a key role in origin and maintenance of pluripotency state of cells [28, 29]. In addition to 5-methylcytosines, 5-hydroxymethyl-2′-deoxycytidine has also been observed in Purkinje cells (constituting 0.6% of total nucleotides) and in granule cells (constituting 0.2% of total nucleotides) but it is absent in present cancer cell lines. These newly identified DNA modifications are now being studied for their implication in healthy and diseased epigenetic regulation.

1.1.1.1 DNA methyltransferases

In mammalian system five members of DNMT family have been reported: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L, but only DNMT1, DNMT3a, and DNMT3b have methyltransferase activity.

DNMT1:

DNMT1, the maintenance methyltransferase is essentially integral to the DNA methylation machinery as it accomplishes the majority of methylation copying to ensure that hemimethylated daughter strands in differentiated somatic cells faithfully maintain and propagate the proper DNA methylation pattern across successive cell generations [30]. DNMT1 is the first mammalian DNA methyltransferase enzyme to be cloned and biochemically characterized [31]. DNMT1 exhibits optimal methyltransferase activity on hemimethylated DNA rather than unmethylated DNA and localizes at the DNA replication foci during the S phase, properties that make it suitable for maintenance methylation. DNMT1 enzyme comprises a large N-terminal domain with regulatory

function and a smaller C-terminal catalytic domain. The regulatory domain harbors different motifs, like a charge-rich domain that interacts with the Dmap1 transcriptional repressor; a nuclear localization signal, a PCNA (proliferating cell nuclear antigen) interacting domain, replication foci targeting region and a cysteine-rich Zn^{2+} binding domain of the CXXC type. The C-terminal domain of DNMT1 contains all the conserved motifs characteristic for cytosine-C5-MTases and harbors the active center of the enzyme. The C- and N-termini are connected via a lysine-glycine repeat hinge region [32, 33]. DNMT1 is regulated by a number of intrinsic and extrinsic control points such as allosteric modulation, post-translational modifications, auto-inhibitory restraints, etc. which ensure dynamic stability and functional competence of the enzyme [34, 35]. The enzyme is focused to specific genomic loci in a cell-dependent manner, and its enzymatic activity is tightly controlled on accomplishing its duty as a transcriptional repressor [36]. DNMT1 plays a unique central role during embryogenesis for epigenetic reprogramming of germ-line and zygotic lineages.

DNMT3A and DNMT3B:

The DNMT3 family consists of DNMT3A and DNMT3B, which are highly related to one another with amino acid sequence and structural similarity but encoded by separate genes. Both proteins transfer methyl groups to hemimethylated and unmethylated substrates at equal rates and without evidence of intrinsic sequence specificity beyond the CpG dinucleotide; DNMT3A has also been reported to methylate CpA sites [31]. DNMT3A and DNMT3B are highly expressed in embryonic tissues and undifferentiated ES cells and down-regulated in differentiated cells. Similar to DNMT1, both DNMT3A and DNMT3B are indispensable for embryonic development in mice [37]. Point mutations in human DNMT3B are responsible for the rare autosomal recessive human disorder known as ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome. The general architecture of both DNMT3 enzymes resembles DNMT1; each possesses an N-terminal regulatory part and a C-terminal catalytic part harboring all the conserved C_5 DNA MTase motifs. However, the N-terminal parts of DNMT1 and DNMT3A/3B are unrelated. In DNMT3A and DNMT3B this section contains two defined domains: a cysteine-rich region called the ADD (ATRX-DNMT3-DNMT3L) domain, also known as the PHD (plant homeodomain) domain, and a PWWP domain. The catalytic domains of DNMT3A and DNMT3B share approximately 85% sequence similarity and in contrast to the catalytic domain of DNMT1, are enzymatically active in their isolated form [38]. DNMT3A and DNMT3B mediate de novo methylation where

new methyl marks are added to previously unmethylated cytosine around the time of implantation and are maintained throughout the lifespan of the organism. During the development of the germ cells, another round of de novo methylation occurs, and the methylation imprints are established in a gender-specific manner [39].

DNMT2:

DNMT2 is a relatively small protein of 391 amino acids and lacks the large N-terminal domains present in DNMT1 and DNMT3 families. DNMT2 is the most widely conserved DNMT protein with close homologs in plants, insects, and *Schizosaccharomyces pombe*, but there are no reports on the genomic sequence found in the genomes of *Saccharomyces cerevisiae* or *Caenorhabditis elegans* [40]. DNMT2 contains all 10 sequence motifs that are conserved among m^5C methyltransferases, including the consensus *S*-adenosyl-L-methionine-binding motifs and the active site ProCys dipeptide. The conservation of the catalytic (cytosine-5) DNA methyltransferase motifs strongly suggests a DNA methyltransferase activity; however, no catalytic DNA methyltransferase activity could be detected for this protein, which was attributed to the insertion of a serine residue into a critical proline-cysteine dipeptide that is essential for DNA methyltransferase activity in other enzymes. The possibility that DNMT2 may have additional enzymatic activities has now been confirmed experimentally proving that DNMT2 is a highly specific RNA methyltransferase (cytosine 38 of transfer RNA^{Asp}) rather than a DNA methyltransferase. In fact, DNMT2 utilizes a DNA methyltransferase mechanism for RNA methylation [41-43]. DNMT2 is involved in genomic stability, organ development, metabolic processes, and aging via indirect regulation of metabolic pathways through RNA methylation. Recently, it has been shown that Dnmt2-mediated tRNA methylation interfere with stress-induced tRNA fragmentation, which suggested roles for DNMT2 during cellular stress responses [44].

1.1.2 Histone core complex

The histone proteins are major elements in the chromatin core complex. Depending on the modifications in the histone proteins chromatin forms euchromatin or active form and heterochromatin or the silent form of chromatin. With the help of linker histones or heterochromatin associated-proteins, nucleosomes are arranged into a diameter of 30 nm compact fibers and high-ordered assemblies whose mechanism and structures are poorly understood. The hierarchical structure of nucleosome is a stable fundamental construction capable of expression and repression of genes by regulating the

activities of enzymes that requires direct access to the DNA template thereby regulating DNA replication, transcription, and translation that forms the primary foundation of a cellular function. Depending on cell condition and stage along with DNA methylation of its cytosine bases within CpG repeats, histones are subject to numerous modifications in their random coil N-terminal tails, and to a lesser extent within their C-terminal tails and globular domain that determine the access of different enzymes to the DNA template for multiple operation like replication, repair and transcription.

1.1.2.1 Histone modifiers

Establishment and deletion of H3K4 methyl mark are involved in euchromatin and heterochromatin rearrangement and formation. Histone modifying enzymes catalyze the accumulation or elimination of an array of covalent modifications of histones and non-histone proteins. The expression of these two subclasses of enzymes is regulated by different signals at the various stages of cell development and also in disease states including cancer [45].

In 1996, two groups first reported the histone modifying enzymes that have sequence homology to previously identified transcriptional regulators in *Saccharomyces cerevisiae*. Applying affinity chromatography, Schreiber, and colleagues isolated a mammalian histone deacetylase (HDAC) that harbors 60% of sequence identity with the yeast transcriptional repressor Rpd3 [46]. After that, different histone modifying enzyme were identified in human, including kinases [47, 48], lysine and arginine-specific methyltransferases [49] arginine deiminases [50, 51], ubiquitinases [52], deubiquitinases [53], and lysine- and arginine-specific demethylases [45, 54], etc. Till now 10 different types of reaction has been identified which are catalyzed by histone modifying enzymes including acetylation (hKAT1/5/7/8, hKAT2A/B, hKAT3A/B, hKAT6A/B, hKAT10/12), deacetylation (SIRT2, Sp Sir2), methylation (hKMT1A/B/C/D/E/F, hKMT2A/B/C/D/E/F/G/H, hKMT3A/B/C, hKMT4/6/7/8), demethylation (hKDM1 hKDM2A/B, hKDM3A/B, hKDM4A/B/C/D, hKDM5A/B/C/D), deimination (PADI4), Proline Isomerization (Sc FPR4), phosphorylation (AuroraB, MSK1/2, HALPIN, CKII, MST1), ubiquitination (RNF20/RNF40, Bmi/Ring1A), ADP-ribosylation (poly-ADP-ribose polymerase (PARP) , Sumoylation [55]. Acetylation of histone is a highly dynamic process that is regulated by the different action of two enzyme families, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Using acetyl-CoA as cofactor HATs catalyze the transfer of an acetyl group to the ε-amino group of lysine and

neutralize the positive charge of Lysine that potentially weakens the interactions between histones and DNA. Till now two major classes of HATs has been recognized: type-A, normally associated with modifying histones that are incorporated into chromatin and located in the nucleus and type-B, predominantly cytoplasmic and acetylating free histones in the cytoplasm [56]. An HDAC enzyme reverses the lysine acetylation and restores the positive charge of the lysine. There are four classes of HDAC including class-I (HDAC1, -2, -3 and -8), class-II (HDAC4, -5, -6, -7, -9 and -10), class-III (SIRT1–7), and class-IV (HDAC11) and mostly associated with gene repression [57]. There are two type of methyltransferase enzymes including histone lysine methyltransferase (HKMT) and protein arginine methyltransferase (PRMT). All methyltransferases catalyzed the transfer of a methyl group from S-adenosylmethionine (SAM) to a lysine's ε-amino group (in the case of HKMTs) and to a ω-guanidino group of arginine (in the case of PRMT) and facilitates differential function in the different cellular state [49, 58]. For example, Trithorax group (TrxG) family facilitate H3K4 methylation and NSD (Nuclear receptor SET-Domain) arbitrate H3K36 methylation that turn on gene expression. In contrast, polycomb group (PcG) members are associated with H3K27 methylation and H3K9 HMT mediated H3K9 methylation are usually allied with gene repression [59, 60]. Two main families of histone demethylases, the amine oxidases (utilize FAD as a cofactor) containing lysine-specific demethylase 1 (LSD1 or KDM1A) [61] and iron-dependent dioxygenases (using Fe(II) and α-ketoglutarate as co-factors) containing Jumonji C (JmjC)-domain, erase methylation marks [62, 63]. Every demethylase has their different action which is related to very specific methylation marks; for example, H3K4 – monomethylation removed by KDM1A or LSD1, KDM1B, KDM5A, NO66; dimethylation removed by KDM1A or LSD1, KDM1B, KDM5A, KDM5B, KDM5C, KDM5D and NO66; and trimethylation removed by KDM2B, KDM5A, KDM5B, KDM5C, KDM5D and NO66 [64]. KMT and KDM proteins partly distinguish between the different methylation states, mono-, di- and tri-methylation for lysine and mono-, symmetrical and asymmetrical dimethylation for arginine residues. After recognition, they finally lead to recruitment of other proteins such as phosphorylated RNA polymerase II, which link individual histone marks to a specific output [65].

Histone phosphorylation is highly dynamic, and it takes place on serine, threonine, and tyrosine bases usually but not entirely, in the N-terminal histone tails. Till now very few histone kinases are known, and all of them transfer a phosphate group from ATP to the hydroxyl group of the target amino acid side chain. Their mode of action and their

recruitment to histone core is still not clear but this additional negative charge of phosphate group influence the separation of histone and DNA that leads to DNA-binding transcription factors recruitment and gene expression [66, 67]. Apart from these significant modifications which are mostly known and studied, very little is known about other types of modifications such as deamination, ADP-ribosylation, ubiquitylation and sumoylation which are also related to histone modification associated gene expression. Deamination is the process of conversion of an arginine to a citrulline group which neutralizes the positive charge of the arginine. Histones are mono- and poly-ADP-ribosylated on glutamate and arginine residues and usually associated with positive regulation of gene expression [68]. During ubiquitylation, ubiquitin (a 76-amino acid polypeptide) is attached to histone lysines via the sequential action of three different enzymes, E1-activating, E2-conjugating, and E3-ligating [66, 69]. Sumoylation is quite related to ubiquitylation and also involves the covalent attachment of ubiquitin-like small molecules to histone lysines via the action of similar enzymes E1, E2, and E3 [70, 71].

Mutations in the different genes of respective enzyme are associated with the development and progression of various cancers. For example, somatic mutation of Lysine acetyltransferase (KAT), p300 to diffuse large B-cell lymphoma and transitional cell carcinoma of the bladder [72-74] Somatic mutation in CBP (KAT) is associated with relapsed acute lymphoblastic leukaemia, diffuse large B-cell lymphoma and transitional cell carcinoma of the bladder [75]. Not only KAT but also a somatic mutation in Lysine methyltransferase, MLL2, NSD2, GLP helps in Non-Hodgkin lymphoma, medulloblastoma, Multiple myelomas, Medulloblastoma and ganglioglioma development respectively [76-79]. Abnormal JARID1C (Lysine demethylase) function due to somatic mutation is correlated with renal cell carcinoma progression [80].

1.1.2.2 Histone tail modification and cellular function

The histone core itself is not enough to uphold the regulation pattern of chromatin. Mainly chromatin remodeler mediated histone tails modifications are the main driver of histone core arbitrate gene regulation. The cell contains various chromatin remodeling activities that can modify histones or move nucleosomes [11]. From birth to death a cell pass through large genetic expression profile which is regulated by DNA and histone modifications. There are two types of chromatin in the genome, silent heterochromatin, and active euchromatin. Each of these chromatin patterns associates with a various set of chromatin marks, tagged on DNA and histones where miRNAs also participate

During the early stages of embryo development in mammals, changes occur in genome-wide DNA methylation and histone modification patterns. During embryo development Trithorax group (TrxG) and Polycomb group (PcG) proteins animatedly regulate Hox genes expression, which is involved in transcriptional regulation that maintains cell proliferation and differentiation in the stem and progenitor cells [81]. Often dysregulation of TrxG, and/or PcG activity leads to aberrant Hox gene expression patterns in cancer [82]. CARM1 (also known as PRMT4), a histone H3 arginine (R) methyltransferase and transcriptional coactivator mediated H3 arginine methylation involved in tropho-ectoderm development, less H3 arginine methylation containing 4-cell stage blastomeres become tropho-ectoderm. Moreover, the pluripotency factors OCT4, NANOG and SOX2 also regulated by CARM1. H3R17 and H3R26 methylation in the promoter region by CARM1 increase expression of these pluripotency factors and maintain the cell pluripotency [83]. In 2006 Bernstein B E et al. reported a novel chromatin modification pattern call “bivalent domains” [84]. Bivalent domains consisting of the activating histone H3K4me3 and the repressive H3K27me3 mark is apparant in many promoters in embryonic stem (ES) cells. These bivalent domains are related to poise expression of developmental genes, such as transcription factors and allowing their activation when the time comes [84, 85].

The germ line and global cellular development and functions are maintained and regulated by histone modification pattern, in mammals; where the silent heterochromatic structure is maintained by low levels of acetylation and high levels of H3K9, H3K27, and H4K20 methylation. Not only the modification mark but also mark associated different protein group recruitment is associated with different structure. The recruitment of Polycomb group (PcG) proteins PC2 (a PRC1 protein complex) to H3K27me preserve the inactive state of X chromosome [86, 87]. Heterochromatin Protein 1 (HP1) recruitment to H3K9me is a requisite to maintain the pericentric heterochromatin. Three histone H3 and one H4 methylation sites are implicated in activation of transcription marks: H3K4, H3K36, and H3K79 and H4K16. H3K4me and H3K36me have been associated with transcriptional elongation. Not only lysine methylation but also H3S10 phosphorylations are related to transcriptional activation. Whereas, the three other histone lysine methylation sites (H3K9, H3K27, and H4K20) and Ubiquitylation of H2AK119 are accompanied by transcriptional repression [55, 88]. More detail of histone modification is explained in Table: 1.1.

It has already been clearly established that anomalies with epigenetic events are a significant regulator of cancer development and progression. Alterations in the function of histone-modifying complexes are believed to disrupt the pattern of normal function and consequently disrupt the control mechanism of chromatin-based routes, eventually headed to oncogenic transformation and cancer development [89]. Various research results suggest that deregulation of histone modifying enzymes are related to different cancer development such as HAT (P300, CBP, pCAF, MOZ, MORF, Tip60), HDACs (HDAC1/2/3/4/5/6/7/8, SIRT1/2/3/4/7), HMTs and PcG Proteins (MLL1-4, NSD1-3, EZH2, BLIMP1, RIZ1, EVI1, PFM9, MEL1, SUV39H1, ZMYND1, HCP1, LBP1, BMI1, SUZ12, CBX7), Histone Demethylase (GASC1, PUT1, LSD1) [90]. Overexpression of HDAC3, HDAC7, HDAC8, SMYD3, SUV39H1 associated with colon cancer development. SMYD3 mediated increase H3K4 trimethylation help to form complexes with HELZ (RNA helicase) and RNA pol-II, which directly form a network by binding with the promoter region binding motif 5'-CCCTCC-3'. This association facilitates transcription of cell proliferation inducing genes such as NKX2-8, in hepatocellular carcinomas, colorectal and breast cancers. Aberrant Wnt/β-catenin signalling mediated SMYD3 overexpression leads to WNT10B upregulation in breast cancer development [65]. Apart from overexpression, downregulation of specific modulators is also associated with colon cancer development. pCAF, HDAC1, HDAC4, MLL3 are either downregulated or mutated in colon cancer. In breast cancer HDAC6, SIRT3, SIRT7, NSD3, HCP1 genes are overexpressed and correlated with oncogene expression [90]. Estrogen receptor (ER) related transcriptional activation is induced by SMYD3 overexpression in breast cancer. SMYD3 mediated increased the level of H3K4me2 and H3K4me3 at the promoter region of ER-targeted genes, which encourages cell growth related transcription in breast cancer [91]. Functional abnormality caused by LSD2 down-regulation increase H3K4 dimethylation and induces oncogene expression in various cancer including different types of leukemia, seminoma, and some classes of ER-negative breast cancers [92]. During early stages of epithelial to mesenchymal transition (EMT), TGF-β activation and promoter CpG methylation reduces expressive H3K4me3, H3K9Ac marks and induces H3K27me3 marks in the promoter region of TSGs, which in turn results in low level of TSG expression including β1 and α4 integrin and E-cadherin expression [93, 94]. The interaction between cistrons and epigenomes play a crucial role in breast cancer development. Breast cancer–associated single nucleotide polymorphisms (SNPs) are enriched in cistrons of a transcription factor such as ESR1 and FOXA1,

binding sites and the epigenome of H3K4me1 in breast cancer. Altered binding affinity and transcriptional efficiency of ESR1 and FOXA1 promotes cancer progression [95]. During prostate cancer development function of HDAC4, CBP, p300, LSD1 (KDM1), JHDM2A (KDM3A), or JMJD2C (KDM4C) enzymes are altered. H3K9me1, H3K9me2, and H3K9me3 are accompanied by the repression of AR target genes in LNCaP cells [96-98]. It is known that overexpression of a demethylase KDM1, specific for H3K4me1 and H3K4me2, significantly decreases AR binding [99] and increased level of H3K4me3 in prostate cancer cells activates the expression of genes involved in cell growth and survival (FGFR1 and BCL2) [100]. Moreover, different cancers are associated with abnormal histone modification including ~10% of human acute leukaemia (acute myeloid leukaemia, acute lymphoblastic leukaemia, or mixed lineage leukaemia), Glioma, B cell lymphomas, Hematologic, pancreatic, ovarian, Thyroid and Squamous cell carcinoma [90].

Table 1.1 Lists of histone modifications

Histone modification	Function	Organism	Reference
H2A on serine 1 phosphorylation	double strand breaks repair	Mouse, human, yeast	[101]
Acetylation of H2AX lysine 5	TIP60-UBC13 complex mediated DNA double-strand break repair	Human	[102]
H2A K5ac and K9ac	TIP60-mediated acetylation of H2A at K5 and K9 facilitates H2A.Z incorporation catalyzed by the small complex and regulate chromatin transcription	Human	[103]
acH2A.Z	Associated active gene transcription	Human	[104]
H2AK119 Ubiquitylation	Bmi/Ring1A mediated H2AK119 Ubiquitylation is associated with transcriptional repression	Human	[88]
H2BK120 ubiquitylation	RNF20/RNF40 and UbcH6 mediated H2BK120 ubiquitylation activate transcription	Human	[105]
H2BE2 ADP-ribosylation (H2BE2ar1)	Associated with transcription but specific function is unknown	Human	[106]
H3 proline isomerization (H3P38)	Catalyzed by enzyme, FPR4 and regulates the levels of H3K36 methylation	Budding yeast	[107]
H3K4me3 than H3K4me2	involving mutually exclusive histone modifications of the same histone residue (H3K4ac and H3K4me)	*Saccharomyces cerevisiae*	[108]
H3K9me	Chromodomain proteins (Chp1/Chp2/Swi6/Clr4) bind and regulate pericentric heterochromatin	Fission yeast	[109]
H3K4me3 than H3K4me2	Sgf29, a histone acetyltransferase, recognizes H3K4 methylation marks and directs the SAGA (Spt–Ada–Gcn5 acetyltransferase) to its rightful position	Humans and yeast	[110]
H3K4methylation	Downregulated by Class I HDACs by inducing specificity protein 1 (SP1) signalling mediated RB binding protein 2 (RBP2) and JARID1 family histone demethylases such as PLU-1 (lysine-specific demethylase 5B), SMCX (lysine-specific demethylase 5C), and LSD1 expression	Human	[9]

H3K4 acetylation	HDAC3 remove acetylation at centromere regions and is also involved in centromeric chromatid cohesion.	Human	[84]
H3K4 acetylation	H3K4 deacetylation facilitates Shugoshin1 (Sgo1) binding to the centromere and induces H3K4 dimethylation in CENP-A (Centromere protein A) rich kinetochore while the specific interaction between H3K4me2 and Sgo1 is not clear.	Human	[84]
H3K27me3 H3K4me3	Maintain bivalent chromatin structure and regulate gene expression in stem and progenitor cells	Eukaryotes	[65]
H3K27 di- and tri-methylation	Helps to increase H3K4 methylation in the promoter region in active gene promoter.	Eukaryotes	[111]
H3K4 methylation H3K27 me2/me3	Recruitment of PRC1 and the monoubiquitylation of histone H2A at the promoter of HOX gene clusters are regulated by a member of the Jumonji C, UTX. UTX- mixed-lineage leukemia (MLL) 2/3 complexes increase H3K4 methylation level by decreasing the H3K27 me2/me3 level	Eukaryotes	[112]
Asymmetric dimethylation of histone H3R2 (H3R2me2a) and H3K4me3	Heterochromatic loci, inactive euchromatic genes and transcriptionally poised or active promoters in the mammalian genome regulation	Eukaryotes	[55]
H3K4me1	MLL3/4-dependent H3K4 mono-methylation required for minor zygotic gene activation	Mice	[113]
H3K4me1	Recruit RSC chromatin remodeling complex to stress-responsive genes.	Yeast	[114]
H3K9 methylation	essential for heterochromatin formation	Eukaryotes	[115]
H3K9me1/2	GLP-mediatedH3K9me1/2 establishment helps in Oct4 and Nanog silencing during differentiation	Mouse	[116]
H3K36me	Accumulate at the 3′-end of active genes and is associated with elongation	Human	[117]
H3K79 methylation	Activation of HOXA9 and it has a role in maintaining heterochromatin	Human	[55]
H3S10 phosphorylation	activate NFKB-regulated genes and “immediate early” genes such as c-	Mammals	[118]

	fos and c-jun.		
Arginines in H3 and H4 converted to citrullines (Deimination)	Prevent arginine methylation	Mammals	[50]
H4K16 acetylation	Negatively regulate the formation of a 30-nanometer fiber and the generation of higher-order structures	Human	[119]
H4S1 phosphorylation (H4S1p)	Catalyzed by Caesin kinase II and help in DNA double-strand break repair	*S. cerevisiae*	[120]
H4K20me	DNA double-strand breaks repair	Yeast	[121]
H4K12Ac	DNA repair	Yeast	[122]

1.1.3 MicroRNA

Ambros and colleagues (1993) identified lin-4, the first miRNA, which participates in regulating the development timing in *Caenorhabditis elegans* [123]. lin-4 was found to regulate the expression of lin-14 mRNA negatively through interaction with a complementary region in 3′UTR of lin-14. With the advancement of technology, thousands of distinct miRNA in the human genome has been discovered.[124]. DNA methylation and histone modifications play a significant role in differential gene expression patterns which in turn is also controlled by miRNA. miRNA plays a key role in regulating the gene expression globally for normal homeostasis. When this circuit is disrupted, it leads to various diseases and harmful manifestations. miRNAs are born from the cosmos of "dark genomic matter" encoded in intergenic or intragenic regions. The miRNA genes are transcribed by RNA polII into pri-miRNA [125], however, C19MC, the largest human miRNA cluster is transcribed by RNApolIII. The length of pri-mRNA which stretches between 1 kb - 3 kb is further processed by Drosha (ribonucleases) and DiGeorge syndrome (22q11.2 deletion syndrome) critical region 8 (DGCR8) or Pasha in the nucleus which forms 70 - 100 base pairs (bps) long hairpin structures "pre-miRNA" [126, 127]. Pre-miRNA, after being transported out of the nucleus by exportin-5, is further processed by an RNase named Dicer. This enzyme contains two RNase III domains, a helicase domain, dsRNA binding domain, a DUF283 domain and a PAZ (Piwi–Argonaute–Zwille) domain [128]. This PAZ domain allows the weak interaction with the 3′ end of ssRNA and 2-nucleotide at 3′ overhang of dsRNAs which is processed by Drosha [129]. This overhang is required for dicer activity. It has been found that the PAZ domain of Dicer finds the cleaved pre-miRNA by Drosha and helps the RNase III domain to chop out the stem and loop from the pre-miRNA and further processes it into variable length (18-25 nt) mature double-stranded miRNA [130, 131] , following which, the double strand gets separated into the guide and passenger strand. The guide or the mature strand gets incorporated into the RNA-induced silencing complex (RISC), whereas the passenger strand commonly denoted as a star (miRNA*) gets degraded. The RISC complex, which is the primary player of miRNA pathway, consists of miRNA, argonaute (Ago) proteins (Ago 1-4) and other protein factors. Ago proteins also play a vital role in miRNA biogenesis and maturation. The guide strand of miRNA unwound by helicase helps in the target recognition and binding of miRNA into the RISC complex [132, 133] (see Fig-1). miRNAs mainly bind to 3′ untranslated region (3′ UTR) of the target mRNAs, but current studies have shown that it also binds to the coding regions and the 5′ UTR of mRNA. This

has been demonstrated that the "seed" region of 7-8 nt in the 5' end of miRNA is necessary for its function [134]. Recent research reports show that miRNA regulate the gene expression probably by targeting the promoter associated ncRNA (paRNA) and direct transcriptional silencing [135]. Investigating the exact role of miRNA in cellular processes during tumorigeneses is a major area of interest. The variation in miRNA profiling in human cancer indicates that it can act both as a classical tumour suppressive gene and an oncogene also. The profiling can also be used for prognosis and early diagnosis in cancer. The variation in the level of expression of miRNA can be utilized in the field of pharmacogenomics to develop anti-cancer drugs.

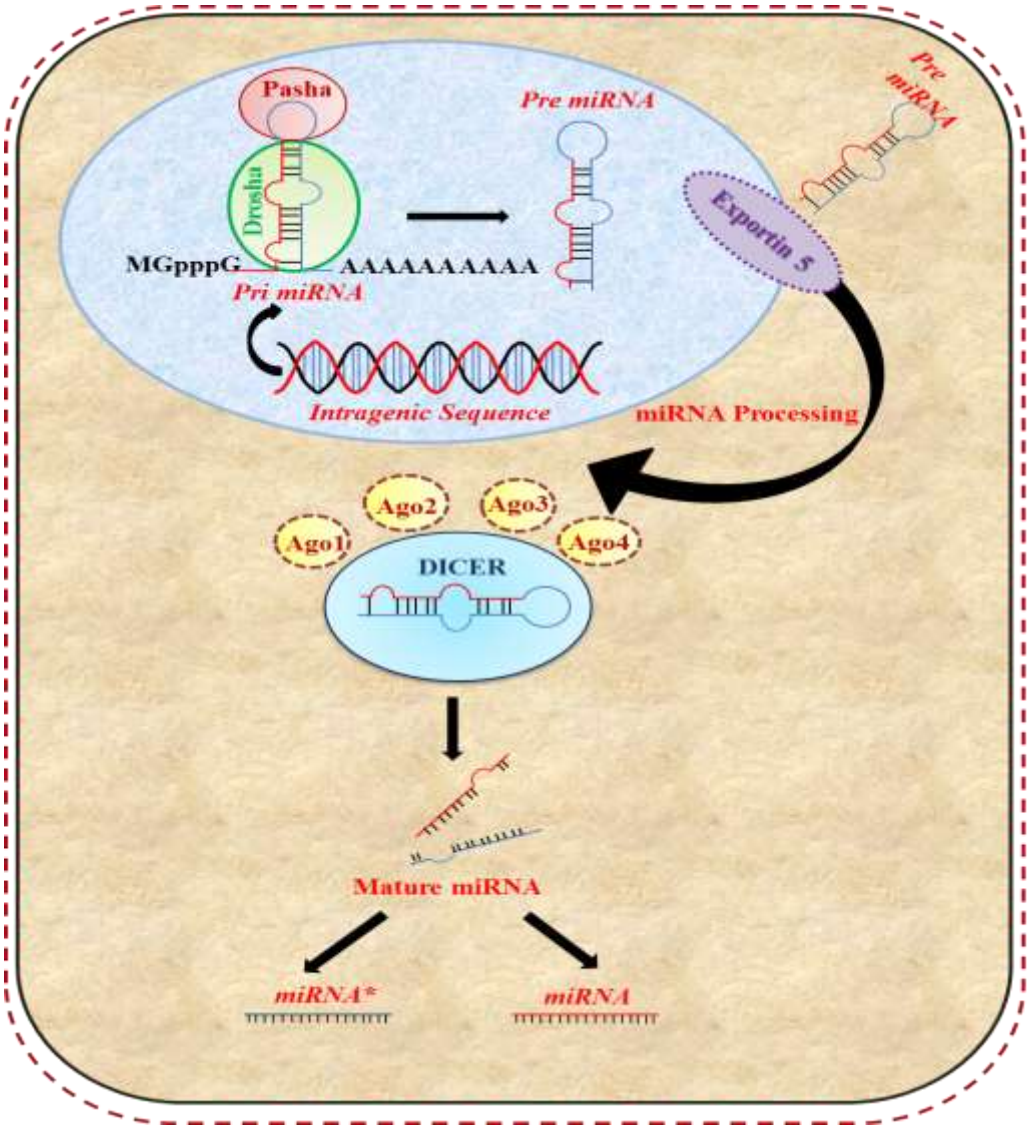

Figure 1.1: Biogenesis of miRNA:
From the miRNA gene RNA polymerase II transcribed pri miRNA. Then it is further processed to pre-miRNA by Drosha and Pasha. Exportin5 then transports the pre-miRNA from nucleus to cytoplasm. Another ribonuclease namely Dicer further processes it into variable length (18-25 nt) mature double-stranded miRNA. Argonaute proteins play a vital role in miRNA biogenesis, maturation, and effector functions. The double strand gets separated into the guide and passenger strand (miRNA*). The guide strand of miRNA unwinded by helicase helps in the target recognition and binding of miRNA into the RISC complex.

1.1.3.1 Emerging roles of miRNA in modulating gene function

The precise mechanism of miRNA-mediated modulation of gene function in development and homeostasis has not yet been completely understood. The main function of miRNA is to repress any gene expression by translational inhibition or degradation of mRNA. However, activation, as well as upregulation of genes, are reported in some cases.

miRNA navigates its regulatory functions through two different pathways; one path leads to the formation of a large gene silencing complex known as RNA-induced silencing complex (RISC) or miRISC complex where Ago protein plays a vital role. This miRISC complex silences gene by binding with the 3′ UTR of the mRNA causing the initiation of translation. It has also been reported that guide miRNA binds with the open reading frame (ORF) of mRNA which splices the transcriptome by spliceosome [136]. When miRNA complementarily binds with the 5′ UTR of mRNA, activation of the transcriptome takes place instead of suppression. In the other pathway miRNA binds with RNA binding protein and prevents them from binding to their target RNA [137] , which also modulates the target gene promoter by directly binding/changing the methylation signature [138].

KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) is a known oncogene with various functions in melanoma. This gene is the target of miR-221 which binds with the 3′UTR of the KIT and downregulates, but this gene is up-regulated in some cancers. A variant of a KIT oncogene is overexpressed during acral melanoma; since its 3′ UTR mismatches with the seed region of miR-221 so it cannot bind to that region. Therefore, without the regulation of miR-221 KIT expression increases up to four fold[139]. Apoptotic Fas-associated factor 1(FAF1) is a component of DISC that interacts with caspase 8 and FADD. The overexpression of FAF1 can induce apoptosis in the absence of extrinsic death signal [140, 141]. miR-24 has a seed region in the ORF of FAF1, which down-regulates the FAF1 so that the apoptosis is not triggered, however, when the miRNA expression is blocked, apoptosis is re-induced [142]. Thus, miR-24 acts as an oncomiR which downregulates the apoptotic genes. Regulation by miRNA also involves binding with 5' UTR of mRNA and activating the translation as seen in the case of hepatitis C virus (HCV). The liver-specific miR-122 binds to the 5′ UTR of genomic RNA up-regulates its transcription and positively regulates its lifecycle. [143]. In another scenario when the cell is subjected to nutrition stress, it halts translation by blocking the synthesis of ribosomal proteins. After the stress is withdrawn, cell restores the ribosomal protein synthesis. miR-10a interacts with 5' UTR of mRNA of ribosomal proteins and improves their translation. miR-10a binds downstream to the regulatory 5' TOP motif and globally enhances the protein translation [144]. The current studies have shown that expression of the gene can also be induced by miRNA. The miR-373 sequence is complementary to promoter site of E-cadherin (E-cad). By transfection, miR-373 induces E-cad expressions whereas knockdown of miRNA represses its expression. Eventually, it has been confirmed that induction of E-cad needed the mature miRNA, not the pre-

miRNA. Another cold shock domain-containing protein C2 (CSDC2), which also contains a putative seed region in its promoter site, is also found to be readily induced by miRNA. miRNA helps the transcription by enhancing the binding of RNA polymerase II to the promoter of the E-cad and CSDC2 [145] (Fig.1.2). MiRNA controls the genetic expression in various circumstances of the cells. These regulations are crucial for the cellular development and normal physiology. Thus,different mechanisms of regulating the gene expression impact miRNA a distinct role in genetic regulation.

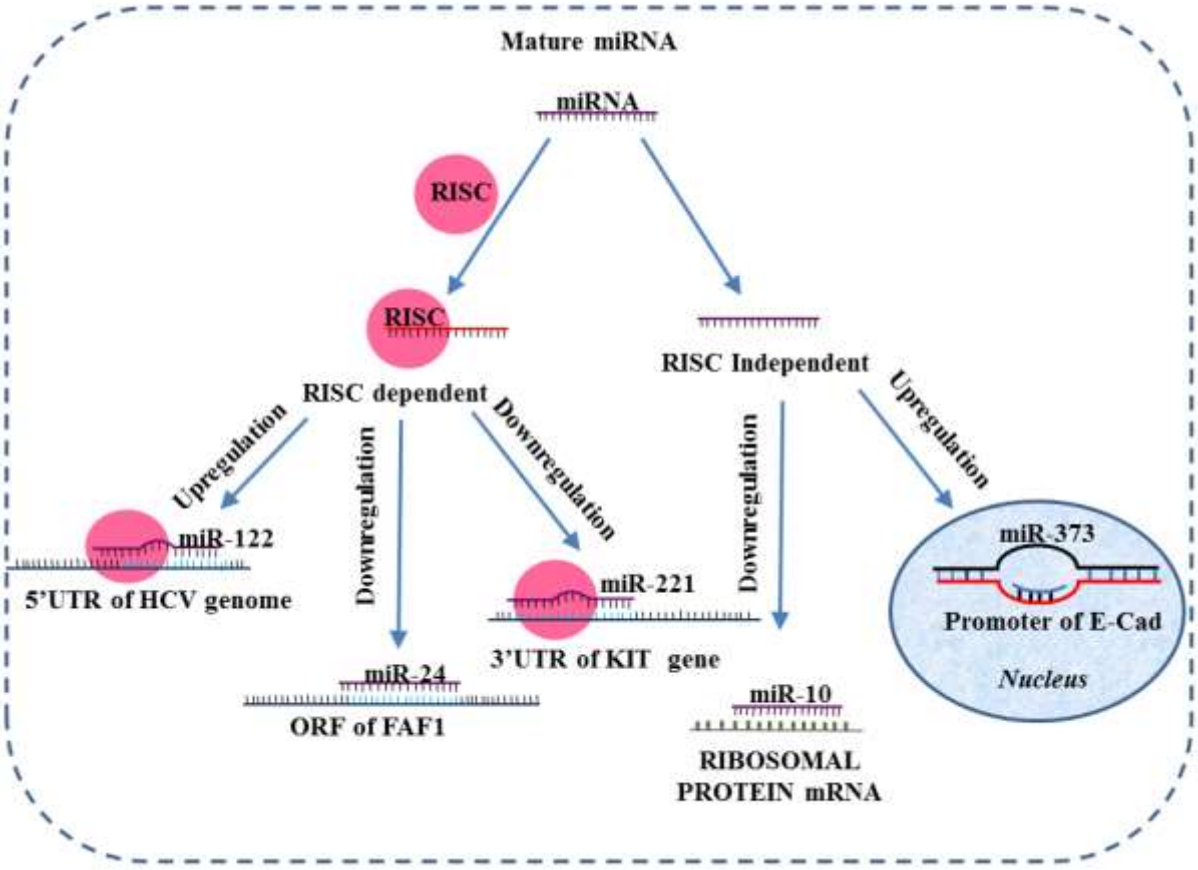

Figure.1.2: Mature miRNA regulate gene expression in different ways:
KIT oncogene transcript degraded by miR-221 which has a seed region at the 3' UTR of the mRNA. The Fas-associated factor 1(FAF1) has the seed region of miR-24 in the open reading frame (ORF) of mRNA which is eventually degraded by the miRNA. miR-122 binds to the 5' UTR seed region of RNA, the genome of HCV and positively regulates its live cycle by activating the translation. miR-10a interact with the mRNA of ribosomal proteins 5′ UTR and improved their translation. The miR-373 sequence is complementary to promoter site of E-cadherin (E-cad), and it induced it expression.

1.1.3.2 Currently known functional miRNA in the human cancer genome

Recent explorations by next generation sequencing have led to the discovery of more than 24,000 miRNA (http://www.miRbase.org). For target prediction of miRNA different computational algorithms has been utilized. It considers the seed sequence and

the number of target sites surrounding mRNA sequence. The algorithms use the parameters like binding free energy to the target sequence and the secondary structure of the target binding sites which helps or prevents the binding of miRNA and mRNA. Through miRNA target prediction and validation has represented a small fraction which regulates 60% of human genes [146]. By improving the prediction algorithms, identification of new miRNA target will be more accurate and precise. As previously stated, miRNA is an inseparable part of cellular gene regulation. High-throughput studies proved that it also has a crucial role in human diseases like cancer. Here, we will try to explain the functional miRNA of the human genome which participates in cancer. The most prominent tumour suppressive miRNAs are miR-15/16. Their target includes BCL2 which induces apoptosis in leukemic cell line model [147]. miR-16 down regulated theCOX-2 gene in colon cancer [148]. In fibroblast cells, miR-16 targets VEGF, VEGFR-2, FGFR1, which governs the cell intrinsic angiogenic activity. Downregulation of miR-15 and miR-16 promotes upregulation of FGF2 and FGFR1 gene in cancer associated fibroblasts [149, 150]. In multiple myelomas, deletion of miR 15/16 increases the level of FGFR1, P13KCA, MDM4, and VEGFa. In breast cancer, miR-16 regulates WIP1 phosphatase during DNA damage response [151]. miR-15a/16 targets Bmi-1, which inhibits cellular proliferation in ovarian cancer [152]. In lung cancer, it induces cell-arrest by targeting CCND1, CCND2, and CCNE1 [153]. Another TS-miRNA, miR-34 also targets BCL-2, NOTCH, and HMGA2 in gastric cancer [154]. In fibroblast cells, it targets MYC and controls a set of cell cycle regulator during cellular senescence. Similarly, in ovarian cancer, it targets MET genes [155]. Another imperative family of miRNA is miR-200 targets ZEB1 and CNNB1 which inhibit cellular growth migration and invasion in nasopharyngeal carcinoma [156]. In pancreatic carcinoma as well as in breast cancer, it targets BM1 and inhibits cellular metastasis. In endothermal cancer, it targets FN1, LEPR, and NTRK2 which inhibits cell mortality and resists anoikis [157]. miRNA let-7 family is the most significant miRNA which targets IL-6, in breast cancer, E2F2 in prostate cancer, BCL-XL in liver and MYC in Burkitt lymphoma which initially inhibits cancer progression [158]. The miRNA discussed above are the TS-miRNA, which remains downregulated in cancer cells due to hypermethylation or deletion of the genes. The further discussed miRNAs are the functional oncomiRs of the human genome. The cluster of miR-17/92 is the most active oncomiR cluster which plays a predominant role in cancer progression. In breast cancer, it targets HBP1 and regulates the invasion and activates WNT/ β-catenin pathway [159]. In myeloid cells, it targets p63 and increases the cell

proliferation. In fibroblast cells, it disrupts cellular senescence by inhibiting p21 [160]. Another family of oncomiRs, miR-222/221 targets p27 and stimulates cellular proliferation in glioblastoma [161]. In small cell lung cancer, its target is PTEN and thus induces TRAIL resistance [162]. In endothelial cells, it targets KIT and causes the formation of new capillaries and angiogenesis. The most upregulated miRNA in most type of cancer is miR-21. It targets multiple tumour suppressive genes like RECK in glioblastoma which resists the cancer cells against diverse therapeutics. MARKS gene in prostate cancer inhibits cellular apoptosis and induces cellular motility and invasion. In breast cancer, it targets TPM1 and PDC4 and influence tumour growth and suppresses cellular apoptosis [163]. miR-155 is overexpressed in lung cancer, breast cancer, acute myeloid lymphoma and CLL, Burkitts lymphoma and Hodgkin lymphoma. It targets HGAL, FOXO3A, SOCS1, JMJD1A in different cancers which contributes to tumour progression and resistance to the chemotherapy [158]. The dysregulation of miRNAs in cancer relative to normal tissue indicates the functional role of miRNAs in tumour cells.

1.1.3.3 miRNA regulation of gene expression

Till few years back it was thought that miRNA can only regulate gene expression by inhibiting the target mRNA and suppressing their protein synthesis. But in the light of recent exploration into the epigenetic regulation of a gene by miRNA shows its different paths of regulating genes. Here we briefly describe some of the pathways recently discovered by researchers which could enlighten the diversity of miRNA regulation study.

Repression of translation initiation by miRNA

The first case of miRNA repression was discovered in *C. elegans***.** Initially, it was shown that lin-4 inhibits the translation of lin-14 without a reduction in the mRNA levels or modifications in polysomes. This discovery leads to the conclusions that miRNA inhibits mRNA at elongation step. The firstmiRNA-mediated repression of translation was observed in HeLa cells where cells were targeted by endogenous let-7 miRNA for mono and bicistronic reporter mRNA [164] . From this experiment, it was found that the level of reporter mRNA was not significantly decreased whereas the translation was inhibited [164]. These results were further supported by the observation in Huh7 where miR-122 targets CAT-1 mRNA [165] and in HEK29 T-cells for a miR-16-targets reporter mRNA [166].

Repression of cap-dependent translation

The miRNA-mediated translational repression is frantically dependent on the mRNA capping. It has been reported that mRNA which contains 5' capping is more susceptible to the miRNA. In HeLa cells it has been observed that mRNA with non-functional ApppG cap structure was less targeted than mRNAs with proper m7G capping [167].To understand the pathway for targeting mRNA with caps it has been found miRNA interfere with either eIF4E function or eIF4Erecruitment to the 5'-cap structure [164] . This pathway was supported by *in vitro* experiments in a cell-free system. It was then confirmed miRNA-mediated translational inhibition occurs during its initiation steps, recognizing the m7G cap of mRNA. These results were further supported by the inability of miRNA targeting IRES-dependent translation or translation from ApppG-capped mRNAs [168-170].

miRNA targeting by suppressing ribosomal assembly

Wang et al. proposed that miRNA repress translational inhibition by inhibiting the joining of 60S subunit. They also found enrichment of 40S subunits in the miRNA-mediated mRNA repression complexes in reticulocyte lysates. Another study concluded that the 60S ribosomal subunits accessory proteins like eIF6 prevent the joining with 40S and regulate translation[171]. These proteins were co-immunoprecipitated with the AGO2-Dicer- TRBP (TAR RNA-binding protein) complex [172]. Repression of eIF6 in human cells or in *C. elegans* inhibits the action of miRNA (let-7 or lin-4) which indicate miRISC complex with eIF6 inhibits 80S complex assembly [173].

Poly (A) tail mediated translation repression

Previously it has been proved that Poly(A)-binding protein (PABP) enhances cap-dependent translation of mRNA by interacting with the eIF4G of the eIF4F complex [174]. Deadenylation of mRNA increases the rate of miRNA-mediated translational inhibition. In HeLa cells, it has been shown that both 5’capping and poly(A) tails are required for optimal translation inhibition [167]. Deadenylation of mRNA increases the miRNA targeted translation inhibition which has been shown in HEK293 cells overexpressing AGO2 and GW182 proteins and let- 7 miRNA [168]. Some groups found in mammalian and *Drosophila* cells miRNA-mediated mRNA translation inhibition occurs in both non-polyadenylated and polyadenylatedmRNAs, but non-polyadenylated are not

as strong as for polyadenylated mRNAs [175, 176]. From the above we can say miRNA works through both poly (A) tail–dependent and –independent mechanisms.

Repression by miRNA at Post-Initiation Steps

Different research groups found that miRNA can inhibit translation at post-initiation step. This analysis was led by the investigation on *C. elagans* that indicates the lin-14 and lin-28 mRNA which are the targets of lin-4 miRNA remains with translating polysomes instead its lower protein level in larval development [177, 178]. In mammals also it has been observed that repressed mRNA were in association with functional polysomes [179]. But the repression in postinitiation step by miRNA the pathway remained a mystery until it has been proposed that, may be, miRNAs antagonize translation elongation by causing premature termination and subsequent ribosome drop-off. This conclusion was made by metabolic labeling and ribosome run-off experiments led by Petersen et al. [180]

1.1.3.4 Regulation of signalling pathways by miRNA- an impact on development and normal physiology

Various structural proteins and signalling pathways involving miRNA maintain a tight cooperation for control of development, including cell adhesion, neurogenesis, brain, eye, liver, and heart development and normal physiology. miRNA-mediated neuronal, and neural stem cell differentiation and function are not clearly described till now. MiR-9/9* and -miR-124 (miR-9/9*-124) -NEUROD2 encourage human fibroblast to convert into neurons. Neurogenic transcription factors such as ASCL1 and MYT1L, enhance this conversion process [181]. Compositional changes of SWI/SNF-like BAF chromatin-remodeling complexes are guided by miR-9* and miR-124. During mitotic exit, a BAF53a subunit of the neural progenitor BAF is repressed by the miR-9* and miR-124 and contribute to the neural fate. The nuclear receptor TLX is a key controller of neural stem cell self-renewal and proliferation. Its interaction with miRNAs controls the neural stem cell differentiation and specification [17, 182, 183]. miR-9 and TLX expression is inversely proportional as the miR-9 expression are increased, and TLX expression is decreased during differentiation of neural stem cell, thus highlighting the fact that miR-9 negatively regulates TLX expression.

miR-132 and miR-134 have a very deep impact in mammalian neurogenesis. Transcriptional activation of CREB and MeF2 is followed by increased miR-132 and

miR-134 levels respectively. miR-132 represses p250 GTPase Activating Protein (p250GAP), and miR-134 suppresses the translational repressor Pumillio2 (Pum) expression, the two important factors of neurogenesis. p250GAP inhibition downregulates Rac activity which causes dendritic growth and branching [184, 185]. Brain development is interconnected with miRNA control mechanism. MiR-9 controls patterning, neurogenesis, and differentiation whereas miR-134 directs neurogenesis and spine growth [186]. The roles of individual microRNAs in vertebrate eye development remain a mystery but in *Oryzia slatipes,* miR-204 regulates multiple aspects of eye development. It targets the transcription factor Meis2 which controls lens formation and dorsoventral (D-V) patterning of the retina which is related to optic fissure coloboma. The experimental data supports the fact that miR-204 also plays a major role in Pax6 pathway in eye development [187].

Involvement of miRNA function during liver development is emerging. miR-106a-363 and miR-17-92 have a regulatory effect on embryonic liver cell proliferation, cell cycle, and apoptosis. In mature hepatocytes, let-7c and miR-23b inhibit cell proliferation and mediates cell cycle arrest. TGF-β signalling has a precious role in adult liver development. In the adult liver cell, let-7c controls TGF-β signalling by supervising the TGFBR1 expression [188].

Cardiomyocytes development and miRNA-signalling strictly depend on each other. In human miR-1, miR-20, miR-21, miR-26a, miR-92, miR-127, miR-129, miR-130a, miR-199b, miR-200a, miR-335 and miR-424 play a vital role in heart development. miR-1 binds with the histone deacetylase 4 (HDAC4) which is a transcriptional inhibitor of muscle genes and induce myogenesis. The repression of serum response factor (SRF) by miR-133 resulted in myoblast proliferation [188-194]. Heart containing 'non-cardiomyocyte' cells including, fibroblasts, smooth muscle cells, endothelial cells, and immune cells, have different miRNA expression pattern. miR-21, miR-16, miR-22, miR-23a, miR-27a, miR-24 are highly expressed in skin fibroblasts and heart, whereas endothelial cells express let-7 family, miR-126, miR-221, and miR-222 [194].

Cell adhesion is interconnected with an expression of different miRNAs. Adhesion molecules expression and biochemical pathways involved in normal cellular adhesion are controlled by miRNA including miR-17, miR-29, miR-31, miR-124 and miR-200. Cytoskeletal dynamics, actin polymerization and depolymerization are controlled by specific small GTPases belonging to Rho superfamily, which includes the Rho, Rac and Cdc42 subfamilies [195]. These subfamilies are controlled by different miRNA pathways.

RhoA and Cdc42 expression can be repressed by miR-133 [196]. Moreover, miR-138 leads to repression of RhoC and the downstream effector kinase ROCK2. These correlations indicate to the miRNA-mediated control of Rho signalling and as well as cytoskeletal dynamics [197]. Expression of Integrin, an essential cell–matrix adhesion molecule, is regulated by miRNA. For instance, Integrin β1 is governed by miR-124, miR183, miR-29, miR451, integrin α5 by miR-31, miR-92a, integrin β3 by miR-30, let-7a. Cell-cell adhesion molecules are also controlled by miRNA-mediated silencing including, E-cadherin by miR-205, miR-200, miR9, miR-10a, miR-192, Intercellular adhesion molecule 1 (ICAM-1) by miR-221, miR-222, miR-339, miR-17 and E-selectin by miR-31, miR-10a [198]. Healthy development and physiology of cells are intimately related to miRNA regulation. Deregulation in the control of miRNA can lead to abnormalities in cellular physiology.

1.1.3.5 Regulation of signalling pathways by miRNA - an impact on cancer

MicroRNA directly or indirectly involved in regulating a number of cell signalling pathways, including Wnt, Notch, Hedgehog, TGF-b/BMP, receptor tyrosine kinase (RTK), Jak/STAT, a nuclear receptor, Hippo pathways [199]. miRNAs upregulate or downregulate the Wnt pathway depending upon the stages of cell development. This tight regulation is entirely disrupted during tumourigenesis. miR-8 and miR-200c control Wnt ligand secretion by negatively regulating TCF and upstream positive modulators respectively in *D. melanogaster* and mouse cells. In mammals, miR-8 inhibit Wnt pathway by repressing the activity of Wingless (Wg) signalling [200]. However, miR-135 activates Wnt signalling by targeting two negative regulators, Axin, and Notum of Wg signalling [201]. Wnt/β-catenin pathway is involved in cell proliferation, metastatic and tumourigenesis. Adenomatous polyposis Coli (APC) expression is down-regulated in the colorectal cancer cell, and this facilitates Wnt/b-catenin signalling. miR-135a and miR-135b are the main culprits behind the repression of APC by targeting the 3′ UTR [202]. MiR-200a represses β-catenin activity in two different ways. One is the silencing of E-cadherin repressor proteins ZEB1, ZEB2, which is followed by an increase in the level of E-cadherin and β-catenin binding and induction of epithelial to mesenchymal transition (EMT) by elevating nuclear β-catenin levels. Another way involves direct silencing of β-catenin expression by binding to its 3′ UTR [203]. During medulloblastoma, miR-125b, miR-326 and miR-324-5p are down-regulated which direct over activation of the Hedgehog pathway [204] whereas miR-92, miR-19a and miR-20 are overexpressed [205].

miR-430 and miR-214 are involved in zebrafish embryogenesis [206]. Akt pathway transmitted growth signals are manipulated by miRNA in glioma. MiR-7 arbitrated suppression of EGFR expression was inhibited by miR-7 downregulation and Akt pathway activation in glioma. Moreover, over-expression of miR-26a target phosphatase and tensin homolog (PTEN) result in Akt signal enhancement [207]. 3'-phosphoinositide-dependent protein kinase-1 (PDK-1) is an essential component of Akt signalling and controls cancer cell survival and proliferation. MiR-375 mediated negative regulation of PDK-1 controls cell proliferation and suppressed tumourigenesis. This is the main reason behind miR-375 low expression in different cancer cells including pancreatic cancer and hepatocellular carcinoma [208]. MiR-146, miR-301a, miR-155, miR-181b, miR-21, miR223, miR15, miR16, miR199a are involved in Nuclear factor κβ (NFκβ) signalling and their deregulation mediates cancer. NFκβ signalling negatively regulates its signal status by IRAK1 and TRAF6 downregulation through MiR-146 up-regulation. Different cancers such as breast cancer, pancreatic cancer, anaplastic thyroid carcinomas, brain tumours, shows NFκβ/miR-146 regulation irregularity [209]. MiR-155, miR-301a, and miR-181b are positive regulators of NFκβ signalling. miR-181b-1 represses CYLD expression, a ubiquitinations, which is followed by the NFκβ activation [210]. MiR-181b-1 overexpression in colon adenocarcinoma, prostate, and hepatocellular cancer is correlated with uncontrolled NFκβ signalling and carcinogenesis. Unlikely MiR-21 represses NFκβ activity by targeting PDCD4 [211]. Increased notch signalling is an important change during medulloblastoma. The function of miR-199b-5p is misbalanced in medulloblastoma cell and leads to HES1 overexpression mediating Notch activation [212]. MiR-375 negatively regulates the Hippo pathway through yes-associated protein (YAP) down-regulation. The downstream target of YAP is the mRNA level of connective tissue growth factor (CTGF). YAP is a downstream regulator of hippo signalling pathway which helps in cell growth, proliferation, invasion and epithelial-to-mesenchymal transition. Lower miR-375 expression in hepatocellular carcinoma cells is followed by Hippo/YAP signalling over-activation [213]. Over-activation of Hippo and downregulation of a large number of tumour suppressor genes by miR-372/373 encourage testicular germ tumourigenesis [214]. C-Src/Mammalian target of rapamycin (mTOR) / fibroblast growth factor receptor 3 (FGFR3)/AKT mediated pathway, epidermal growth factor/Ras/mitogen-activated protein kinase (MAPK) pathway are a critical oncogenic pathway. miR-99a downregulation is directly correlated with the up-regulation of these pathways [215]. K-Ras and N-Ras arbitrate RTK/MAPK signalling pathway is controlled

by the let-7 during cancer cell differentiation [214]. miRNA regulation of different signalling pathway in cancer cell gives them a crucial role in the cell proliferation and differentiation. The oncomiR and tumour suppressive miRNA give the researcher a new dimension for an understanding of the complexity of deregulations of the genetic expression during carcinogenesis.

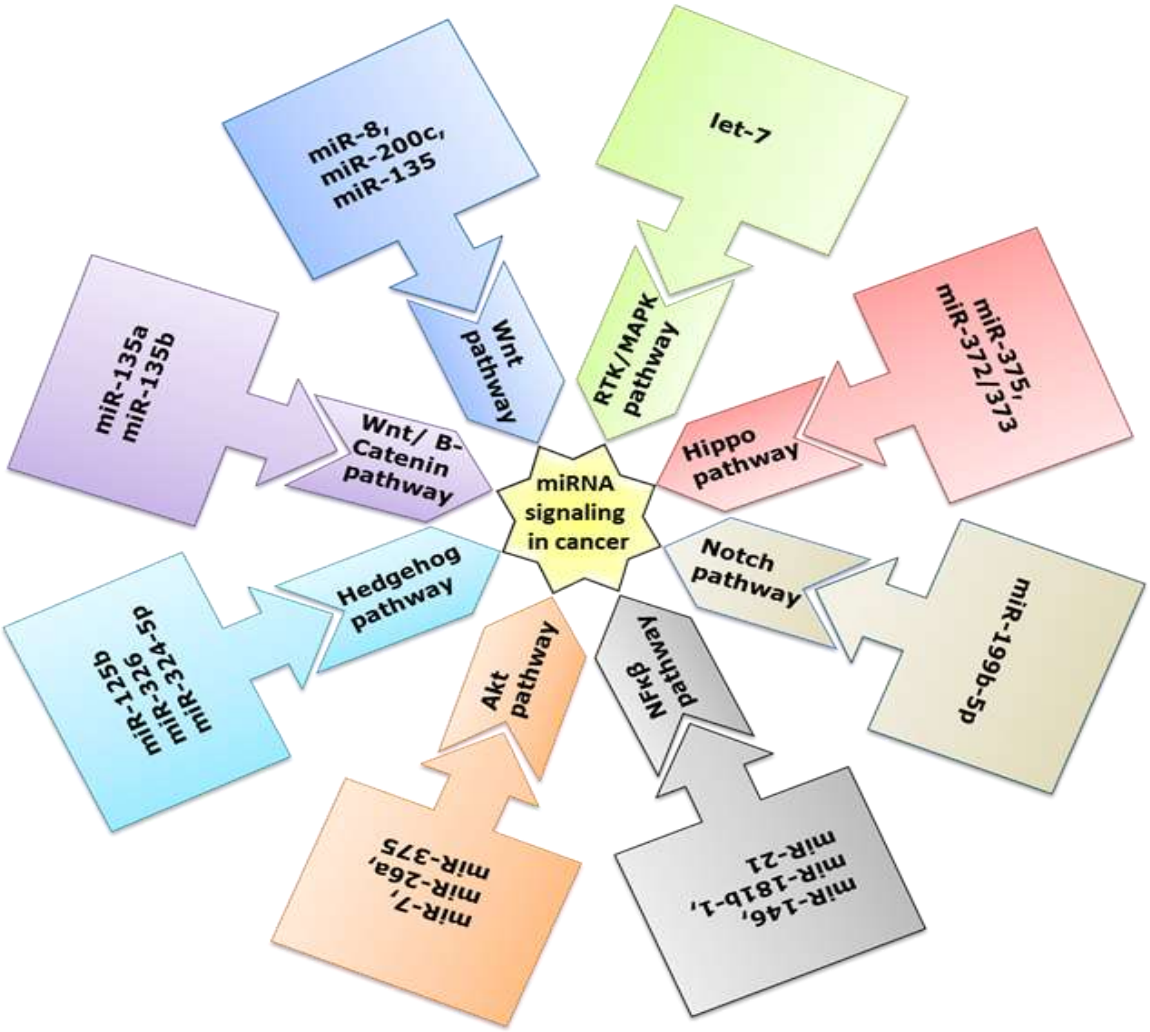

Figure.1.3: miRNA regulation of different pathways in cancers:
In cancer cells Hedgehog pathway by miR-125b, miR-326, miR-324-5p Wnt/ B-Catenin pathway by miR-135a, miR-135b Wnt pathway by miR-8, miR-200c, miR-135 RTK/MAPK pathway by let-7 Hippo pathway by miR-375, miR-372/373 Notch pathway by miR-199b-5p. Akt pathway by miR-7, miR-26a, miR-375, NFκβ pathway by miR-146, miR-181b-1, miR-21 is regulated.

1.1.3.6 miRNA as therapeutic drug for cancer

In the new generation of therapeutic approaches against cancer, miRNA plays an inherent part. miRNA can work as an oncomiR which downregulates the tumour suppressive gene and as tumour suppressive miRNA (TS-miRNA) which downregulate

oncogenes [216]. By modulating, the expression of miRNA, it is able to regulate the cellular gene expression. For therapeutic uses there are mostly two kinds of miRNA; (a) miRNA mimics, and (b) anti-miRNA oligonucleotides (AMOs).

miRNA mimics

TS-miRNA is remaining down-regulated in cancer cell because it inhibits the expression of an oncogene. So it is possible that inhibition of dysregulated oncogenes by using synthetic miRNA mimics can be a very useful prognostic tool for cancer. Recently in AML cell lines e forced expression of miR-29a and -29b induced apoptosis as well as inhibit cell growth by downregulating Mcl-1 protein [217]. This data shows miR-29 role as a TS-miRNA and provide us a basic to use synthetic miR-29 as a novel therapeutic in the case of AML. miR-124 and miR-203 remains downregulated by hypermethylation in HCC which restores these miRNA by mimics significantly reduced the cell proliferation in all the HCC cell line tested. miR-124 transfected cell shows reduced CDK6, SET and MYND domain containing 3 (SMYD3), vimentin (VIM), and IQ motif containing GTPase activating protein 1 (IQGAP1) at protein level and ATP-binding cassette, subfamily E, member 1 (ABCE1) and the protein level of CDK6 were decreased in miR-203 transfectants [218]. But their miRNA-mimic oligonucleotides have flaws that it has a transient effect. It is not stable, and it requires repetitive deliveries. Liang et al. reported miR-155 targets CXCR4 in MDA MB 231 and silenced it. This gene interacts with SDF-1 and helps in the phosphorylation of Akt. The CXCR4 gene was completely silenced by the miRNA which cannot be reversed by additionally adding CXCR4. These data shows the effectiveness of synthetic microRNA mimics as therapeutics [219]. In another cancer miR-26a which is predominantly present on average level remain downregulated in human and murine liver tumours. It directly targets downregulated cyclinD2 and E2, inducing G1 arrest in human cancer cells *in vitro*. A construction of miR-26a in scAAV vector system has been shown an improvement in the tumour treatment [220]. But their miRNA-mimic oligonucleotides have flaws that; it has a transient effect, it is not stable, and it requires repetitive deliveries.

ANTI-microRNAs 2'-*O*-Methyl Anti-microRNA Oligonucleotides

The premier and simplest way of oligonucleotides modification is 2'-O-methyl (2' OM) group addition. This group contributes to nuclease resistance and improve the binding affinity to RNA. This has been used earlier to knock down miR-125b in prostate

and cervical cancer cell line. The cell lines show reduced rate of proliferation [221]. In a library of 2'-O-methyl anti-microRNA oligonucleotide (OMe-AMO) inhibitors was used for screening and identifying of miRNA which are responsible for cell proliferation and apoptosis. It was validated by luciferase assay using vector barring the miRNA target site [222]. It can target and knock down miRNA with some limitation. First, a direct procedure to ensure the decrease of miRNAs is hard because it binds to the miRNA and sequester it from its target other than the degradation. Therefore, the only method to ensure the decrease in a number of miRNAs is to measure the level of expression of a reporter gene containing the target sequence of the miRNA. In addition, adding back miRNA in the presence of the 2'-O-methyl antisense oligonucleotide cannot rescue the knockdown phenotype. The most important drawback is that a single miRNA can target hundreds of gene so knocking down of a miRNA can cause off-target effect broadly in miRNA therapeutics [223].

2'-*O*-Methoxyethyl Anti-microRNA Oligonucleotides

2'-O-methoxyethyl (2'-MOE) groups of oligonucleotides possess higher affinity and specificity toward RNA than OME analogues. Esau et al. showed that a panel of 86 miRNAs in preadipocytes can be inhibited by using 2'-O-methoxyethyl anti-microRNA oligonucleotides (MOE-AMOs) which inhibit adipocyte differentiation [224]. The comparison study for miRNA expression profile in adipocytes (differentiated versus non-differentiated) showed that miR-143 was involved in the differentiation by regulation of ERK5 protein. Transfection with MOE-AMO, complementary to miR-143 successfully inhibited the process compared with the miRNA negative control [224].

The Locked Nucleic Acid Antisense Oligonucleotides

Locked nucleic acid (LNA) of antisense oligonucleotides has been successfully used to inhibit miRNA in different cancer cells. Knockdown of miR-21 using locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) shows effectiveness in breast cancer and reduced tumour growth [225]. Targeting miR-21 by LNA in glioblastoma and breast cancer cells which suppressed the cell growth and enhanced the caspase activity of cells [226, 227]. In spite of these recent developments, the effective and safe approaches for therapeutics miRNA still remain challenging for human trails.

microRNA Decoy

Recent findings have undergone a revolution that an endogenous miRNA can be saturated by the vectors expressing miRNA target sites thwarting the downregulation of its natural target. This new technology has been named as decoy [228], sponge [229], eraser [230], and antagomiR utilizing various gene delivery systems incorporating plasmids and vectors based on adenoviruses, retroviruses, and lentiviruses [231]. High vector copy, active promoters, or stable transcript can facilitate in overexpression of a miRNA target. The pitfalls of antagomiR approach can be improved by using the sponge strategy in several ways. Primarily, the most powerful tool to identify miRNA and its function is through gene knock out approach, but these knockouts are very time-consuming and are limited to studies on mice. Secondly, maximum miRNA genes are present in functional protein-coding genes creating instability, and thirdly an effect of a miRNA family can be repressed by a single member due to the possession of same seed sequence. When the decoy vectors are based on lentiviral vectors they can efficiently antagonize a miRNA in the absence of multiple administrations just the reverse of oligonucleotide-based miRNA knockdown. A recent stable miRNA sponge strategy was initialized by Valastyan et al (2009) to inhibit miR-31 in vivo in non-invasive MCF7-Ras cells using retroviral miRNA sponges that carried miR-31 recognition motifs in their 3' UTRs. This miR-31 sponge diminished the function of miR-31, 2.5 times without affecting the activity of other known anti-metastasis miRNAs [232]. This advancement in technique allowed the visualization of the ability of nonaggressive breast cancer cell to metastasize. But this decoy vector method also has some restrictions. The target genes often when overexpressed could be highly toxic for the cells and also the determination of the degree of miRNA inhibition mediated by a sponge vector is very uncertain and challenging. Therefore, gene-based knockout are approvable to detect a complete failure of miRNA activity [233].

1.4 Motivation

In this new era, epigenomics has taken the central character of the scientific research field. In the last decade, the extensive work has been done in this area. Groundbreaking discoveries in this area opened a new avenue for cancer research. The reason for aberrant expression of genes during diseases like cancer enticed lots of unanswered queries which are now solved by the epigenetics. But still, the lots of dark unsolved problem remain hidden in this mysterious side of the cell. To endeavor these questions, this research has been stated. Here it was trying to correlate the path of different epigenetic modification and resolve the reason of aberration of epigenetic marks.

Chapter 2
Literature Review

2 Literature Review

Epigenetics at the molecular level is mainly classified into two major classes DNA methylation and histone modifications. The current research added the non-coding RNAs in this field of epigenetics due to its role in gene regulation without hampering the DNA sequence. For cellular gene expression these three mechanisms regulate each other but the connections among them were not well studied. From this point of view this thesis is embedded with the correlation between miRNA functions and other epigenetic mechanisms events

2.1 miRNA meets other epigenetics modifications

miRNA itself is consider as an epigenetic tool which plays a significant role in regulating other epigenetic modifications like DNA methylation and Histone modification. On the other way, DNA methylation is a crucial factor for miRNA gene regulation. In the next three subsequent sections, we focus the connection between the epigenetic modifiers, miRNA and chromatin modifications.

2.2 DNA methylation and its regulation by miRNA

In the new era of epigenetics, the most comprehensively studied modification is probably DNA methylation in mammals. The methylation occurs in the cytosine residue of the CpG sequence [234-237]. In the human genome, there is unevenly distributed short stretches of CpG dinucleotides known as ‘CpG islands'. Most of the CpG islands are located at the 5′ end of the genes and conquer about 60% of the gene promoters [236-238]. Methyl layer in DNA is occupied by MBD causing silencing of transcription [235, 237, 239]. Methylation represses gene expression, mainly by preventing or supporting the regulatory transcriptional proteins, activators, or repressors respectively by binding of MBD with the methylated DNA. In this scenario, the MBD proteins get access to the DNA after the removal of acetyl groups by histone deacetylases [9, 240]. It has been discussed earlier in this thesis. DNMT1 maintains the DNA methylation signature after replication by methylating the hemimethylated daughter strands of DNA; DNMT3A and DNMT3B act as *de novo* methyltransferases to generate replication-independent new methylation patterns [10, 241].

The DNMT family is regulated by different kinds of miRNA. It has been demonstastrated that miR-29 family regulates the DNMT3A and DNMT3B [242]. Overexpression of miR-29b decreases the expression of DNA methyltransferases

DNMT1, DNMT3A and DNMT3B in both RNA and protein level. This leads to the global hypomethylation of DNA, but this upregulates some gene in acute myeloid leukaemia (AML) cells. In AML, miR-29b directly target the 3' UTR DNMT3A and DNMT3B downregulates their expression, whereas for DNMT1 it targets its transactivator Sp1and reduces its expression [217] (Fig 2.1). In colorectal cancer, DNMT1 is over-expressed and plays a crucial role by hypermethylated tumour suppressive genes like ADAM23, Hint1, RASSF1A, and RECK. MiR-342, which is found down-regulated in colorectal cancer directly, targets 3'UTR of DNMT1. Overexpression of miR-342 prevents the proliferation of cell and demonstrates inhibition of tumour invasion and metastasis in lung carcinoma in nude mice [243]. In human, glioma 524 hypermethylated and 104 hypomethylated regions were identified, among which 216 hypermethylated and 60 hypomethylated regions were of known gene promoters, whereas promoters of only eight genes (ANKDD1A, GAD1, HIST1H3E, PCDHA8, PCDHA13, PHOX2B, SIX3, and SST) were reported to be hypermethylated in initial stage of glioma. Overexpression of miR-185 reduced the global methylation pattern in glioma by directly targeting DNMT1 [244]. DNA methylation not only plays a role in cancer but is also reported to be associated with other diseases, such as Systemic lupus erythematosus, a complex autoimmune disease which is caused by genetic and epigenetic disorders. MiRNA controls the gene expression by regulation of methylation by targeting DNMT1. Overexpression of miR-21 and miR-148a in $CD4^+$ T cells enhanced hypomethylation by repression of DNMT1 in both patients with lupus and lupus-prone MRL/LPR mice [245]. In Scleroderma endothelial cells, the alteration of miRNA significantly changes the expression of DNMT1. Up-regulation of miR-152 reduced the expression of DNMT1 at mRNA and protein level while nitric oxide synthase 3 (NOS3) gets highly expressed. However, it was importantly noted that the reduced level of miR-152 expression improved DNMT1 expression with the reduction of NOS3 [246]. In breast cancer, miR-194 regulates the expression of tumour suppressors, such as cyclin G2, p27Kip1, and ADAM23 by regulating DNMT3A. Studies show that in breast cancer cell line and patients, the level of miR-194 is related inversely to DNMT3A mRNA or protein level. MiR-194 inhibits breast cancer cell motility without modifying the cell cycle distribution. The results have been supported by breast cancer tissue array, quantitative real-time PCR, and immunohistochemical staining data [247]. Recent work describes that miR-143 directly regulate DNMT3A by binding with the 3′UTR of DNMT3A in colorectal cancer [248]. The different splice variants of DNMT3b have a conserved region in the target site

of miR-148. This region is conserved in splice variants DNMT3b1, DNMT3b2, and DNMT3b4, but not expressed in variant DNMT3b3. miR-148a regulates DNMT3b1, but it does not control the expression of DNMT3b3 due to the lack of target sequence. This example reveals the importance of splice variant and miRNA specificity in gene regulation [249]. DNA methylation is an inseparable part of the epigenetic regulation. MiRNA regulates the DNMTs and maintains the cellular homeostasis of gene expression.

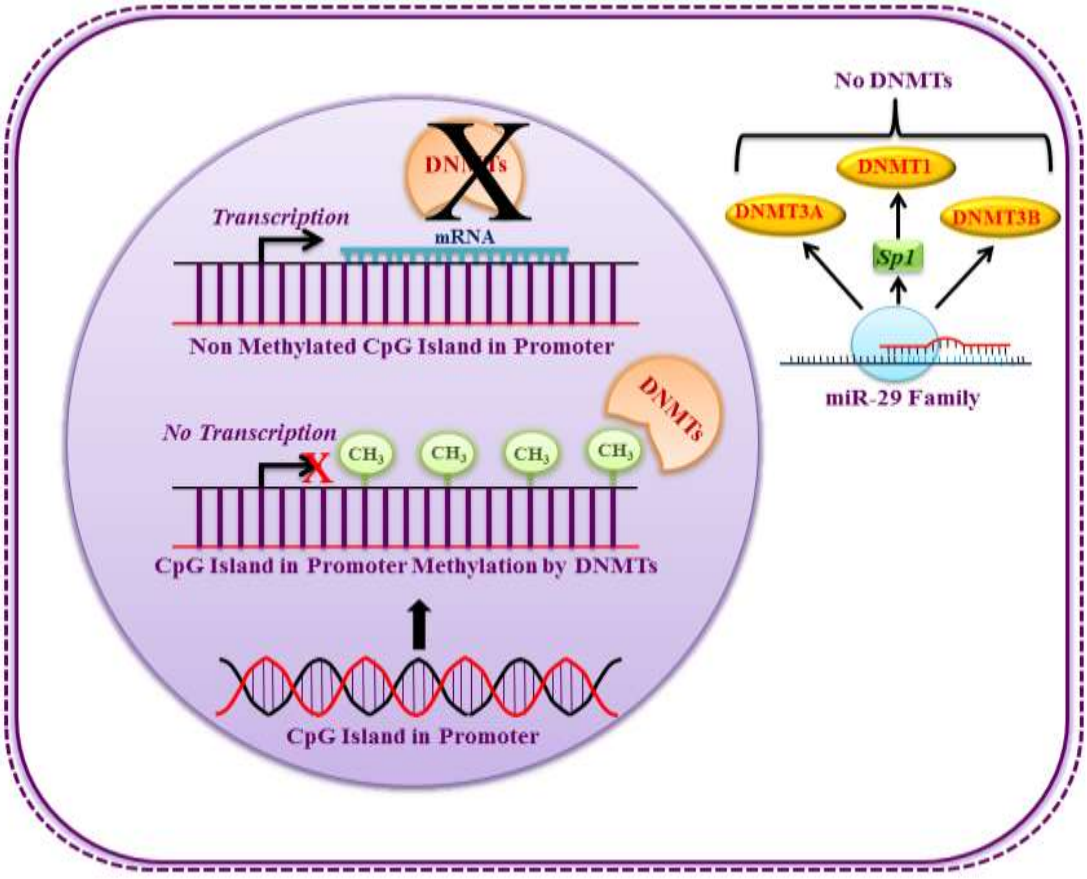

Figure 2.1: miR-29 family regulates the DNA methyltransferase (DNMTs) expression: DNMT mediated DNA methylation repress transcription. MiR-29 family blocks translation of DNMTs by directly interacting with DNMT3A and DNMT3B mRNAs and indirectly affects DNMT1 mRNA, which results to passive demethylation of CpG island. Unmethylated CpG island induced transcription by RNA polymerase and other transcription factor binding at promoter region. Hence, miRNA indirectly induced expression by inhibiting methylation of various genes.

2.3 miRNA gene regulation by DNA methylation

The expression of miRNA in colon cancer cell line HCT 116 and its derivative, which has a double, knock down of DNMT1 and DNMT3B was monitored. It demonstrated that about 10% of miRNA expression is regulated by DNA methylation, and partial reduction of DNA methylation cannot re-express the miRNA. miR-10a, miR-200b, miR-222, and miR-130a were upregulated in the double knockdown cell line [250]. From

the analysis of the miRNAs methylation signatures in cancer metastasis and from different cancer cell lines it was confirmed that miR-148a, miR-34b/c, and miR-9 can be reactivated in cells by using 5-aza-2′-deoxycytidine, which is the inhibitor of DNA methyltransferase [251]. miR-34b/c is mostly downregulated in multiple myelomas due to epigenetic repression by promoter methylation [252]. miR-203 which is a tumour suppressive miRNA is inactivated in hematological malignancies by hypermethylation in the promoter region of the gene of miR-203 [253]. Another tumour suppressive miRNA also get repressed in different hematological cancer and solid tumour formation by methylation. miR-124-1 in many samples of haematological cancers, including acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML), chronic lymphoblastic leukaemia (CLL), multiple myeloma (MM), B-cell, T-cell, and NK-cell NHLs [254] get repressed by hypermethylation. miR-29a methylation in Anaplastic large cell lymphoma leads to progression of cancer [255]. Philadelphia-negative (Ph-ve) myeloproliferative neoplasms (MPNs) comprise of more CpG island methylation of miRNA gene than normal cells. Homozygous miR-34b/c methylation is the cause of its repression in those cells. miR-34a, miR-124-1 and miR-203 are also down-regulated by hypermethylation of promoter of the respective miRNA genes in Ph-ve MPN's cells [256] . Sometimes oncogene expression is enhanced by repression of miRNA. Oncogene MLL-AF4 expression is enhanced by methylation-mediated repression of miR-143. The miR-143 expression is epigenetically repressed by promoter hypermethylation in MLL-AF4-positive primary blasts and cell lines, but not in normal bone marrow cells and MLL-AF4-negative primary blasts, which was directly associated with expression of the MLL-AF4 oncogenes [257]. From this, it is apparent that all the epigenetics modifications are inter-related with others modifications. Epigenetic modifications not only directly regulate gene expression of cell but also it indirectly affects cellular gene expression by modifying other modifications. Such studies in both breast and prostate cancers are lacking so here this thesis try to focus on this aspect of epigenetics.

2.4 Histone modification and its regulation by miRNA

The histone coding pattern is regulated by enzymes that add or remove covalent modifications in a specific position of histones. Addition and removal of acetyl group are done by enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively, whereas methyl group addition or removal is done by histone

methyltransferases (HMTs) and histone demethylases (HDMs) [258, 259]. Several histone modifiers work together with DNA modifier for epigenetic regulation of genes like different HMTs including G9a, SUV39H1, and PRMT5, which can directly recruit DNMTs for methylation in specific gene promoter for stable silencing [260, 261]. DNMTs can also recruit HDAC and methyl-binding protein for chromatin condensation and gene silencing [262, 263] for example H3K9 methylation which leads to heterochromatin formation can be directed by MeCP2 recruited by DNA methylation [264].

Histone modifiers and microRNA (miRNA) make a networking circuit to control various gene expressions. In some cases, miRNA regulates gene expression by regulating the histone modifiers, whereas in other cases they both work together and control gene expression. In various cancers, HDACs are overexpressed which promote cell proliferation and help them in escaping apoptosis. It has been demonstrated that miR-449a was down-regulated in prostate cancer tissue, but when miR-449a was introduced into the cells, apoptosis, cell-cycle arrest, and senescence were enhanced. Further analysis proved that HDAC 1 is a putative target of miR-449a which is frequently overexpressed in cancer [265]. miR-206 and miR-9* is hypothesized to regulate histone acetylation by targeting HDACs and HATs. In Waldenstrom Macroglobulinemia (WM) cells having over-expressed miR-206 and down-regulated miR-9* are characterized by imbalanced expression of HDACs and HATs that leads to deacetylation in H3 and H4 by increased activity of HDAC. Restoration of miR-9, it downregulates HDAC4 and HDAC5 and promotes acetylation [266]. In lung cancer, HDAC1 has been found to be overexpressed which plays a crucial role in tumourigenesis. It is seen that the down-expression of miR-449a/b is one of the main reason for that. miR-449a/b directly bind with the untranslated region of HDAC1 and down-regulates its expression thus inhibiting cell growth and anchorage-independent growth of cancerous cells [267]. Alteration in C_{18} ceramide generation by repression of ceramide synthase (CerS1) results in drug-resistant tumour cells. HDAC1 and miR-574-5p are the key controller of CerS1 gene. The promoter region of the gene is repressed by HDAC1 through inhibition of the transcription factor Sp1, which is recruited in the GC box region and miR-574-5p targets the 3'UTR region of the mRNA to degrade it. In this scenario, HDAC and miRNA work together to control the gene expression [268]. Human pathogenic Kaposi's sarcoma-associated herpesvirus (KSHV) targets the primary endothelial cells (LECs) and induces the expression of miR-132 which regulates antiviral immunity by targeting p300 transcriptional co-activator. Inhibition of miR-132 re-establishes p300 expression that helps in innate antiviral

immunity [269]. In murine myogenic C2C12 cells, miR-26a induces creatine kinase activity which is evidently increased during myogenesis. Using *in-silico* approaches, it has been found that miR-26a targets the 3′ UTR of histone methyltransferase, *Enhancer of Zeste homolog 2* (*Ezh2*) and down-regulates it [270]. In prostate cancer cells, miR-101 inhibits Ezh2 expressions. The expression of miR-101 is also regulated by the androgen treatment and HIF-1α/HIF-1β induction [213] (see Fig-2.2 and Table-2.1). It is now well proved by scientists that deregulation of miRNA in cancer is also caused by epigenetic modification. Regulation of miRNA in cells attained a great attraction for scientists for understanding the epigenetic phenomena of controlling miRNA expression.

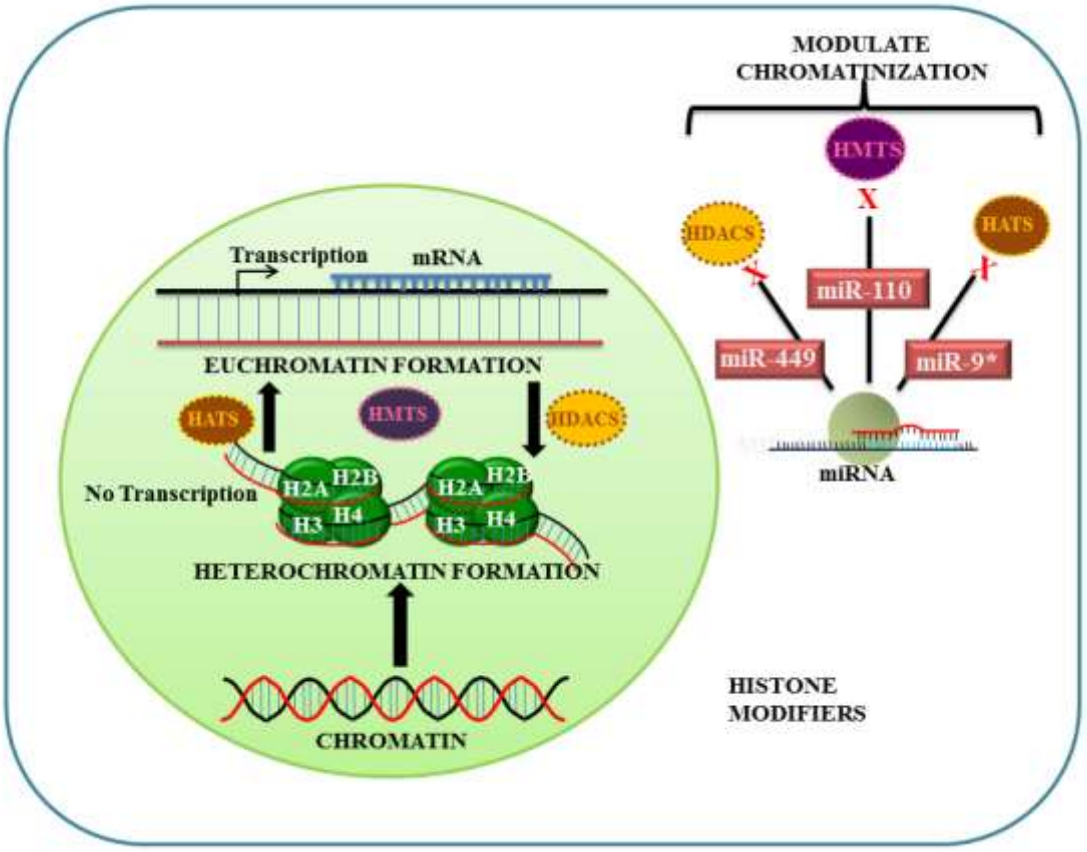

Figure 2.2: miRNA-mediated degradation of histone modifiers and transcriptional control: HATs, HDAC, HMT's and HDM's control gene transcription and heterochromatinization by different histone modifications. miRNA repress the expression of histone modifying enzymes. miR-449a, MiR-206 inhibits HDAC expression, miR-9*, miR-132 control HAT expression. miRNA arbitrated repression of HDAC, specific HMT (H3K9 methyltransferase, H3K27 methyltransferase) and specific HDM induce transcription. miR-110 inhibits the expression of HMT's (Ezh2) in the different cancer cell. The miRNAs regulate chromatinization by modulating the chromatin modifiers

Table 2.1 Regulation of epigenetic modifiers by miRNA

Name of microRNA	Type of cancer/cell	Targeted gene	Regulation	Reference
miR-342	Lung	DNMT1	Downregulated	[243]
miR-185	Glioma	DNMT1	Downregulated	[244]
miR-21	$CD4^+$ T Cells	DNMT1	Downregulated	[245]
miR-148a	$CD4^+$ T Cells	DNMT1	Downregulated	[245]
miR-152	Scleroderma Endothelial Cells	DNMT1	Downregulated	[246]
miR-194	Breast	DNMT3A	Downregulated	[247]
miR-143	Colorectal	DNMT3A	Downregulated	[248]
miR-148	Embryonic Stem Cells	DNMT3B	Downregulated	[249]
miR-29b	AML	DNMT3A, DNMT3B,	Downregulated	[217]
miR-449a	Prostate Cancer	HDAC 1	Downregulation	[265]
miR-206	Waldenstrom Macroglobulinemia	HATs	Downregulation	[266]
miR-9*	Waldenstrom Macroglobulinemia	HDAC4 HDAC5	Downregulation	[266]
miR-449a/b	Lung Cancer	HDAC1	Downregulation	[267]
mR-132	Primary Endothelial Cells	p300	Downregulation	[269]
miR-26a	C2C12 Cells	*Ezh2*	Downregulation	[270]
miR-101	Prostate Cancer	Ezh2	Downregulation	[213]

2.5 miRNA regulation of Chromatin remodelling

The mystery of cellular functions is hidden behind the wrapping of a 2 m long DNA in every cell. This topologically impossible task is made possible by chromosomal remodelling. It comprises of a dynamic balance between the genomic accessibility and packaging. This intensive packaging is done by histone proteins and histone deposition, removal, and modification by a different subset of enzymes. These sets of proteins also

work in chromatin remodelling complexes [271]. These remodellers use the energy of ATP hydrolysis for opening, removing or reconstructing the nucleosome, the basic unit of chromatin [272, 273]. The chromatin remodeller complexes have been divided into four families. These complexes are different in the context of their domain, active site, and specialized purpose of remodeling, but they all have five basic properties. (a) They have more affinity for the nucleosome than DNA. (b) These remodeller complexes have a covalent histone modification recognition domain. (c) A DNA-dependent ATPase domain, which helps to break the DNA–histone contact. (d) A regulatory domain which controls the ATPase activity. (e) Domains for interaction with other chromatin or transcription factors. Together, all these common properties permit the nucleosome for its selection, remodelling, and engagement. Although the remodeler families share common properties but depending on their unique flanking domain, they have been grouped [274]. Individually every family of protein complexes shares conserved regions from yeast to human, although slight variations have been found in protein sequence in deep protein sequencing. SWI/SNF family is composed of 8 to 14 subunits. In eukaryotes, SWI/SNF family remodeler consists of two related catalytic subunits. One is ATPase domain containing helicase-SANT and post helicase-SANT, and another is bromodomain. The main function of this family is to slide and eject nucleosome from many loci. But this complex does not help in chromatin assembly [275]. ISWI family of remodeler contains 2 to 4 subunits. This family primarily promotes nucleosome assembly and represses transcription, whereas some complexes like NURF helps in RNAPII activation [276]. The CHD (chromodomain, helicase, DNA binding) family of remodelers consist of 1 to 10 subunits. CHD remodelers enhance transcription by sliding or ejecting nucleosome [277]. The INO80 (inositol requiring 80) family remodelers contain more than 10 subunits. This family of remodeler has various functions including DNA repair and transcription. It has a unique ability to reconstruct the nucleosome by removing canonical H2A-H2B dimers with H2A.Z-H2B dimers [278]. The recent intensive study shows miRNA regulation of chromatin remodeler complexes in different cancer. miR-221 binds in the 3'UTR of the CHD5 protein and down-regulates its protein expression in colon cancer cell line [279]. miR-9* and miR-124 promotes the replacement of SWI/SNF subunits, Baf45a, and Baf53a with Baf45b and Baf53b in mice which helps in post-mitotic differentiation in neurons [280]. Another miRNA miR-84 promotes regulation of development in *Caenorhabditis elegans* by regulating SWI/SNF proteins [281]. miR-99 family targets the SWI/SNF chromatin remodelling factor SNF2H/SMARCA5, which is a component of the

ACF1 complex [282]. Therefore, chromatin remodelling by miRNA gives us an overview of the direct and indirect intrinsic role in their genetic expression.

2.6 *In-silico* Identification of miRNAs that targets DNMT1 and MLL1 using miRNA target prediction algorithms

In animal system miRNAs are predominantly target the 3' untranslated regions (3'UTRs) of respective target genes. Occasionally, it also targets the 5' UTR ORF or promoter region of the gene [283] . The actual prediction of miRNA-mRNA interactions in the animal system remains challenging due to the complexity and incomplete knowledge of the principles of these processes. Hence, it is important to take advantage of the newest findings in miRNA biology and their targets prediction algorithms to find possible miRNA-mRNA interactions. In recent years various target prediction algorithms were developed exploring different approaches, and many methods were developed to experimentally validate them**.** The target sites of the miRNA in animals are not evenly distributed within the 3′ UTR rather they tend to cluster at both ends of the 3' UTR [284] . In the case of a gene having short 3' UTR, it remains in the 5' part of the 3' UTR [285]. There are also some miRNA having multiple target sites of the same miRNA [135, 286, 287] . Multiple target sites enhance the degree of degradation of mRNA [288], and many target prediction algorithms use this feature for searching and scoring. If two miRNA same or different, target sites are located closely they can act synergistically [289] . It can be surely said that no single model can predict all miRNA-mRNA interactions due to their relative heterogeneity. The classification of defining miRNA targets is based on complimentary sequence within the 5' and 3' of miRNA and three types of sites: (1) canonical, (2) 3'-supplementary and 3) 3'- compensatory sites [124]. The canonical site is known to have a complete paring within the seed region which determines the certainty of the interaction. There are three types of canonical sites [290] : the 7mer1A, that has an adenine in position 1 at the 5' end of miRNA, the 8mer having matched adenine in position 1 and an additional match in position 8 and the 7mer-m8 that has a match in position 8. Most of the validated conserved targets are 7mer canonical sites which are highly conserved for miRNAs [146]. But the degree of gene silencing is higher with the adenine opposite position 1 of miRNA [291] . There are also known sites with shorter, 6-nt seed, but they are thought to have limited functionality. All of these groups can have an additional pairing within 3′ part of miRNA and corresponding nucleotides of the transcript (3'-supplementary sites), but it usually has a less profound effect on target recognition and

its efficacy [287]. The effectiveness of miRNA-mRNA target predictions depends on at least 3-4 nucleotides consecutively paired in positions 13-16 of miRNA. Sometimes a mismatch in the seed can be compensated by additional extended pairing in 3′ part of miRNA (3′-compensatory sites). These algorithms not only depends on miRNA-mRNA sequence matching, it also takes account for the orthologous sequences alignment, UTR context or free energy of complexes' for efficient target prediction [134]. It restricts their search in conservative sequences of human transcriptome with comparatively evolutionally distant species such as the mouse, the dog or the fish. It was based on the presumption that the target sites of miRNA will be kept unchanged by evolutionary force [146, 292]. MiRNA families are also highly conserved between closely related species, having many conserved targets [290].

In this scenario, various algorithms were used to find miRNA which can efficiently target DNA and histone H3 modifications in cancer. DNA methylation is the key epigenetic mechanism which facilitates cell-specific gene regulation [293]. In other hand, MLL1 is a methyltransferase which methylates H3K4 and regulates gene expression [294]. DNMT1 works as a repressor of a gene and MLL1 work as an activator of genes. In cancer, both marks remain dysregulated and cause aberrant gene regulation. From this point of view, miRNA which can target DNMT1 and MLL1 in cancer were identified using *in-silico* approaches.

2.6.1 miRanda algorithm predicted miRNA targeting DNMT1 and MLL1

This algorithm compares the miRNAs complementarity to 3' UTR regions of the mRNA [295]. The final results depend on the binding energy of the duplex structure, the conservation of the whole target site and it's positioning within the 3' UTR. Then the algorithm calculates the weighted sum of the match and mismatch scores based upon the base pairs and gap penalties. It doesn't allow the wobble pairing in the seed region which is compensated by the matches at the 3' end of miRNAs. It promotes the miRNAs with multiple binding sites within the 3' UTR which help to increase the specificity, but it underestimates the miRNA with single but perfect base pairing. It takes into account the evolutionary relationships of interactions more globally focusing on the conservation of miRNAs, relevant parts of mRNA sequences, and the presence of a homologous miRNA-binding site on the mRNA.

For identifying the potential miRNA that target DNMT1 and MLL1 miRanda miRNA target identification tools were used. This algorithm, it predicts 15 potential miRNAs.hsa-miR-185, hsa-miR-148a, hsa-miR-148b, hsa-miR-152, hsa-miR-374b, hsa-

miR-342-3p, hsa-miR-448, hsa-miR-153, hsa-miR-217, hsa-miR-155 207, hsa-miR-377, hsa-miR-379, hsa-miR-410, hsa-miR-140-5p, hsa-miR-340 which can be used for down regulating DNMT1. Then the target sequence was analyzed for the conserved region of DNMT1 3′ UTR. All the data were tabulated in this chapter. From the list, those miRNAs were picked which target the conserved region of DNMT1 with the highest score predicted more than others by this tool. Using this algorithm, four miRNA hsa-miR-185, hsa-miR-148a, hsa-miR-148b, hsa-miR-152 are fulfilling the criteria. For MLL1 it predicts more than 50 miRNAs. From the highest score and binding energy, three miRNAs were chosen for targeting MLL1, which are miR-148a, miR-152, and miR-193a.

2.6.2 Targetscan algorithm predicts miRNA targeting DNMT1 and MLL1 mRNA

These algorithms [290] use different approach to the prediction of interactions of miRNAs with mRNAs. Its search for the full complementarity in the miRNA seed region, defined as 6-nt long (nucleotides 2-7) and then it extends search up to 21-23 nucleotide-long fragments representing effective interactions. Then the outcome is classified into three groups on the basis of the exact matching of the length and occurrence of the adenine base at the first position of mRNA target site which seems to be evolutionarily conserved that act as an anchor for the RISC complex. Numerous parameters were identified in signal –to-noise outcomes based on the results of experimentally validated datasets. It considers seed matching, pairing contribution outside the seed region, AU content 30 nt upstream and downstream of predicted site and the distance to the nearest end of the annotated UTR of the target gene. The fundamental importance of the outcome score depends on the conserved miRNA binding region among 3′ UTRs seed region. The newest version also predicts less conservative miRNA-mRNAs interactions with wobble pairings and bulges, especially within 5′ region of miRNA [296].

Targetscan algorithm was used for identification of potential miRNAs which targets DNMT1 and MLL1. This prediction tool uses the complementary region between miRNA and mRNA for prediction miRNAs. Using this tool, 3 highly conserved miRNA having seed region in the DNMT1 and MLL1 3' UTR was predicted. For DNMT1 all three miRNA has 8 mer binding sites in the UTR but for MLL1 only 1 miRNA-binding region was 8 mer and others are 7 mer. Those miRNAs also target the conserved region of DNMT1 and MLL1, which make these miRNA more potential target of DNMT1 and MLL1 in mammals. For DNMT1 the miRNAs which were further analyzed are miR-148a miR-152, miR-148b and for MLL1 miR-193a, miR-148a, miR-193b.

2.6.3 DIANA-microT algorithm predicted miRNA targeting DNMT1 and MLL1

This algorithm [36] predicts target by using a 38 nt-long frame which moves along the 3′ UTR and analyses the minimum energy of predicted miRNA binding and mismatches. Then after every shift, it measured and compared the energy of 100 per cent complementary sequence bound to the 3′ UTR region with the predicted duplex. It searches for 7-9 nt long complimentary sites in 5'region of the miRNA with the canonical central bulge. It also considers 6 nt complimentary with 1 wobble pairing if it shows additional pairing in the 3′ region of the miRNA [60]. DIANA-mciroT consider both conservative alignment and considers non-conservative sites for scoring the duplex. It provides a unique signal-to-noise ratio (SNR) a ratio between a total of predicted targets by single miRNA in search 3′ UTR and a total of predicted targets by artificial miRNA with a randomized sequence in search 3′ UTR. It also gives the percentage probability of the presence of the results by its pairing and conservation profile.

This tool predicted more than 27 miRNA targeting DNMT1 3′ UTR. The cut-off value was set at 0.9 to more specific results. These miRNA has more than one binding site in 3′ UTR of DNMT1. From those miRNAs, few miRNAs were used for further work which binds to the conserved region and predicted similarity by different other algorithms. But when MLL1 searched with this algorithm does not show any potential miRNA with higher cut-off score. So when the cut-off score has been lowered, it shows miRNA which has a potential binding site in MLL1 3' UTR. All the results were analyzed and further processed for experimental validation.

From the pools of miRNAs, miR-152, miR-148a, and miR-193a were chosen for further works. On the view of this following objective are decided to execute.

Objectives

Objectives

As demonstrated in the introduction and literature review sections the study of molecular epigenetics is at a juncture where DNA methylation aspect is beginning to settle, histone modification aspects are in progress, and miRNA related phenomenology's are beginning to emerge. Introduction of a new thesis in the field of epigenetics is thrilling, for there are ample scope to decipher the epigenome and interaction of miRNA and chromatin modifiers. In view of the prevailing concepts and lacunae in the knowledge of these fields the objectives of this THESIS are

- How the microRNA, miR-152 gene is regulated and modulates the expression of DNMT1and DNMT1target genes in breast cancer.

- How the microRNA, miR-148a gene is regulated and targets DNMT1and other genes in human prostate cancer progression.

- To decipher the role of miR-193a in regulation of histone modifier MLL1 and a comparison with DNA methylation in prostate cancer

Chapter 3
Objective 1

3 How miR-152 gene is regulated and influences the expression of DNMT1 and DNMT1 target genes in breast cancer

3.1 Introduction

MiRNAs, DNA methylation, and DNMT1 impact gene expression by destroying mRNA, silencing transcription and coordinating DNA – histone crosstalk signalling. Breast cancer is one of the most abundant cancers and leading cause of cancer related deaths in women [297]. In last few decades, it has been reported that breast cancer progression has been coupled with signalling pathways and specific molecular transcriptional processes [298, 299]. To develop new approaches for prevention, detection, and treatment of the disease, we tried to understand the aberrant expression of genes in breast cancer.

Breast cancer cells lacking estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2) are known as the triple-negative breast cancer (TNBC) cells [300-302]. Nearly 20.8% of young breast cancer patients develop TNBC. In most of the cases due to its poor prognosis, it rapidly metastasizes within three years leading to the death of the patient. More than 90% of cancer-related death is due to metastasis. The primary tumours tissues break away from the neighboring cells and detached cells enter the circulation system and invade other distant tissues and organs directly or through lymph nodes. This metastasis process is regulated by both proteins and miRNAs. This is a multistep process initiated by different signalling pathways, causing carcinoma cells to undergo an epithelial–mesenchymal transition (EMT) [303].

miRNAs are small non-coding RNAs consisting of 18-25 nucleotides, regulate gene expression by binding to the 3' UTR region of mRNAs, and subsequently, lead to its degradation [12, 134, 304]. A single miRNA can target hundreds of mRNAs, and eventually down-regulates corresponding protein expression. Regulation of miRNA gene by aberrant promoter hypermethylation is an emerging field [305]. miRNA which actively down-regulates the oncogenes in cells are regarded as tumour suppressive miRNA (TS-miRNA). Although many miRNAs are encoded in the intragenic regions of genes, there are miRNA encoded by the respective genes. Among different extensive ongoing research works, epigenetic modifications in cancer cells is a fascinating one and a challenging topic [306, 307]. DNA hypermethylation of tumour suppressor genes, alteration in histone modifications and miRNA-mediated gene regulation cause the early manifestation of cancer [308]. Recent research elicited that high degree of DNA methylation present in the

CpG islands of miR-152 gene. miR-152 remains down-regulated in breast cancer cell lines in comparison to the immortalized breast epithelial MCF-10A cells [309] . However, the role of miR-152 in cancer progression or regression or cell death is not clearly understood. In gastric cancer, it was illustrated that tumour size and stage depends on the extent of down-regulation of miR-152. Recently, miR-152 gene down-regulation was reported to be associated with tumour stage and lymph node metastasis in breast cancer [310]. miR-152 act as a tumour suppressive miRNA by targeting IGF-IR and IRS1, which leads to inhibition of PI3K/AKT and MAPK/ERK signalling pathways, HIF-1a and VEGF expression in breast cancer. miR-152 regulates various genes, including estrogen receptor α gene by destroying/inactivating DNMT1 mRNA [311]. E-cadherin (CDH1) is a transmembrane glycoprotein that forms the core of adherent junctions between adjacent epithelial cells and linked with actin cytoskeleton [312, 313]. Down-regulation of CDH1 promotes metastasis, enabling the dissociation of carcinoma cells from one another in the tumour. This role of CDH1 has been demonstrated by both *in-vitro* and *in-vivo* experiments [314-316]. We searched databases and have experimentally demonstrated earlier that DNMT1 regulates gene expression by DNA hypermethylation-mediated silencing mechanism in breast cancer [317].

In view of this and to focus a better molecular logic in light of epigenetic tools we attempt to elucidate the correlation of miR-152, DNMT1, and CDH1 function. Results observed in this study, illustrate that, CDH1 downregulation is directly proportional to the suppression of miR-152 function in breast cancer cells. Reduced expression of DNMT1 in terms of mRNA and protein levels were observed owing to upregulation in expression of miR-152, which might be due to loss of DNMT1 mRNA through physical association of miR-152 and mRNA of DNMT1. Recently we have shown that CAV1 gene is regulated by histone modifications irrespective of DNA methylation switch in breast cancer [318]. Therefore, we traced the modification markers on DNA and histone 3 surrounding the promoter DNA of miR-152 gene by ChIP analyses. Stunning results were obtained, implicating that there might be a paradoxical role of DNA methylation immediately adjacent to histone 3 lysine 4 trimethylation (enrichment of H3K4me3) active marks to silence the miR-152 gene. Additionally, when DNMT1 protein function is blocked by application of inhibitor, miR-152 expression prevails and destroys the residual of DNMT1 mRNA. In this condition there will be no DNMT1 protein. Thus DNMT1 target genes will be expressed which was shown here as DNMT1/miR-152 switches for on/off of DNMT1 target genes. Further to this setup, it was defined that DNMT1 downregulation mediated

upregulation of CDH1 (hereafter, DNMT1/CDH1 axis) in the presence of overexpressed miR-152, prevents migration of TNBC cells. Our data provides novel insights into the epigenetic regulation mechanism of transcription of both microRNA and mRNA coding genes and enhances the amplitude of cancer epigenome.

3.2 Materials and methods

3.2.1 Search for miR-152 target

The previous report implicated that miR-152 plays a tumour suppressor role and remains silenced by DNA hypermethylation in endometrial cancer [319]. To understand the potential molecular mechanism of the miR-152 function we searched for the potential target using different databases for miRNA predictions tools such as TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and microRNA.org (http://www.microrna.org/microrna/home.do). Among the approximately 100 targets predicted by the prediction depending on the miRNA binding sites with at least 8 mer seed-pairing, high context++ score percentile (98) [296] and certain free energy for the miRNA binding to be predicted targets of miRNAs we have found that miR-152 potentially targets the 3′ UTR of the DNMT1 mRNA.

3.2.2 Analysis of DNMT1 and CDH1 expression in breast cancer

We have collected information from different databases to analyze the expression of miR-152 in breast cancer tissue samples and to find out the role of miR-152 in cell survival. The effect of miR-152 on cell survival was plotted in Kaplan-Meyers plot. The plot was drawn from miRNA database MIRUMIR [320]. To understand the correlation between DNMT1 mRNA and extent of DNA methylation of genes we explored different online databases. For methylation analysis of genes we extracted data from MethHC database [321] and for correlation between DNA methylation and DNMT1 mRNA expressions, we have used IST online database (http://ist.medisapiens.com/). The stage-specific expression of DNMT1 was also analyzed. The correlation graph between DNMT1 and CDH1 has been established using cBioportal databases (http://www.cbioportal.org/) [322, 323].

3.2.3 Cell Culture

MDA-MB-231, MCF-7, and BT-474 breast cancer cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS), India. The cells were grown in Leibovitz's L-15 Medium, Minimum Essential Media and Dulbecco's modified Eagle's medium (GibcoAuckland, New Zealand,) respectively, supplemented with 10% fetal bovine serum (FBS) (16000–44; Gibco, Carlsbad, CA), and 100 U Penicillin & 0.1 mg Streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

3.2.4 Construction of vectors for luciferase assay

3′-UTRs of DNMT1 containing an entire miR-152 recognition sequence was amplified by PCR from genomic DNA. The PCR product was subcloned into a pcDNA Luciferase Vector (A gift from Dr. Patha Sarathi Ray, IISER-Kolkata, West Bengal, India). A pcDNA luciferase construct containing the mutations in the seed sequence of DNMT1 3′-UTR was synthesized using GeneArt® Site-Directed Mutagenesis System (Catalog number: A13282, Thermo fisher scientific). The wild-type construct containing the seed sequence was "CAUGCACUG". In the mutated constructs, three nucleotides within the seed sequence "CAU***CGU***CUG" were mutated, which was denoted in italics. All constructs were confirmed by sequencing and transfected as previously mentioned procedure.

3.2.5 Luciferase reporter assay

The cells were transiently transfected with 100 ng of reporter plasmids alone or co-transfected with different concentrations of (10nM, 20nM and 30nM) miRNA mimics using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen) in 96 well plates. The activities of reporter genes with Renilla luciferase and the internal standard firefly luciferase were quantified by a Dual-Luciferase Reporter Assay System (Promega). Independent triplicate experiments were done for each plasmid construct. Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

3.2.6 Transfection with miR-152 mimics, DNMT1 siRNA, and DNMT1 overexpression vectors independently and in the desired combination

For analyzing the effect of miR-152 on cell survival and the existence of DNMT1 mRNA, miR-152 mimics (Qiagen) was used. Variable concentrations of miRNA mimics

were used to analyze the effective concentration of miR-152 for transfection. Apparently, 30nM of miR-152 efficiently inhibits the expression of DNMT1 at the protein level. To observe the role of DNMT1, DNMT1 siRNA (DNMTi) (Santa Cruz Biotechnology, Inc.) was used to suppress the expression of DNMT1. To overexpress, DNMT1, pcDNA3/Myc-DNMT1 vector (Addgene) was used. miR-152 mimics, DNMTi, the pcDNA3/Myc-DNMT1 vector, was used to transfect MDA-MB-231 cells along with Lipofectamine 2000 transfection reagent (Invitrogen) and optimem transfection media (Invitrogen). Further, cells were incubated at 37°C, 5% CO_2 for 48 h to carry out different assays. As a control, we used control miRNA mimics, siRNA control, and pcDNA3/Myc vectors.

3.2.7 RNA extraction and quantitative reverse-transcription (qRT)-PCR

Total RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions. qRT-PCR was performed utilizing the cDNA prepared from 1 µg of total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentas) and SYBR® Green JumpStart™ TaqReady Mix in the Realplex4Eppendorf system. The PCR were carried out using standard protocols, and the cDNA was amplified under the following conditions: 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 59.8 °C for 30 s and 72 °C for 30 s. We analyzed DNMT1 and CDH1, after transfection with miRNA mimics, siRNA, and vectors. The mRNA level was normalized to the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. miRNA was isolated by using miRVana™ miRNA Isolation Kit, with phenol (Invitrogen) following manufacturer's protocol. From the isolated miRNA, cDNA of miRNA was prepared using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) followed by analysis using qRT-PCR.
For qRT-PCR of miRNA, primers were designed by Invitrogen miRNA primer designing protocol. The qRT-PCR of miRNA was done by NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen.) The cDNA of miRNA was amplified under the following condition 50^oC for 2 min, 95^oC for 2 min 40 cycles of 95^oC for 15 s, 60^oC for 30 s. Then all the results were interpreted using Eppendorf qRT PCR software. Expression of miR-152 was analyzed by normalizing with the expression of U6 small RNA. All the primers sequences are mentioned in Table 3.1 Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

Table 3.1 Primer list

Gene	Forward Primer	Reverse Primer
DNMT1	CCATCAGGCATTCTACCA	CGTTCTCCTTGTCTTCTCT
CDH1	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAACGCA
miR-152	CCGTCAGTGCATGACAGAACTTGG	Universal Reverse Primer for miRNA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT

3.2.8 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was done using H3K4me1 Rabbit anti-Human Polyclonal Antibody (Invitrogen, 49-1003), H3K4me2 Rabbit anti-Human Polyclonal Antibody (Invitrogen, 49-1004), H3K4me3 Rabbit anti-Human Polyclonal Antibody (Invitrogen, 49-1005), Rabbit anti-Human Polyclonal H3K9me3 (49-1008, Invitrogen), rabbit Anti-Histone H3K27me3 antibody (ab6002, Abcam), and the experiment was performed according to the manufacturer's instructions of Imprint Chromatin Immunoprecipitation Kit (Sigma). PCR and Real-time PCR techniques were used to analyze ChIP precipitated DNA. For RT-PCR (Realplex^{4}Eppendorf) analysis SYBR® Green JumpStart™ TaqReadyMix (Sigma) was used. Sonicated DNA was used as PCR template and analysis with PCR condition 95°C for 2 min, followed by 40 cycles of 95°C for 20s alternating with 58°C for 30s annealing and 72°C extension for 30s. miR-152 5'-AGAGGAGGCCTGTCCTGAGT-3′ (sense) and 5'-CGGGTAGACTCCAGAAGCAT-3' (antisense) and GAPDH 5'-CAATTCCCCATCTCAGTCGT-3' (sense) 5'-TAGTAGCCGGGCCCTACTTT-3'(antisense) were amplified for analysis. Anti-mouse IgG precipitated DNA was used as a template for negative control. Nonspecific antibody (mouse IgG) precipitated DNA was used for normalization. % Input has been calculated by following formulas: ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log_2 (Input Dilution Factor))

Where, Input Dilution Factor = (fraction of the input chromatin saved)$^{-1}$

The default Input fraction is 1% which is a dilution factor of 100 or 6.644 cycles (i.e. log2 of 100). Thus, subtract 6.644 from the Ct value of the 1% Input sample as mentioned in the equation above. % Input = $2^{(-\Delta Ct\ [normalized\ ChIP])}$

3.2.9 Confocal microscopic analysis of DNMT1 and CDH1 expression

For confocal microscopic observations, MDA-MB 231 cells were grown on 18 mm coverslips. Cells were transfected with miR-152 mimics, DNMTi, pcDNA3/Myc-DNMT1 vector; To analyze DNMT1 and CDH1 expression, the cells were then fixed with ice cold methanol for 10 min, permeabilized with 0.1% Triton X-100 in PBS (PBST) and blocked with 1% BSA-PBST for 30 min at RT. For DNMT1 and CDH1 protein localization, cells were incubated with anti DNMT1 primary antibody [Dnmt1 (H-300) sc-20701 Santa Cruz Biotechnology] and anti-E Cadherin antibody [(ab15148)Abcam] at 1:500 dilution in 1% BSA in PBST for 1h at RT, followed by staining with goat anti-rabbit IgG-FITC [goat anti-rabbit IgG-FITC sc-2012, Santa Cruz Biotechnology] for 30 min. After wash, cells were stained with DAPI and dry coverslips mount with ProLong® Gold Antifade Mountant (Thermo fisher) and observed by Laser scanning confocal microscope (Leica) with 63x magnification and digital zoom. Three independent experiments were done to validate the results (mean±S.D, $P < 0.05$).

3.2.10 Western blotting

The cells were grown up to 80–85% confluence and then transfected with miRNA mimics, siRNA, and vectors for 24 hours before harvesting. The cells were then harvested, washed with PBS and lysed using RIPA buffer (Sigma). Then the cells were kept in 4^{o}C for 10 min to complete the cell lysis, and lysate was centrifuged at 8000 g for 10 min at 4^{o}C. Same amount of protein was loaded in 10% SDS-PAGE for electrophoresis. Then separated proteins were transferred onto PVDF membrane. After blocking the membrane with 5% BSA in PBST solution, the membrane was incubated with specific primary antibody [Dnmt1 (H-300) sc-20701 Santa Cruz Biotechnology and anti-E Cadherin antibody (ab15148) Abcam] overnight at 4^{o}C. Subsequently, the membrane was washed three times; 10 min/each time with PBST buffer, and incubated again with an appropriate HRP conjugated secondary antibody [Goat anti-rabbit IgG-HRP sc-2004 Santa Cruz Biotechnology] at 37°C for 2 h. The membranes were washed with PBST buffer and were developed by Supersignal West Femto-chemiluminescent substrate (Thermo Scientific). β Actin protein levels were used as a control for adequacy of equal protein loading. Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$)

3.2.11 Analysis of cell migration by wound-healing assay

For analysis of cell migration, cells were plated onto the 60-mm dish to create a confluent monolayer and incubated at 37 °C, allowing cells to adhere and spread on the substrate completely. The cells grew up to 90% confluence, and then a scratch was made on a uniform layer of cells using a sterile micropipette tip. The debris was removed, and the edge of the scratch was smoothed by washing the cells once with 1 ml of the growth medium and then replaced with 3 ml of medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition markings were created to be used as reference points close to the scratch. The reference points can be made by etching the dish lightly with a razor blade on the outer bottom of the dish or with an ultra fine tip marker. After the reference points had been made, the plate was placed under a phase-contrast microscope, and reference mark was left on the image capture field but within the eyepiece field of view. The first image of the scratch was taken at 0 hours. The dish was incubated 24 h for different concentration of miR-152 mimics. After the incubation, the dish was placed under a phase-contrast microscope; the reference point was matched; the photographed region was aligned to acquire the second image. Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

3.2.12 Chromatin condensation assay

After treatment with different concentration of miR-152 mimics for the specific time, cells were stained with Hoechst 33342 stain (1 mg/ml) and incubated for 10 min at 37 °C. Images were observed under ~460 nm emission of Hoechst 33342 dye using Epifluorescent Microscope (Olympus IX71). Three independent experiments were done to calculate P value and validate the results (mean±S.D, $P < 0.05$). In each condition, 5000 cells were counted to analyze the result.

3.2.13 Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using the Student's t- test by SPSS software. Values of $P < 0.05$ were considered as significant value.

3.3 Results

3.3.1 Database screening for expression profile of miR-152, DNMT1, and CDH1 in clinical samples and cell lines

To test the impact of TS-miR-152 in cell survival, we have searched the expression pattern of miR-152, DNMT1, and CDH1 in breast cancer database. We have analyzed the miR-152 level and survival rate from those breast cancer samples by Kaplan-Meir survival analysis. We find that the high expression of miR-152 is correlated with increased survival of patients (Fig.3.1 A).

Thereafter, we have analyzed the miR-152 level in Luminal A, Luminal B, and basal subtype cell lines, MDA-MB-231, which is an invasive TNBC cell line. It is apparent that miR-152 was significantly downregulated in breast cancer tissues and in all the three cell lines. The extreme level of down-regulation is noticed in MDA-MB-231, which is critical for breast cancer progression. Extent of downregulation of miR-152 was 1.3, 2.1and 5.4 folds MCF-7, BT474 and MDA-MB-231 cell lines respectively, keeping HaCaT cell line as control (Fig.3.1 B).

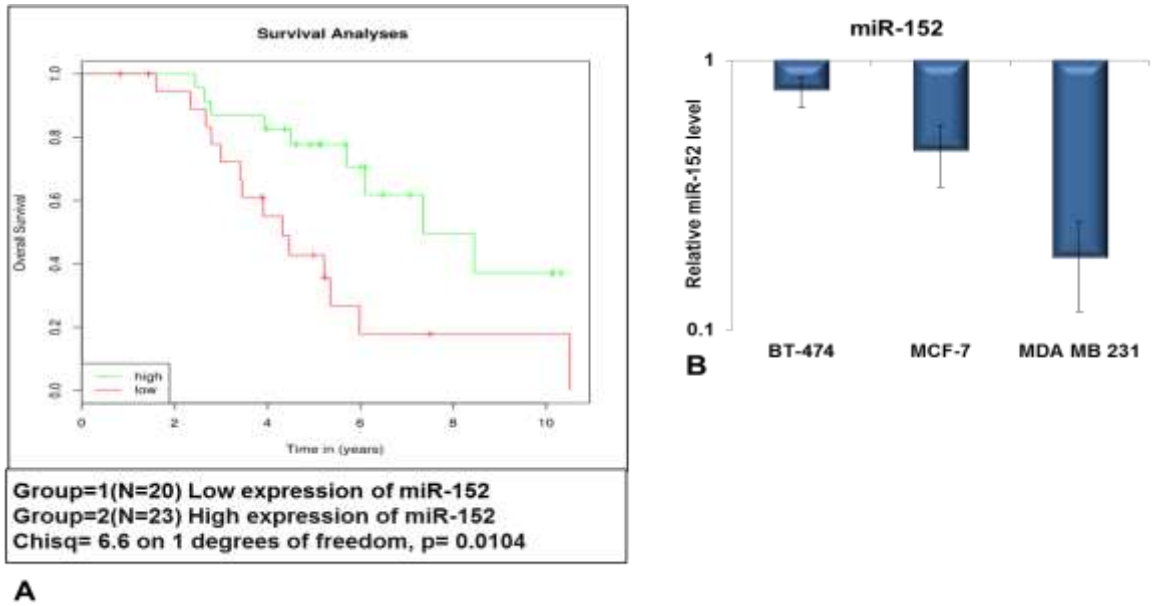

Figure 3.1: miR-152 expression and breast cancer development:
[A] Overall survival was analyzed by Kaplan–Meier plot. [B] miR-152 expression level in BT-474, MCF-7 and MDA MB 231 cell lines compared to HaCaT cells, (n=3 independent experiments, mean±S.D.), $P < 0.05$.

3.3.2 Poor prognosis is associated with higher DNMT1 expression

To define the role of DNMT1 in breast cancer according to the current objectives we have analyzed DNMT1 mRNA expression status from the clinical samples utilizing Genesapience database. The results implicate that DNMT1 expression is highly stage-specific as breast cancer progresses. It is also noticed that trend of DNMT1 expression increases as breast cancer progresses from stage 1 to stage 3 (lower grade to higher grade). We have analyzed further that, although the expression of DNMT1 increases with the grade of cancer, it exhibits differential expression pattern in basal, Her2, Luminal A and Luminal B stages of breast cancer. DNMT1 expression increases with the lower grade to higher grade cancer stage. With respect to normal cells, the DNMT1 expression gradually increases in luminal A, Luminal B and basal type of breast cancers. DNMT1 expression is highest in basal type cells. The association of DNMT1 and aberrant DNA methylation in cancer progression is beyond doubt; however, apart from DNA methylation, how DNMT1 controls the expression of a distinct set of genes as cancer progresses from one stage to other remains to be resolved (Fig. 3.2 A).

3.3.3 DNMT1 and CDH1 expression are inversely correlated with breast cancer progression

CDH1 expression was reported to be inversely proportional to the DNMT1 in breast cancer. CDH1 gene remains silent in metastatic cells. Most of the publicly available database (Fig. 3.2 B) and heat map expression analysis in most of the cases confirmed that where DNMT1 is highly expressed, CDH1 is downregulated (Fig. 3.2 C). Highest level of CDH1 downregulation was observed in basal type cells with respect to normal, luminal A, Luminal B-type cells. This is also associated with poor survival rate. Moreover, analysis by correlation curve established that DNMT1 and CDH1 expression is inversely proportional in breast cancer cells. Correlation curve of DNMT1 and CDH1 expression in clinical breast cancer samples shows that higher CDH1 expression was correlated to lower DNMT1 level (Fig. 3.2 D). From these analyses, it was further confirmed that in breast cancer DNMT1 plays a crucial role to downregulate CDH1 gene (Fig. 3.2). miR-152 was also downregulated in breast cancer, but no report direct or indirect was there on the involvement of miR-152 in CDH1 gene regulation; the link was missing. We hypothesize that CDH1 is regulated by miR-152/DNMT1 switch in breast cancer. To evaluate the hypothesis, we performed several experiments with MDA-MB-231, the same cell line which exhibited lowest miR-152 level.

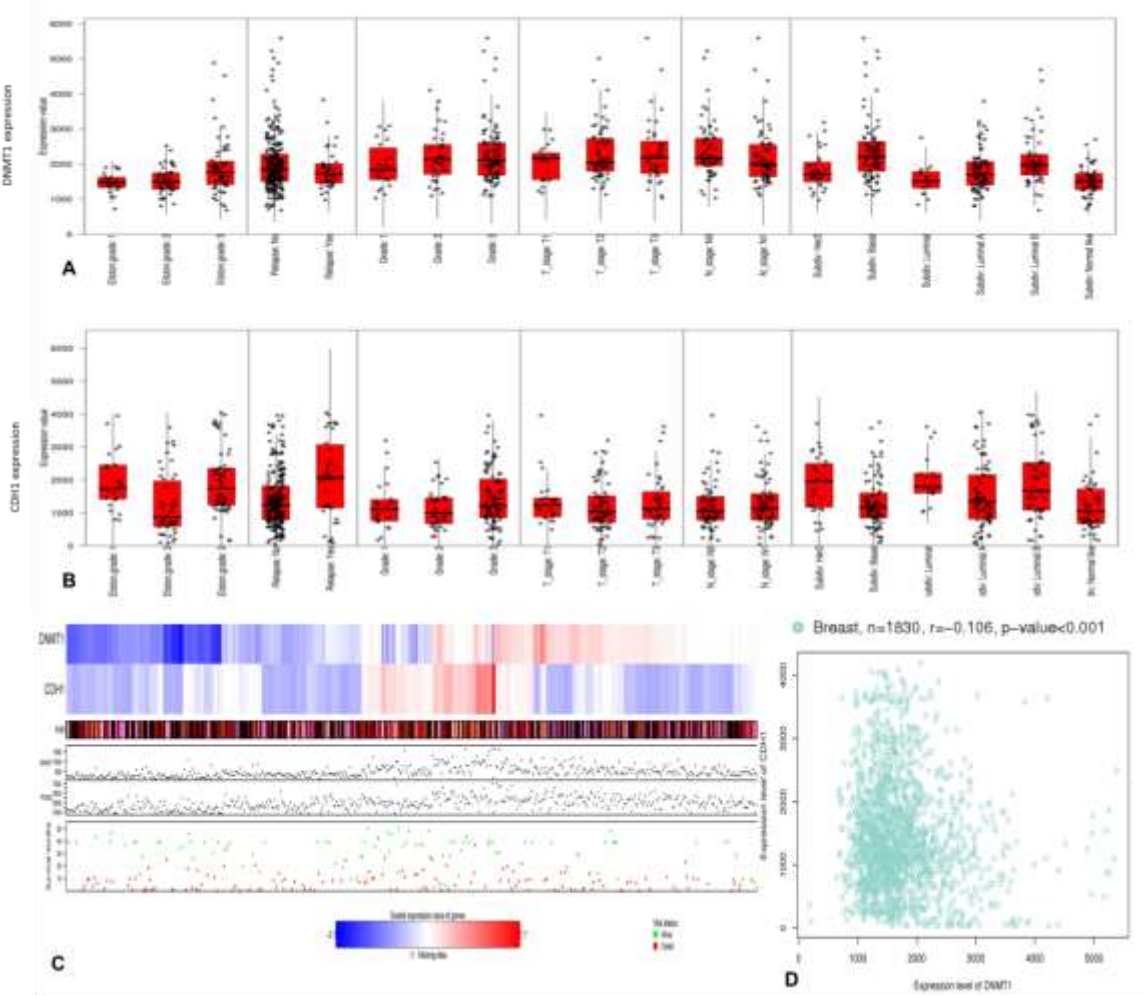

Figure 3.2: DNMT1 and CDH1 expression and their correlation:
Panel [A] Database analysis of DNMT1 expression in different stages of breast cancer. The differences are analyzed statistically, and P value is highly significant P< 0.001. [B] Database analysis of CDH1 expression in different stages of breast cancer. [C] Heat map expression analysis of DNMT1 and CDH1 expression in prostate cancer samples. [D] Correlation curve of DNMT1 and CDH1 expression in clinical breast cancer samples. CDH1 expression (x- axis) correlated to DNMT1 level (y-axis). P value is highly significant P< 0.001

3.3.4 H3K4 tri-methylation (H3K4me3) is not sufficient to overcome the repressive DNA methylation signal of the miR-152 gene promoter

In quest of the correlation between miR-152 and DNMT1 expression, first, it was demonstrated that DNMT1 expression is very high in MDA-MB-231 cell line at both mRNA and protein levels, while miR-152 gene remains silent. To understand the reason and mechanism of silencing of the miR-152 gene we evaluated the promoter DNA methylation and occupancy of modified histone 3 marks. DNMT1 is a well-established enzyme for its ability to methylate DNA and silence gene expression [29]. http://methhc.mbc.nctu.edu.tw/php/index.php database analysis confirms that promoter region of miR-152 is methylated in clinical breast cancer samples. This database has

stored information regarding the correlation between expression of genes and DNA methylation % at promoter region. Data mining from above database indicates a negative correlation value. The -0.11 correlation value represents that % of DNA methylation at promoter region, and miR-152 expressions are inversely correlated. Further, the P value (P= 0.005) is confirming that our evaluation is statistically highly significant (Fig. 3.3).

To clearly focus on the histone marks enrichment at miR-152 gene promoter region, ChIP analyses on H3K4me, H3K4me2, H3K4me3, H3K9me3 and H3K27me3 revealed that miR-152 gene remains silent along with the enrichment of H3K4me3 expressive mark in the promoter region [32]. Surprisingly, the occupancy of the repressive marks, H3K9me3 and H3K27me3 were low. The level of H3K4me, H3K4me2, H3K4me3, H3K9me3, and H3K27me3 marks were 0.11, 0.11, 1.19, 0.06 and 0.18 folds respectively (Fig. 3.3 A and B). From these observations, it was concluded that miR-152 gene is regulated mainly by its promoter DNA methylation. However, it was essential to confirm that DNMT1 activity is essential to regulate the miR-152. Hence, MDA-MB-231 cells were transfected with DNMT1 siRNA to knock down the DNMT1 expression and found that the miR-152 expression restored (Fig. 3.3 C and D). These results on the role of DNMT1 in repressing miR-152 gene are validated by DNMT1 overexpression upon transfection of cells with DNMT1 vector. DNMT1 overexpression associated downregulation of the miR-152 gene is apparent (Fig. 3.3 E). The expression levels of miR-152 were 5.49 and 0.55 in DNMT siRNA treated and pcDNA3/MycDNMT1 transfected cells, respectively. Results of these crucial experiments validate that miR-152 gene remains silent by DNMT1 overexpression in breast cancer apparent (Fig. 3.3 F).

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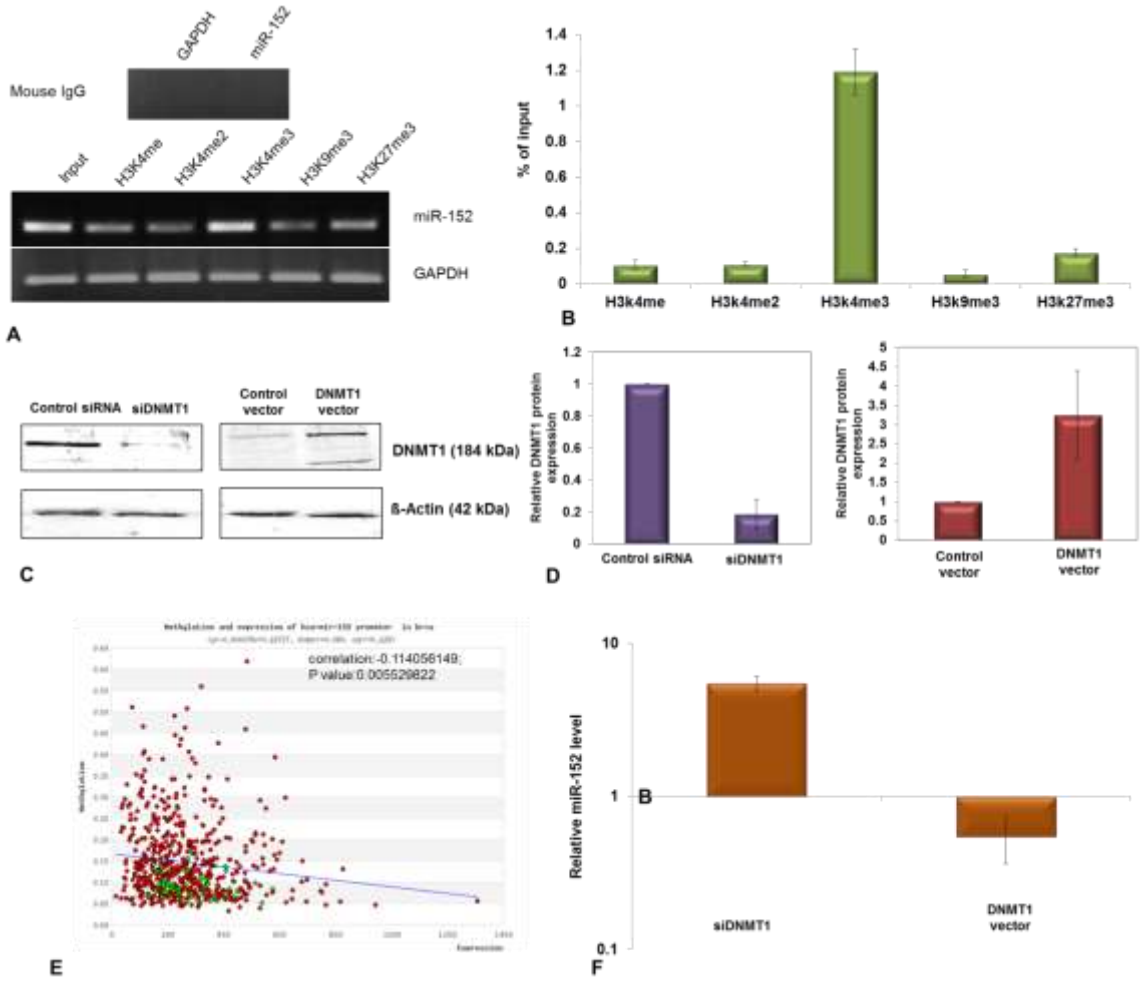

Figure 3.3: Epigenetic regulation of miR152 by DNMT1:
[A] Histone posttranslational modifications, H3K4me1, H3K4me2, H3K4me3, H3K9me3, and H3K27me3, in the promoter region of miR-152 was analyzed by PCR amplification. Where mouse IgG precipitated DNA used as negative control. [B] RT-PCR analysis of H3K4me1, H3K4me2, H3K4me3, H3K9me3, and H3K27me3 occupancy in the promoter region of miR-152. (mean±s.e., n=3 independent experiments with 3 replicas in each experiment). $P < 0.05$. [C] MDA-MB-231 cells were transfected with control siRNA, DNMT1 siRNA, pcDNA3/Myc, pcDNA3/Myc-DNMT1 and DNMT1 expression were anlyzed by western blot. β-actin was used to confirm equal protein loading. [D] Relative DNMT1 expression represented graphically (n=3 independent experiments, mean±S.D.), $P < 0.05$. every western blot images were analyzed by Image J software. [E] Bioinformatic analysis of promoter DNA methylation and miR-152 expression in clinical breast cancer samples from http://methhc.mbc.nctu.edu.tw/php/index.php database. [F] Transfected MDA-MB-231 cells were analysed for miR-152 expression by RT-PCR where the miR-152 level in control siRNA for DNMT1 siRNA and pcDNA3/Myc for pcDNA3/Myc-DNMT1 transfected cells was normalized to 1, (n=3 independent experiments, mean±S.D.), $P < 0.05$.

3.3.5 miR-152 expression restores CDH1 expression via DNMT1 downregulation

DNMT1 overexpression and miR-152 gene downregulation are associated with invasive breast cancer progression. To explore the functional consequences of DNMT1, miR-152 and CDH1 different experiments was done using miR-152 mimics. To determine the effective concentration of miR-152 mimics, three different concentrations of mimics

were used for transfection to downregulate DNMT1. We noticed that all three different concentrations 10 nM, 20 nM and 30 nM of miR-152 mimics gradually decrease the level of DNMT1 expression in respect to control mimic-treated cells. The DNMT1 mRNA level was decreased up to 0.78, 0.55 and 0.25 folds in 10 nM, 20 nM and 30 nM of miR-152 mimics treated MDA-MB-231 cells respectively (Fig. 3.4 A). Further, no change in cellular morphology was observed by most effective concentration (30 nM) of miR-152 mimics. Hence, cellular extracts from treatment of cells at 30 nM of miR-152 mimics was used for protein expression analysis. Significantly, there was negligible DNMT1 protein expression (Fig. 3.4 B and C).

To validate that miR-152 physically interacts with DNMT1 mRNA, luciferase activity assay was performed. MDA-MB-231 cells were co-transfected with control mimic or miR-152 mimics and luciferase DNMT1 3'UTR or luciferase mutated 3'UTR of DNMT1 vectors (Fig. 3.4 D). After 24 hours of transfection, luciferase activity was measured from 10 nM, 20 nM and 30 nM of the miR-152 mimics where no distinct expression was noted in mutated 3'UTR vector and miR-152 (30nM) co-transfected cells with control (Fig. 3.4 A). However, control mimic transfected both types of cells (DNMT1 3'UTR and DNMT1 mutated 3'UTR containing cells) exhibited no variation (Fig. 3.4 B and E).

CDH1 gene is downregulated in MDA-MB-231 TNBC cell line. At the above-mentioned concentrations of miR-152 mimics, MDA-MB-231 cells were treated for RT-PCR and western blotting analysis of CDH1 gene products. The mRNA levels of CDH1 gene was increased by 1.5, 3.0, and 6.39 with respective dosages of 10, 20 and 30nM of miR-152 mimics (Fig. 3.5). The CDH1 protein level at 30nM of miR-152 mimics treatment caused 9.47 folds overexpression (Fig. 3.5 C and D). It is worthwhile to note here that miR-152 does not have any seed region in the CDH1 mRNA, so one can easily predict that miR-152 treatment induced upregulation of CDH1 is caused via downregulation of DNMT1. Inactivation/downregulation of DNMT1 release the DNA methylation stress and helps in the expression of CDH1; which was confirmed by reverse experiments, where cells were transfected with DNMT1 siRNA and DNMT1 overexpression vector, and CDH1 gene expression was analyzed.

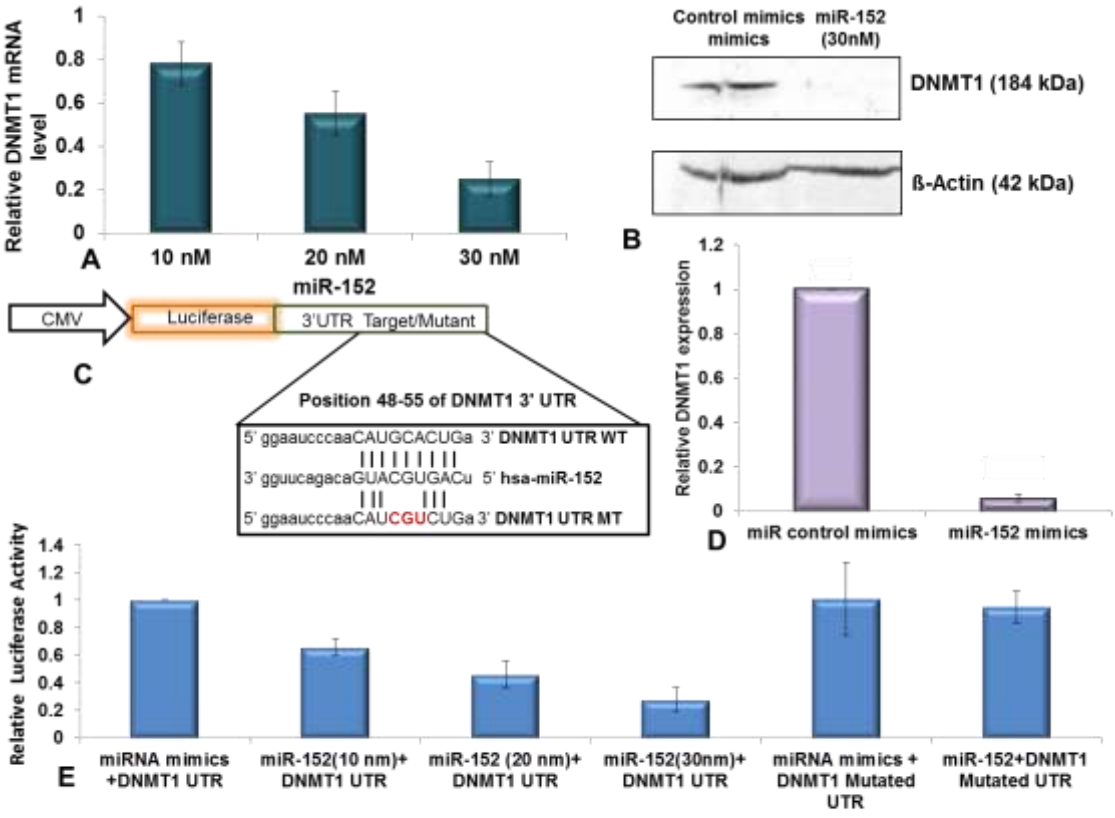

Figure 3.4: DNMT1 expression is regulated by miR-152:
Panel [A] DNMT1 mRNA level was measured by RT-PCR in control miR-mimic and miR-152 transfected MDA-MB-231 cells. The DNMT1 level in control miR-mimic treated cells was normalized to 1, (n=3independent experiments with 3 replicas in each experiment, mean±S.D.). P < 0.05. [B] DNMT1 expression level in miR- control mimic and miR-152 mimic-treated cells was observed by western blot analysis. β-actin was used to confirm equal protein loading. [C] Graphical representation of relative DNMT1 expression was analyzed by Image J software (n=3 independent experiments, mean±S.D.). P < 0.05. [D] Three different luciferase vector were constructed by using specific sequence in luciferase vector. Seed region of wt-DNMT1-UTR was used to construct DNMT1 3′ UTR WT (5'-CAUGCACUG-3'), has-miR-152 (5'-GUACGUGAC-3') and DNMT1 3′ UTR-MUT (5'-CAUCGUCUG-3') luciferase vectors. Red colour indicated the mutated bases in mut-DNMT1 UTR vector. [E] Luciferase activity was measured in microRNA mimics and pcDNA expression constructs co-transfected MDA-MB-231 cells. Renilla luciferase activity was normalized first to firefly luciferase activity and then to the values measured for the parental vector pcDNA. (n=3 independent experiments, mean±S.D.). P < 0.05.

3.3.6 In vitro analysis of DNMT1 and CDH1 expression after DNMT1 vector, DNMT1 siRNA, and miR-152 transfection

DNMT1 and CDH1 expressions in MDA-MB-231 and MCF-7 cells were analyzed after transfecting the cells with pcDNA3/Myc-DNMT1 vector, DNMT1 siRNA and miR-152 (30nM). After 48 h of treatment cells were visualized under a confocal microscope, and it is distinctly noticeable that after transfection with DNMT1 vector, DNMT1 protein

expression was higher than pcDNA3/Myc transfected cells (Fig. 3.6). After treatment with DNMT siRNA DNMT1 expression level was much lower than control siRNA-transfected cells (Fig. 3.6). Similarly, after treatment with miR-152 mimics cells also exhibit a lower level of DNMT1 expression (Fig. 3.6). The expression pattern of CDH1 is also visualized after the respective treatment as mentioned above. It was noted that, miR-152 (Fig. 7) and DNMT1 siRNA (Fig. 3.7) treatment induced expression of CDH1 than respective control cells. Noticeably, in DNMT1 overexpressing cells (pcDNA3/Myc-DNMT1 transfected cell) CDH1 expression was not significant compared to the respective control cells (Fig. 3.7). Collectively, these data clearly demonstrated that DNMT1 downregulation restores CDH1 mRNA and protein expression and DNMT1 overexpression repress CDH1 gene.

To confirm these findings MCF-7 cells were used where CDH1 expression is present. When cells were transfected with DNMT1 vector it supresses CDH1 expression but DNMT1 siRNA and miR-152 mimics shows higher expression than control cells. It confirms that CDH1 expression is regulated by DNMT1 and can be restored by inhibiting DNMT1 expression.

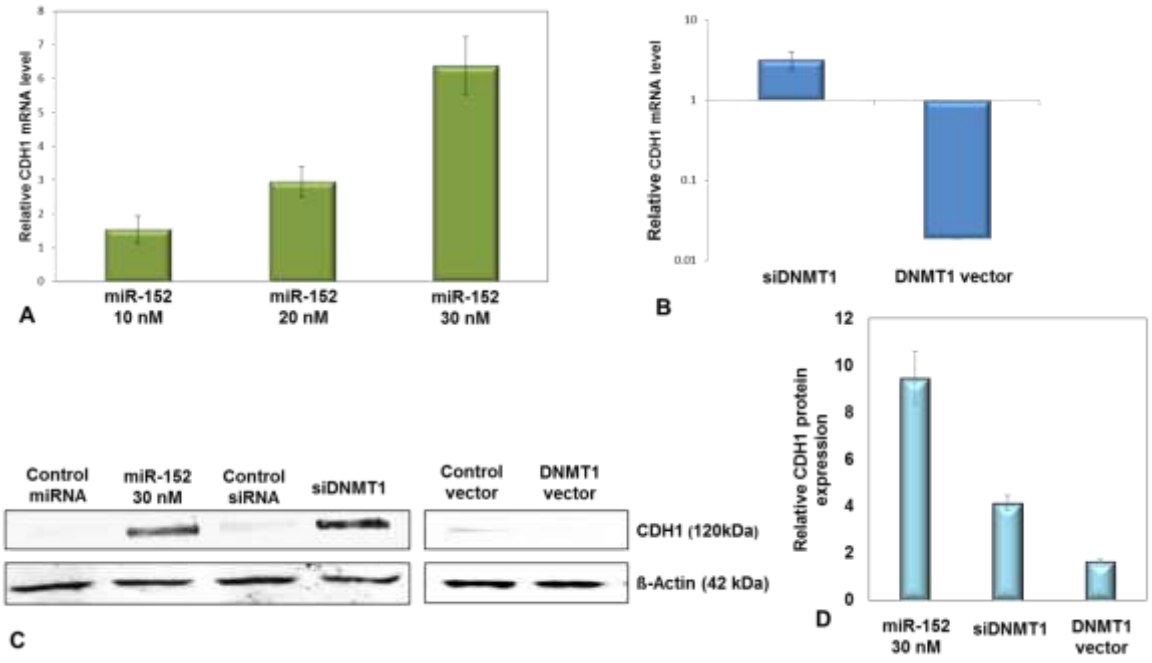

Figure 3.5: CDH1 is regulated by DNMT1 and miR-152 expression:
Panel [A] CDH1 mRNA level was analysed by RT-PCR on treatment with control miR-mimics and miR-152 mimics at 10, 20 and 30nM concentrations in MDA-MB-231 cells. The CDH1 level in control miR-mimic treated cells was normalized to 1, (n=3 independent experiments with 3 replicas in each experiment, mean±S.D.), $P < 0.05$. [B] MDA-MB-231 cell was transfected with control siRNA, DNMT1 siRNA, pcDNA3/Myc, pcDNA3/Myc-DNMT1 and was analysed for CDH1 mRNA expression by RT-PCR. The CDH1 level in control siRNA for DNMT1 siRNA and pcDNA3/Myc for pcDNA3/Myc-DNMT1 transfected cells was normalized to 1 (n=3 independent

experiments with 3 replicas in each experiment, mean±S.D.). P < 0.05. [C] Control miR-mimic, miR-152 mimic (30nM), control siRNA, DNMT1 siRNA, pcDNA3/Myc and pcDNA3/Myc-DNMT1 transfected MDA-MB-231 were subjected for western blot analysis to observe the CDH1 level. β-actin was used to confirm equal protein loading. [B] Graphical representation of CDH1 protein expression with respective control was analysed by Image J software (n=3independent experiments, mean±S.D.)

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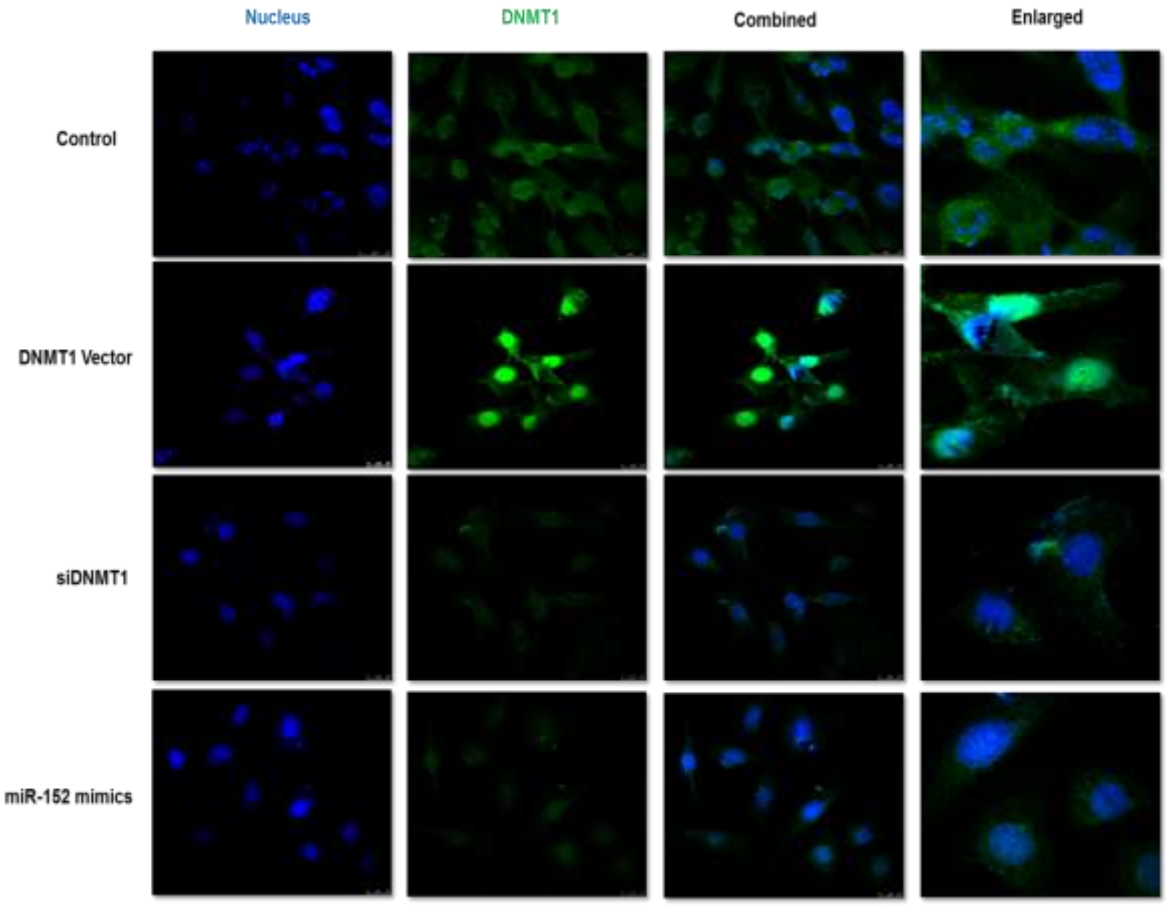

Figure 3.6: Immunofluorescence imaging of DNMT1 in MDA-MB-231 expression by confocal microscopy:
Panel DNMT1 expression were analysed after transfected with pcDNA3/Myc-DNMT1, DNMT1 siRNA, and miR-152 mimic. DNMT1 expression is in green (FITC conjugated secondary anti-rabbit antibody), and DAPI standing shows nucleus in blue.

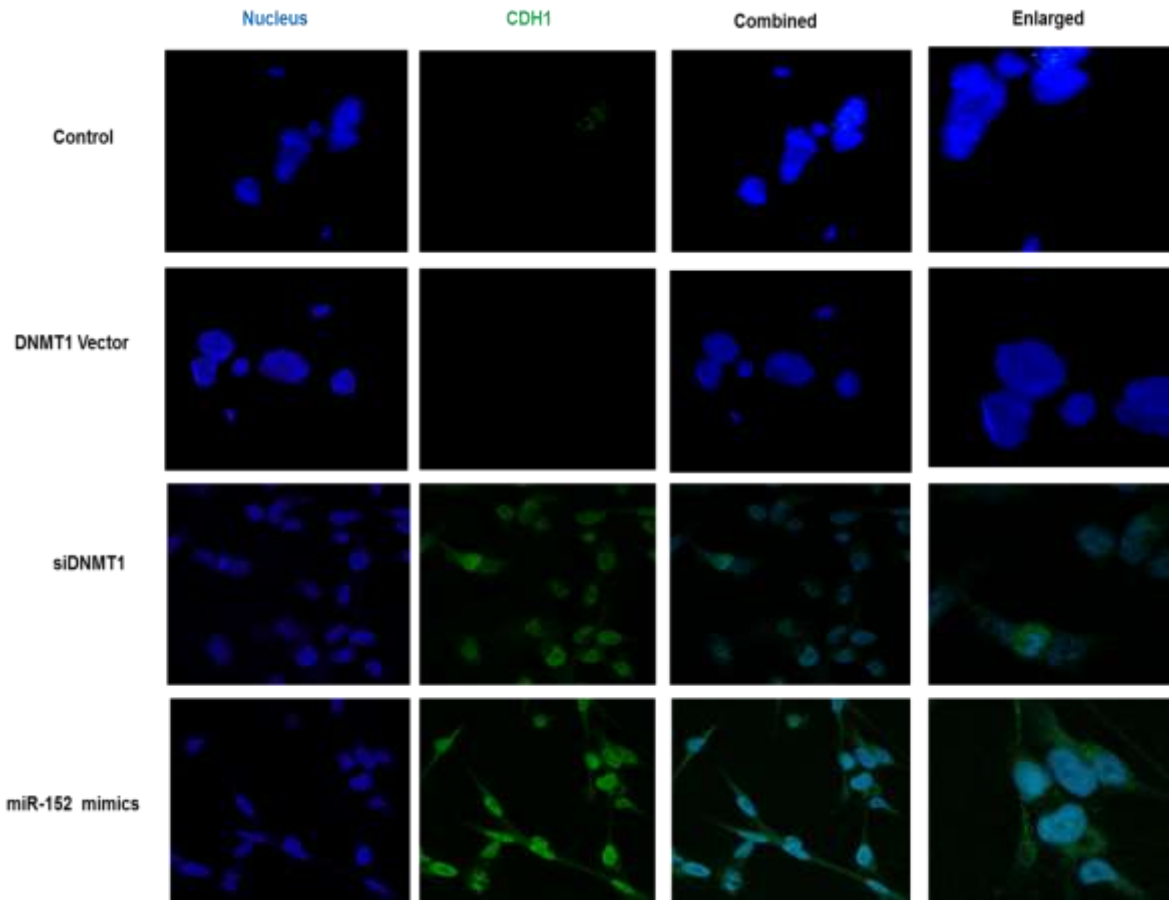

Figure 3.7: Immunofluorescence imaging of CDH1 in MDA-MB-231 expression by confocal microscopy:
CDH1 expression were analysed after transfected with pcDNA3/Myc-DNMT1, DNMT1 siRNA, and miR-152 mimic. CDH1 expression is in green (Alexa fluor 488 conjugated secondary anti-rabbit antibody) and DAPI standing shows nucleus in blue.

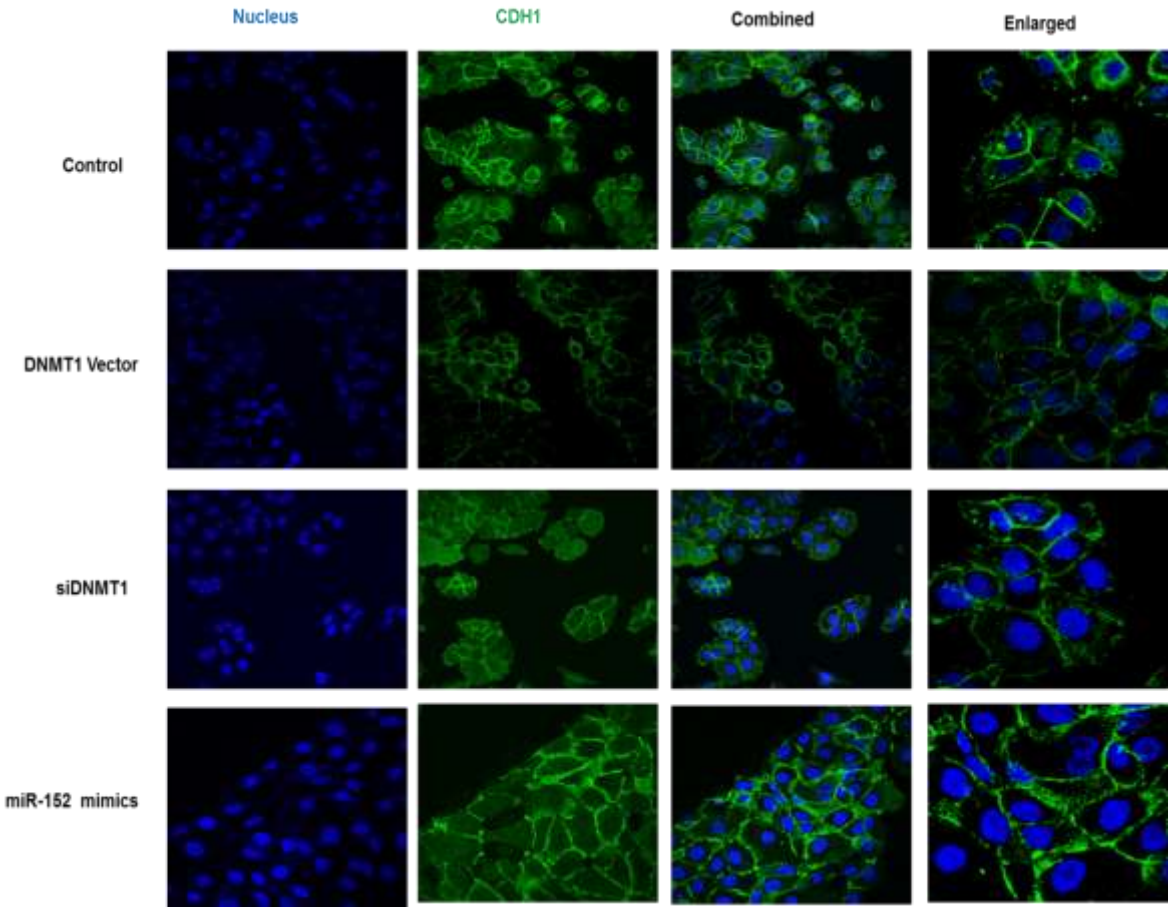

Figure 3.8: Immunofluorescence imaging of CDH1 in MCF-7 expression by confocal microscopy:
CDH1 expression were analysed after transfected with pcDNA3/Myc-DNMT1, DNMT1 siRNA, and miR-152 mimic. CDH1 expression is in green (Alexa fluor 488 conjugated secondary anti-rabbit antibody) and DAPI standing shows nucleus in blue.

3.3.7 Ectopic expression of miR-152 inhibits cellular migration of MDA-MB-231

Based on our bioinformatics search and experimental results, that miR-152 is functionally linked with the restoration of CDH1 protein; our next goal was to sort out the role of miR-152 in cellular migration. The cancer cells exhibit migration properties which cause the progression of the disease [33]. In MDA-MB-231 cells the CDH1 gene is fully suppressed. This cell line shows a higher migratory rate than other breast cancer cell lines, MCF-7, and BT-474. All these cell lines were transfected with different concentration of miR-152 mimics, and migration efficiency of the cells are visualized. At 30mM of miR-152 mimics, highest inhibition is traced. These data demonstrated that miR-152 mimics can inhibit cell migration effectively (Fig.3.8 A and B).

3.3.8 Chromatin decondensation level increases after miR-152 mimics treatment

It was previously reported that chromatin condensation increase the rate of cellular migration [324]. So, observing that miR-152 is inhibiting cellular migration by DNMT1 downregulation and CDH1 upregulation, it was determined that the effect of miR-152 on chromatin condensation. As previously described, the cells were transfected with different concentration of miR-152 mimics and observed the chromatin condensation using Hoechst 33342 stain. We found out that the rate of decondensation was highest at 30nM of miR-152 mimics (Figure 3.8 C and D).

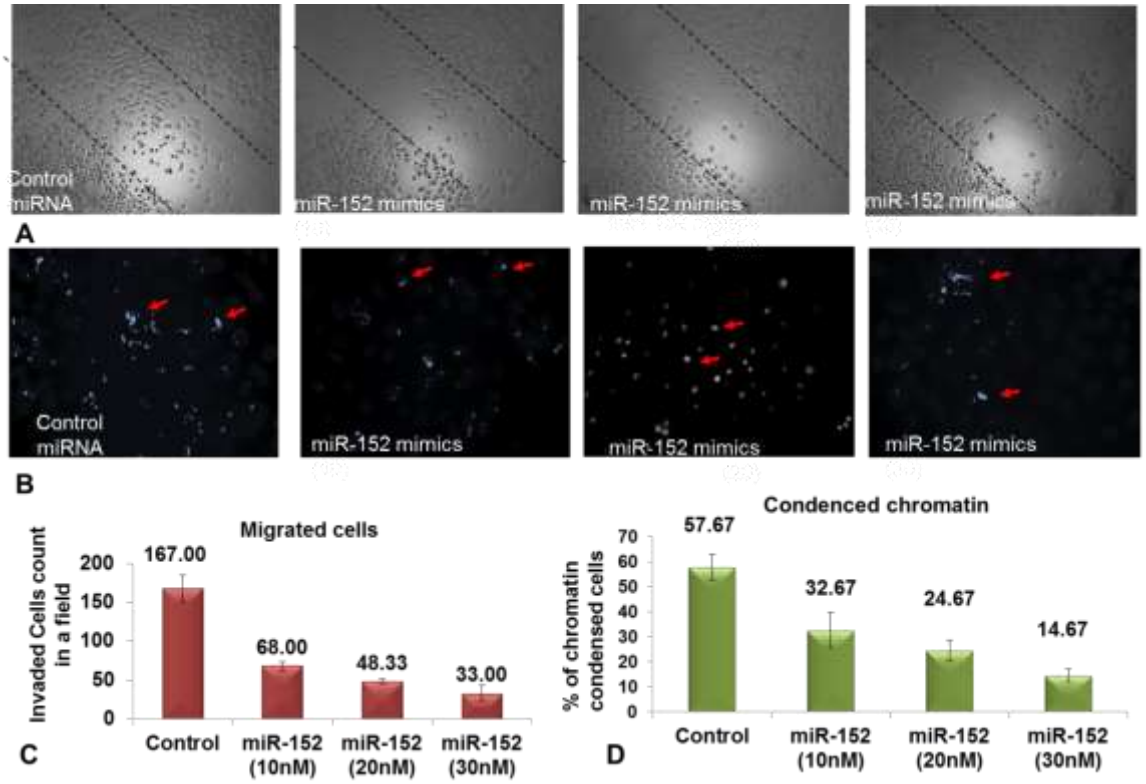

Figure 3.9: Effect of miR-152 on wound healing and chromatin condensation
MDA-MB-231 after transfected with three different concentrations (10nM, 20nM, 30nM) of miR-152 mimic. After 48h results were analysed. [A] The wounded areas and [B] condensed chromatin were analysed under 100x and 200x respectively with an inverted microscope. The representative images of Hoechst 33342 stained highly condensed nuclei (red arrow) are shown.Panel [C] Quantity of migrated cells that presents an average from three independent experiments was counted. Statistical analysis (n=3, mean±S.D.) $P<0.05$. [D] Percentage of condensed nuclei in miR-152 mimic transfected MDA-MB-231 cells represented graphically [A] (n=3 independent experiments, mean±S.D.). $P < 0.05$.

3.4 Discussion

During the last two decades, discoveries on epigenetic regulation of genes by DNA and histone methylation, histone acetylation and other types of molecular modifications of the euchromatin has strengthened the understanding of tumour development and cancer progression. Investigations on the role of the epigenetic factors in regulating the onset and progression of TNBC is also given major emphasis; since this type of cancer accounts for highest mortality among the breast cancer patients. Prime factor for cancer progression is aberrant DNA methylation; genome-wide hypomethylation and gene-specific promoter hypermethylation of tumour suppressor genes. This hypermethylated part of the genomic DNA makes complex with MBD proteins to precipitate as inactive chromatin and thus facilitates gene repression, tumour development, and aggressive progression of cancer [9, 94, 325]. In the case of leaky transcription of the genome, mRNAs are destroyed by second line of the guard, the miRNAs. The role of miRNA and underlying molecular mechanism in regulating the progression of cancer has drawn special attention. Previous reports on the breast, and hepatocellular carcinomas clearly depicted the role of miR-152 as a putative tumour suppressor that destroys DNMT1 mRNA [309, 326]. Moreover, inhibition of cell proliferation, motility, and induction of apoptosis by Wnt-1, ERK1/2, AKT and TNFRS6B signalling was linked with miR-152 function [326]. However, the mechanism of silencing of miR-152 during breast cancer progression was an unexplored issue (Fig. 3.1). In this study, it was reported that molecular epigenetic regulation pattern of miR-152 in light of DNMT1 knockdown and overexpression conditions (Fig 3.2 and Fig. 3.3). Results obtained on luciferase assay suggest that miR-152 is a potent as well as a direct modulator of DNMT1 in MDA-MB-231 cell line (Fig.4). It is well established that hypermethylation of genes is associated with high DNMT1 expression and silencing of the miR-152 gene.

In many cases, cancer progression is associated with the aberrant expression of the developmental genes, and upregulation of housekeeping genes. The aberrant expression of epithelial to mesenchymal transition (EMT) related genes is well documented in tumourigenesis [327]. EMT genes provide us a new basis for understanding cancer cell progression during metastasis. CDH1 gene is one of the essential genes for EMT, which plays a crucial role as a ‘gatekeeper of the epithelial cells'. The partial loss of CDH1 gene expression was observed due to aberrant DNA methylation in various forms of breast cancer. Thus, restoration of CDH1 gene expression can be crucial for controlling the cancer progression and metastasis [328]. It was previously reported that CDH1 gene is

repressed by promoter DNA methylation [329]. It was hypothesized that DNA demethylation of CDH1 gene promoter might enhance CDH1 protein expression. The decrease of DNMT1 protein by an increase of miR-152, inactivation of enzymatic function of DNMT1 would be alternative ways of reducing DNA methylation of genes, which is known as passive demethylation of DNA [237, 330]. Analysis of CDH1 promoter using bioinformatics tools showed that DNA hypermethylation is associated with CDH1 down-regulation in breast cancer (Fig. 3.2). In light of these, it was tried to establish that the relationship between CDH1 gene methylation and DNMT1 protein expression. In TNBC MDA-MB-231 cell line, CDH1 gene is repressed. The expression levels of CDH1 gene after the ectopic expression of miR-152 were analyzed. Results directly indicated that increasing concentration of miR-152 mimics helps in the restoration of CDH1 gene expression dose dependently at both mRNA and protein levels (Fig.5). To further strengthen the findings, CDH1 expression after DNMT1 overexpression and DNMT1 downregulation were analyzed. It is apparent that down-regulation of DNMT1 helps in restoration of CDH1 gene expression, whereas, in DNMT1 overexpression condition CDH1 gene remain silent (Fig. 3.5 Panel C, Figs. 3.6 & 3.7). It was concluded that in *in vivo* conditions miR-152 helps to restore CDH1 function via modulation of DNMT1 enzymatic activity. This result also implicates additional functions, other than DNA methylation, is played by DNMT1 in TNBC.

Confirmation of CDH1 protein function is demonstrated directly by inhibition of cellular migration and also, indirectly, by chromatin de-condensation. CDH1 acts like bridges between adjacent cells that hold cells together and prevent motility. During cancer progression, loss of CDH1 function is directly proportional to the ability of cell migration [331]. In this chapter, it is reported that increasing amount of miR-152 mimics proportionately decrease DNMT1 protein, increase CDH1 protein expression, and inhibit cell migration (Fig 3.8). This data confirms that loss of CDH1 function in TNBC is not due to mutation but by reversible DNA methylation. Inhibition of cell migration is accompanied with chromatin de-condensation. De-condensation of chromatin was also observed after ectopic expression of miR-152, comparison to the control (Fig 3.8).

Chapter 4
Objective 2

4. How the microRNA, miR-148a gene is regulated and targets DNMT1 and other genes in human prostate cancer progression.

4.1 Introduction

Prostate cancer is the most common cause of death due to cancer among men. It was estimated that 180,890 new cases of prostate cancer would appear in 2016, as reported this year, among them 26,120 deaths are predicted in the US according to NIH report. Currently, prostate-specific antigen (PSA) testing, digital rectal examination and histopathological evaluation of prostate needle biopsies are in use for detection and monitoring the progression of cancer. Prostate cancer Gleason score (Gleason grading system) was evaluated by the degree of loss of normal glandular tissue architecture of the cancer patient. However, sometimes prostate cancer varies and behaves uniquely than their corresponding Gleason score group [332]. In the current era of epigenetics, it has been emerged that, post-transcriptional regulation of genes by miRNA plays a vital role in translational biology that regulates physiological functions. miRNAs are small non-coding RNA that consists of 18-25 nucleotides, which binds to the target mRNA and in most of the case degrades it or in few cases increase its self-life. miRNA can bind in the 3' UTR or protein coding region where it degrades it and inhibits protein expression or in 5' UTR where it helps in the protein expression. miRNA regulates some biological process including development, proliferation, differentiation and apoptosis. The dysregulation of miRNA expression and functions has been observed in various cancers. The miRNAs that targets oncogenes are known as tumour suppressive miRNA and remain downregulated during tumour development and cancer progression. There are oncomiR which targets tumour suppressive genes are upregulated in cancers. Previously it was suggested that miRNA can act as a diagnostic marker for cancer which shows a specific profile [333]. But more studies discover that miRNA can be used as therapeutic agents for cancers treatment [334]. In solid tumours like colon [335], breast [336], bladder [337] and pancreatic cancer, miRNA expression follows a specific pattern. But in the case of prostate cancer, miRNA profiles are inconsistent and shows occasional upregulation and in general downregulation [338]. While more than 50 miRNAs have been linked with prostate cancer, only a few of them are related to disease pathogenesis [339]. miR-148a is reportedly downregulated in several cancers like breast and gastric cancers [340, 341]. DNA hypermethylation has been associated with silencing of miR-148a in different

metastatic cell lines, like colon, head, and neck cancers suggesting its role in cancer progression. miR-148a directly targets different growth factors like TGFB induced factor homeobox 2 (TGIF2), DNA (cytosine-5-)-methyltransferase 3 (DNMT3b) and pregnane X receptor (PXR) [249, 251, 342]. Nevertheless, its definite role in prostate cancer is yet to decipher. A major group of the prostate cancer patients initially respond to the androgen ablation, but after continuation, they become resistant to the treatment. However, patients diagnosed with hormone-refractory prostate cancer are usually treated with taxane anti-cancer drugs, such as docetaxel and paclitaxel, but the results were not satisfactory. From this scenario, it is urgent to find the reasons and develop new therapeutic approaches for the patients with hormone-refractory, drug-resistant prostate cancer. In this chapter, it was demonstrated that miR-148a has been remaining downregulated in hormone-refractory prostate cancer compared to other normal cells. Then the effect of miR-148a has been observed by ectopic expression of miR-148a in malignant phenotypes of hormone-refractory prostate cancer PC3 cells and metastatic prostate cancer cells DU-145 where it shows its apoptosis inducer role. DNMT1 is one of the major methyltransferases which regulates several genes in prostate cancer. The role of DNMT1 is still ambiguous in different cancers, but the aberrant DNA methylation in prostate cancer makes it an attractive therapeutic target for the disease. Inhibition of DNMT1 shows suppression of cell proliferation [343]. Recent studies have suggested that miR-148a serves as a tumour suppressor by targeting DNMT1 in the bladder and gastric cancer where ectopic expression of miR-148a suppresses cellular proliferation and also to downregulate DNMT1 [344, 345]. In this chapter, the repression of miR-148a and its potential role as a novel therapeutic target for treatment of hormone-refractory prostate cancer has been described.

4.2 Material and methods

4.2.1 Cell Culture, Plasmids, siRNA, and Transfections

PC3, and DU145 human prostate cancer cells, and the human immortalized keratinocyte cell line, HaCaT, were purchased from NCSS, Pune and maintained in Ham's F-12 Nutrient Mixture and Dulbecco's modified Eagle's medium (DMEM) respectively; all medium supplemented with 10% fetal bovine serum. For analyzing the effect of miR-148a on cell survival and the existence of DNMT1 mRNA, we used miR-148a mimics (Qiagen). Variable concentrations of miRNA mimics were used to analyze the effective

concentration of miR-148a for transfection. Apparently, 30nM of miR-148a efficiently inhibits the expression of DNMT1 at the protein level. To observe the role of DNMT1 we used DNMT1 siRNA (DNMTi) (Santa Cruz Biotechnology, Inc.) to suppress the expression of DNMT1. To overexpress DNMT1, we used pcDNA3/Myc-DNMT1 vector (Addgene). We used miR-148a mimics, DNMTi, pcDNA3/Myc-DNMT1 vector to transfect PC-3 and DU-145 cells along with Lipofectamine 2000 transfection reagent (Invitrogen) and optimem transfection media (Invitrogen). Further cells were incubated at 37°C, 5% CO_2 for 48 h to carry out different assays. As a control, we used control miRNA mimics, siRNA control, and pcDNA3/Myc vectors.

4.2.2 RNA Extraction and Quantitative PCR

Total cellular RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions. qRT-PCR was performed utilising the cDNA prepared from 1 μg of total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentas) and SYBR® Green JumpStart™ TaqReady Mix in the Realplex4Eppendorf system. The PCR were carried out using standard protocols, and the cDNA was amplified under the following conditions: 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 59.8 °C for 30 s and 72 for 30 s. We analyse DNMT1 after transfection with miRNA mimics, siRNA, and vectors. The mRNA level was normalized to the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. miRNA was isolated by using miRVana™ miRNA Isolation Kit, with phenol (Invitrogen) following manufacturer's protocol. From the isolated miRNA, cDNA of miRNA was prepared using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) followed by analysis using qRT-PCR.ForqRT-PCR of miRNA, primers were designed by Invitrogen miRNA primer designing protocol. The qRT-PCR of miRNA was done by NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen.) The cDNA of miRNA was amplified under the following protocol 50^{o}C for 2 min, 95^{o}C for 2 min 40 cycles of 95^{o}C for 15 s, 60^{o}C for 30 s. Then all the results were interpreted using Eppendorf qRT-PCR software. We analyse the expression of miR-148a, normalized to the expression of U6 small RNA. All the primers sequence was mentioned in Table 4.1. Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

Table 4.1 Primer List

Gene	Forward Primer	Reverse Primer
DNMT1	CCATCAGGCATTCTACCA	CGTTCTCCTTGTCTTCTCT
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAACGCA
miR-148a	AAAGTTCTGAGACACTCCGACT	Universal Reverse Primer for miRNA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT

4.2.3 Cell Viability Assays

Approximately 500 cells/well were seeded in 96 well plates for cell viability assay. After 24 hrs cells were transfected with miR-148a mimics at 5nM, 10nM 20nM, 30nM and 50nM concentration with lipofectamine and using optimem medium. After 6 hrs transfection media was removed and fresh media was added. To detect the cell viability MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] working solution was prepared by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS to the final concentration of 0.8mg/ml.100 µl of MTT working solution was added to each well and incubated for 24 hrs in CO_2 incubator. After incubation, the media was removed carefully without disturbing formazan precipitate and dissolved in 100 µl of 100% DMSO. An incubation of 15 min was carried out in the dark and the colorimetric estimation of formazan product was observed at 570nm in a microplate reader. The data was plotted against drug concentration, and non-linear regression curve fitting was performed using software to calculate the optimal growth inhibitory concentration (LC50) of the drugs.

4.2.4 Colony Formation Assay

The colony-forming potential of adherent cells was deliberate as previously described[346]. After the transfection of cells with miR-148a mimics, cells were re-seeded onto 12-well plates at 200 cells/well. After 2 weeks, colonies were fixed with 100% methanol for 15 min and stained with crystal violet for 20 min. After taking photographs, the number of colonies with a diameter more than 1.5 mm was counted. Only adherent cells were used for the colony-forming potential experiment. Plating efficiency (PE) and the number of colonies that arise after treatment of cells, surviving fraction (SF) was measured by following formulas.

PE= (no. of colonies formed/no. of cells seeded) x 100

SF= [(no. of colonies formed after treatment/(no: of cells seeded x PE)] x 100

Three independent experiments were done with three replicas to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

4.2.5 Soft agar assay

PC3 Cells (10^4) were mixed with 0.3% agarose in growth medium, plated on top of a solidified layer of 0.5% agarose in growth medium, in a 6-well plate, and fed every 3 days with growth medium. After 2 weeks, the colonies were dyed with Cristal Violet (0.01% solution), washed with PBS, and imaged by using Epifluorescent Microscope (Olympus IX71). Three independent experiments were done with three replicas to calculate the P value and validate the results (mean±S.D, $P < 0.05$)

4.2.6 Construction of vectors for luciferase assay

PCR from genomic DNA of HaCaT cells amplified the 3′ UTRs of DNMT1 (1526bp) containing an entire miR-148a recognition sequence and the PCR product was subcloned into a pcDNA Luciferase Vector (A gift from Dr. PathaSarathi Ray, IISER-Kolkata, West Bengal, India). A pcDNA luciferase construct containing the mutations in the seed sequence of DNMT1 3′ UTR was synthesized with using GeneArt® Site-Directed Mutagenesis System (Catalog number: A13282, Thermo fisher scientific). The wild-type construct contains the seed sequence "CAUGCACUG" In the mutated constructs, three nucleotides within the seed sequence "CAU*CGU*CUG" were mutated which is denoted in italics. All constructs were confirmed by sequencing and transfected as previously mentioned procedure.

4.2.7 Luciferase reporter assay

The cells were transiently transfected with 100 ng of reporter plasmids alone or co-transfected with different concentrations of (10nM and 30nM) miRNA mimics using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen) in 96 well plates. The activities of reporter genes with Renilla luciferase and the internal standard firefly luciferase were quantified by a Dual-Luciferase Reporter Assay System (Promega). Independent triplicate experiments were done for each plasmid construct. Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

4.2.8 Western Blot

The cells were grown up to 80–85% confluence and then transfected with miRNA mimics, siRNA and vectors for 24 hours before harvesting. The cells were then harvested, washed with PBS and lysed using RIPA buffer (Sigma). Cells were kept in 4^{o}C for 10 min to complete the cell lysis, lysate was centrifuged at 8000 g for 10 min at 4^{o}C. Same amount of protein was loaded in 10% SDS-PAGE for electrophoresis. Then separated proteins were transferred onto PVDF membrane. After blocking the membrane with 5% BSA in PBST solution, membranes were blocked with 5% BSA in PBS-T and left to incubate with primary antibody overnight at 4^{o}C. The following day, membranes were incubated with secondary antibody conjugated to HRP and development was carried out using Supersignal West Femto-chemiluminescent substrate (Thermo Scientific). Loading was assessed by ß-actin. The following antibodies were used: cleaved PARP (Abcam, USA), BCL-2 (Abcam, USA), and BAX (Abcam, USA), DNMT1, and actin (Santa Cruz Biotechnology, Dallas, Texas).

4.2.9 Immunocytochemistry of DNMT1 after miRNA transfection

For immunocytochemistry, PC-3 cells were grown on 18 mm coverslips. Cells were transfected with two different concentrations (10nM and 30nM) miR-148a mimics; To analyse DNMT1 expression, the cells were then fixed with ice cold methanol for 10 min, permeabilized with 0.1% Triton X-100 in PBS (PBST) and blocked with 1% BSA-PBST for 30 min at RT. For DNMT1 protein localization, cells were incubated with anti-DNMT1 primary antibody [Dnmt1 (H-300) sc-20701 Santa Cruz Biotechnology] at 1:500 dilutions in 1% BSA in PBST for 1h at RT, followed by staining with secondary antibody conjugated to HRP for 30 min. The development of protein was done using 3, 3′-Diaminobenzidine (DAB) mount with DPX and observes in bright-field of Epifluorescent Microscope (Olympus IX71). Three independent experiments were done to calculate P value and validate the results (mean±S.D, $P < 0.05$).

4.2.10 Flow cytometry

For cell cycle analysis, 10^5–10^6 PC-3 cells were incubated and then trypsinized, followed by centrifugation (500 x g) for five minutes at 4°C. Next, cells are washed twice with PBS and fixed at -20°C in 90% ice cold methanol. After 1 h of incubation, cells were centrifuged and suspended in PBS with RNaseA (500 U/mL) to digest the residual RNAs followed by PI (10 µg/mL) staining and incubation at 37 °C for 30 minutes. The analysis

was performed on Becton-Dickinson fluorescence-activated cell sorter (FACS). Three independent samples of treated samples were analyzed, and descriptive statistics of the results are reported as (mean±S.D, $P < 0.05$).

4.2.11 Chromatin condensation assay

After treatment with different concentration of the miR-148a mimics for the specific time, the cells were stained with Hoechst 33342 stain (1 mg/ml) and incubated for 10 min at 37 °C. Images were observed under ~460 nm emission of Hoechst 33342 dye using Epifluorescent Microscope (Olympus IX71). Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$). In each condition, 5000 cells were counted to analyse the result.

4.2.12 Statistical analysis:

All data are presented as mean ± SD. Statistical analysis was performed using the Student's t- test by SPSS software. Values of $P < 0.05$ were considered as significant value.

4.2.13 Ethical Approvals

This study was deemed exempt from ethics approval from the National Institute of Technology and consent was not required due to use of cell lines

4.3 Results

4.3.1 MiR-148a plays an important role in prostate cancer patient cell survival, and recurrence remains downregulated in prostate cancer cell lines

Previously it was observed that miR-148a remain downregulated in different cancer types [347, 348]. Here it is shown that, the altered miR-148a shows an effect in prostate cancer patient survival and reoccurrences. In the case of survival, the patient with altered miR-148a expression shows the 35% decrease after 40 months and without altered patient survives more than 110 months. In case of disease-free survival of altered miR-148a expression group the recurrence occurs after 70 months but with normal expression of miR-148a can stop the recurrence until 160 months (Fig.4.1 A). In the overall survival of the prostate cancer patient miR-148a help to survives for the patient. The expression level of miR-148a in PC3 and DU-145 hormone-refractory prostate cancer cell lines

remained downregulated by 6.3 folds and 8.9 folds respectively with respective to the expression of miR-148a in HaCaT human immortalized keratinocyte cell line (Fig. 4.1 B)

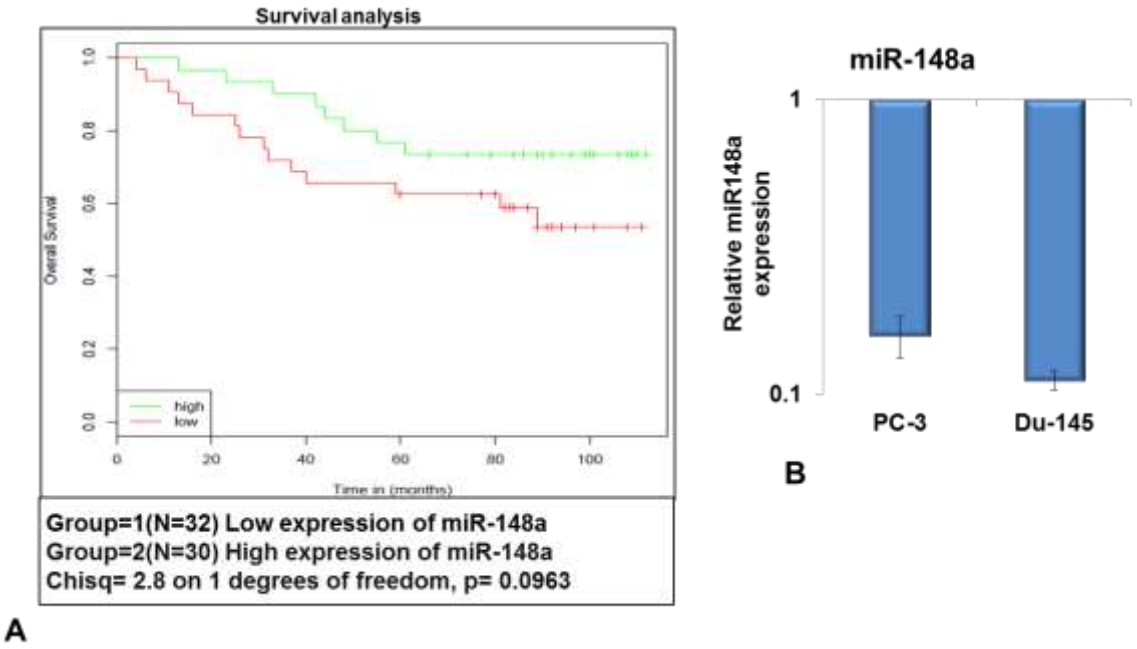

Figure 4.1: Kaplan–Meier analysis plot and mRNA expression of miR-148a:
[A] Overall patient survival was analysed by Kaplan–Meier plot. Total 62 numbers of samples were analysed from miRumiR GEO database. P- value was highly significant [p= 0.0963]. [B] mRNA level expression of miR-148a was analyzed in PC3 and DU-145 cell lines compare to HaCaT cell line. (n=3 independent experiment, mean±S.D.) $P < 0.05$.

4.3.2 Ectopic Expression of miR-148a Inhibited Cell Growth in PC3 Cells

For analysing the effect of decreased expression of miR-148a in malignant phenotypes of prostate cancer the ectopic expression of miR-148a was done in PC3 cells. Cells were transfected with miR-148a mimics and observed the effects after 24 hrs. It was clear from results that miR-148a overexpression inhibited the cell growth (Fig. 4.2). The increased level of miR-148a mimics represses the growth of PC3 and DU-145 cells. For further analysis of inhibition by miR-148a colony formation assay was performed. The results of colony formation assay of PC3 cell line clearly show the inhibitory effect of miR-148a; the number of colonies reduced in PC3 cells transfected with miR-148a compared with those transfected with a controlled precursor (Fig. 4.3 A and B).

4.3.3 Ectopic Expression of miR-148a inhibits anoikis independent cell growth

The programmed cell death that occurs after they detach from the surrounding extracellular matrix (ECM) of anchorage-dependent cells is known as anoikis. Transformed cells have the ability to grow independently of any solid surface. Using soft

agar colony assay the malignant transformation of cells can be measured [349]. The PC3 cells were transfected with miR-148a and soft agar assay were done, and the results show the miR-148a transfected cells formed less colony than control cells [Fig 4.3.A]. This indicates that miR-148a plays an important role in inhibition of cell growth and initiates anoikis cells [Fig 4.3.C].

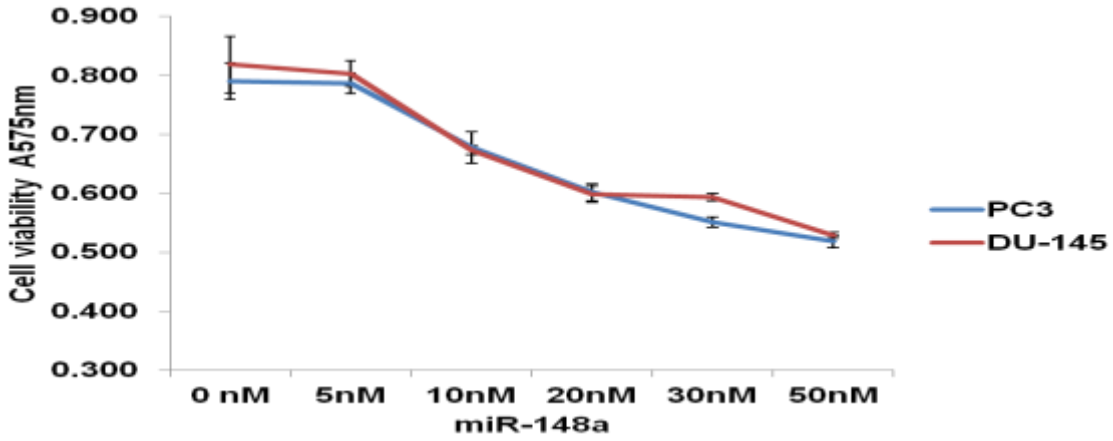

Figure 4.2: Concentration-dependent effect of miR148a mimic on PC3 and DU-145 viability: Cells were transfected with various concentrations of miR148a mimic for which cell viability was measured using MTT assay. Cell viability decreased with increase concentration of miR-193a. (n=3 independent experiment with three replica, mean±S.D.) $P < 0.05$. Cell viability is represented in terms of absorbency.

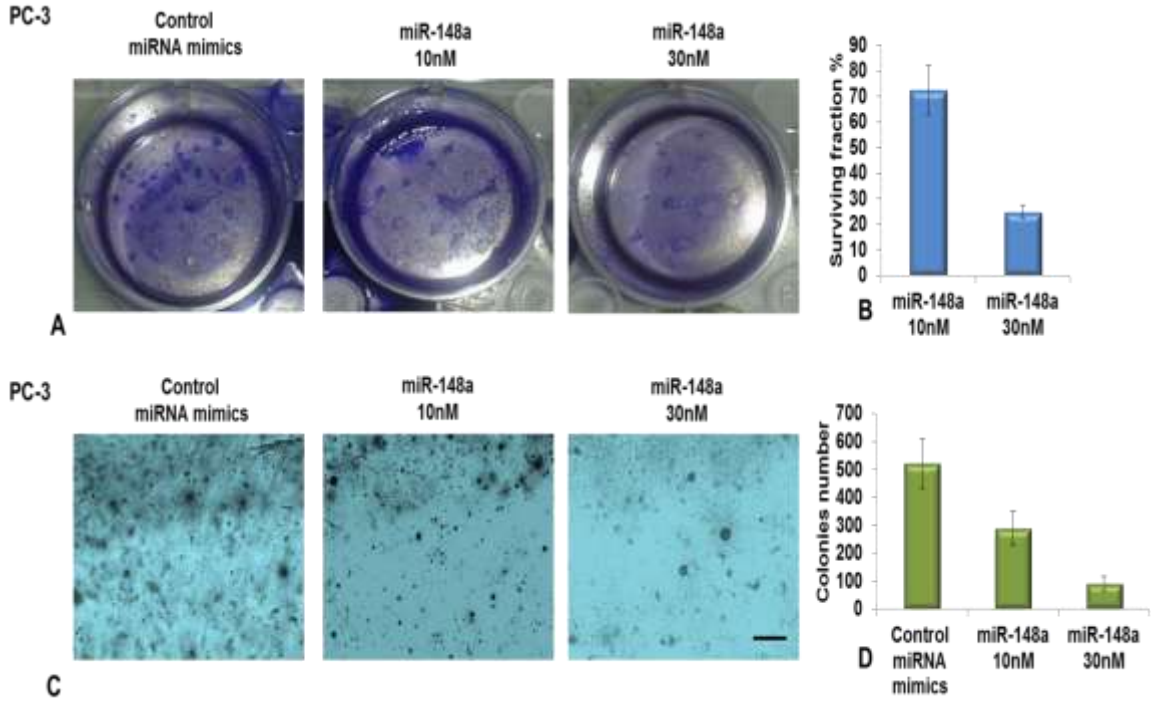

Figure 4.3: Clonogenic and anchorage-independent survival analysis:
[A] Clonogenic survival was analyzed after transfection with miR-148a 10nM and 30nM. [B] Graphical presentation of clonogenic efficiency (n=3 independent experiment, mean±S.D.). $P < 0.05$. [C] Colony formation of PC3 cell lines on semisolid soft agar plates were examined after 3 weeks culture. Then colonies were stained and visualized microscopically. A representative view

of each condition is shown. (D) Quantification of colony formation data derived from colonies was counted in a colony counter. Results from one representative experiment are shown. (n=3 independent experiment, mean±S.D.). $P < 0.05$.

4.3.4 DNMT1 is overexpressed in prostate cancer patients and cell lines

For better understanding, the role of DNMT1 in prostate cancer the mRNA level of DNMT1 was analysed from the clinical sample database Genesapience. The results analysis shows that DNMT1 expression was higher in prostate cancer tissue sample than the normal tissue sample. This increase of DNMT1 expression follows a pattern. With respect to normal cells, the DNMT1 expression gradually increases in Stage T2 to stage T3 cancers. Moreover, during lymph node (N0 to N1) and distance (M0 to M1) metastasis DNMT1 expression also increase with higher metastasis rate. Higher DNMT1 expression is also associated with prostate cancer recurrence. The increase of DNMT1 is related with the progression of cancer. It also shows different expression in hormone refractory cells. DNMT1 expression also depends on the family history of the disease. This dataset suggests DNMT1 can be used for the prognostic marker. The highest expression is observed in the metastatic stage of cancer. Differential expression of DNMT1 is responsible for aberrant DNA methylation and disruption of normal gene expression (Fig 4.4.A). The survival curve analysis shows disease-free survival of the patient with altered DNMT1 expression is higher than the wild-type DNMT1 expressing patients. Aberrant DNMT1 expression associated where less survives than without alteration of DNMT1 expression patients. After obtaining the expression pattern from datasets DNMT1 expression at transcriptome level were analysed in prostate cancer cell lines where it was found that both PC3 and DU-145 cell lines overexpress DNMT1 in comparison to HaCaT cell line (Fig. 4.4C) and Benign prostatic hyperplasia (BPH) [10].

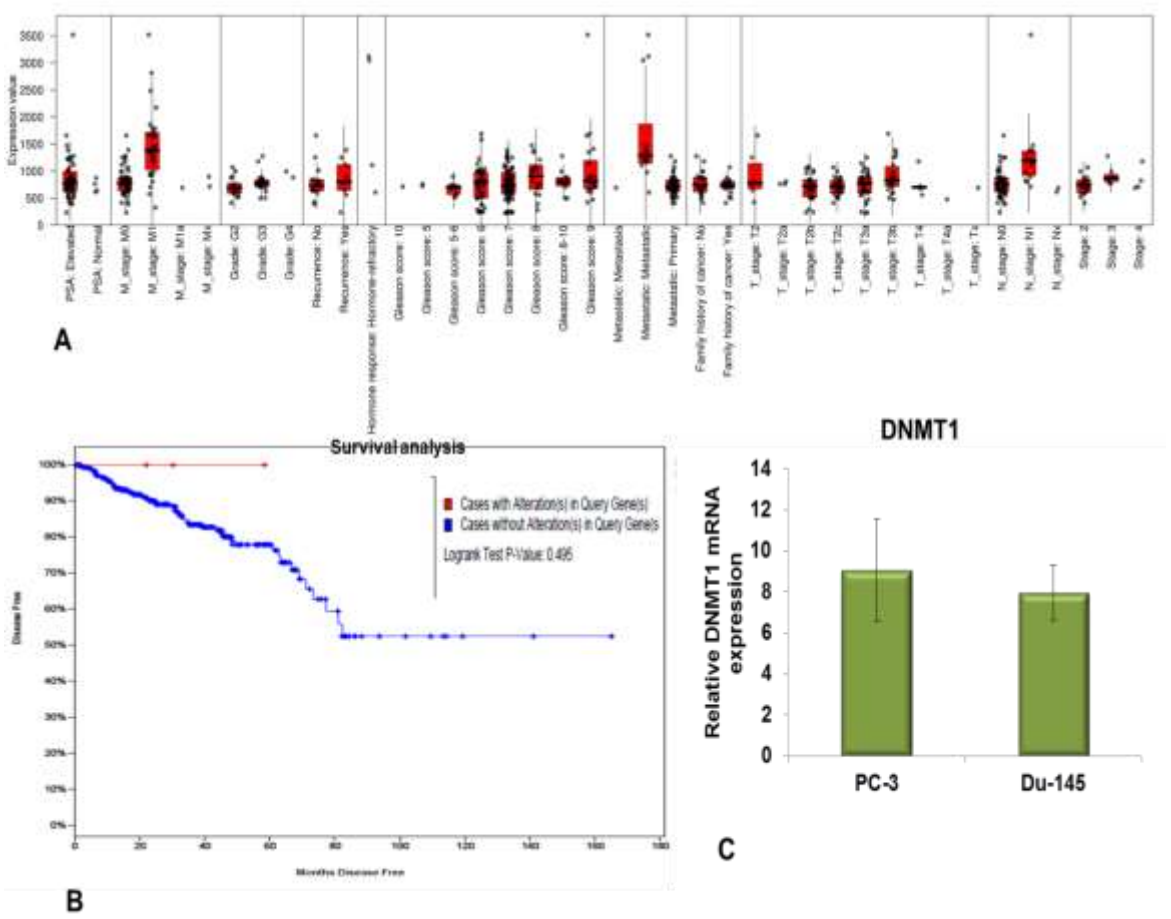

Figure 4.4: DNMT1 expression and disease free survival analysis:
[A] Database analysis of DNMT1 expression in different stages of prostate cancer samples. [B] Overall prostate cancer patient survival was analyzed by Kaplan–Meier plot. P value is $P < 0.459$. [C] mRNA level expression of DNMT1 were analysed by RT-PCR in PC3 and DU-145 cell lines compare to HaCaT cell line. mRNA level of DNMT1 in HaCaT cell line is normalized to 1 (n=3 independent experiment, mean±S.D.) $P < 0.05$.

4.3.5 miR-148a downregulates DNMT1 expression by targeting the 3′ UTR of DNMT1 gene

The initial analysis showed that, DNMT1 is overexpressed in metastatic prostate cancer and prostate cancer cell lines. This indicates DNMT1 play an oncogenic role in development of prostate cancer. For identification of DNMT1 as potential target, different target algorithms were used, and found miR-148a is having highest score among the predicted miRNAs. It has an 8 mer binding site without any wobble. Additionally, the predicted DNA sequence of miR-148a target site within DNMT1 3' UTR was highly conserved among species including chimpanzee, rhesus rabbit, and mouse (Fig 4.5 C). This *in-silico* data suggested that miR-148a could be involved in regulation of DNMT1 in prostate cancer. Downregulation of miR-148a and upregulation of DNMT1 in prostate cancer this inverse correlation is apparent in PC3 and DU-145 cells as well. As shown in

Fig 4.6 A, B when PC3 and DU-145 cells were transfected with miR-148a mimics in 10nM and 30nM, and it downregulates DNMT1 protein expression. In both the cell lines increasing the miR-148a mimic concentration reduces DNMT1 protein expression and the graphical representation shows that DNMT1 expression is lowest in 30nM miR-148a transfected cells in both cell lines. In PC3 cell line immunocytochemistry was done after transfection with miR-148a mimics and it also shows downregulation of DNMT1. To prove that miR-148a directly targets DNMT1 a firefly luciferase reporter gene containing the DNMT1 3' UTR (DNMT1 3′ UTR WT) with predicted target sites was prepared. Another mutant version was also prepared with a mutation in the target sites (DNMT1 3′ UTR MT). Co-transfection of miR-148a mimics with reporter vectors in PC3 cells decreased the relative luciferase activity in increasing miR-148a concentration. But the DNMT1 3′ UTR MT shows no such effects with miR-148a transfection. This assay confirms that, DNMT1 is a potential target of miR-148a (Fig 4.7).

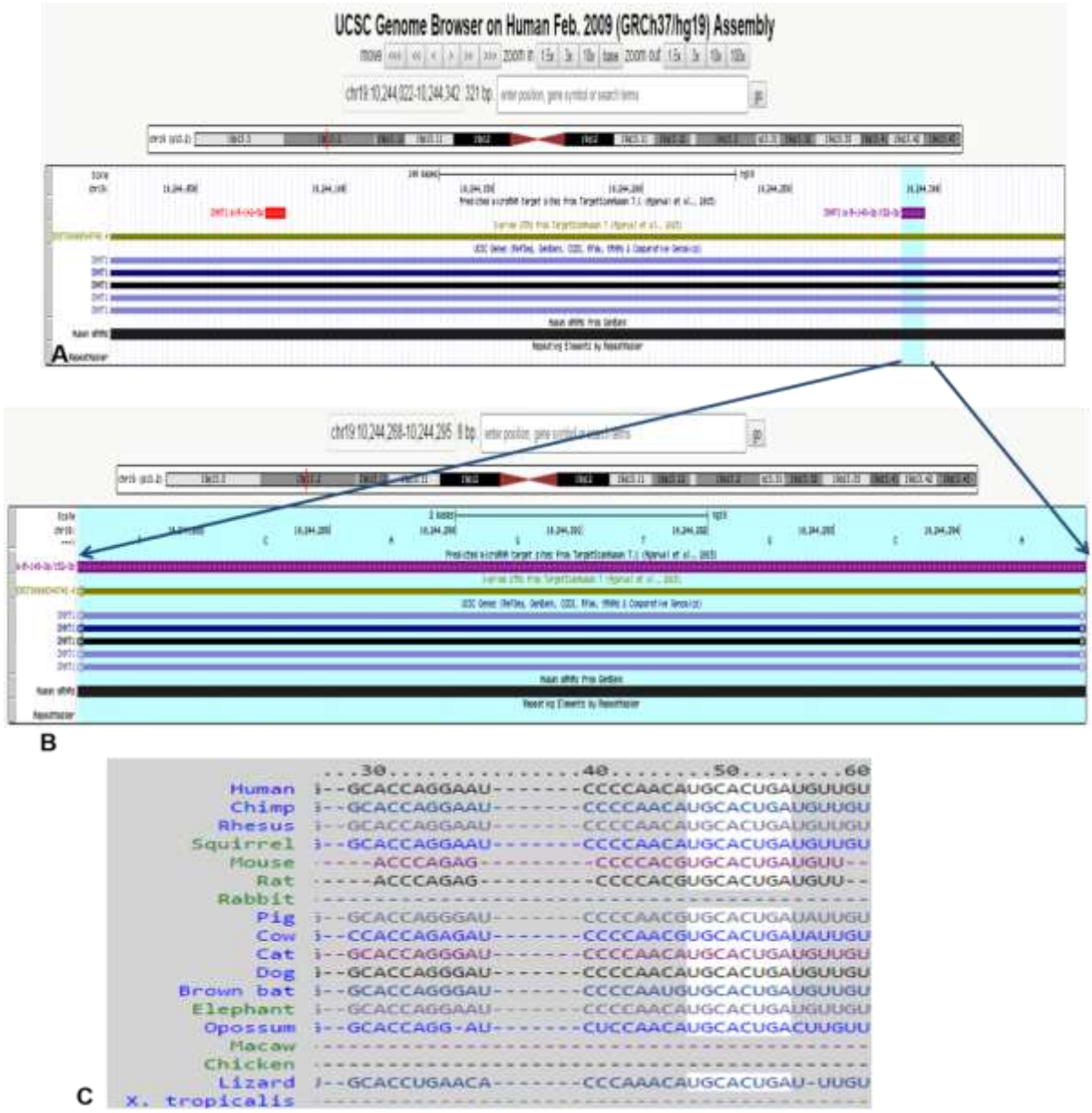

Figure 4.5: Characterization and binding site of the DNMT1-3'-UTR with miR-148a:
[A] CSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly view of 3'UTR of DNMT1. The highlighted area shows the binding site of miR-148a with 3' UTR of DNMT1. [B] Enlarged view of the highlighted area. The 3' UTR has a 8mer binding site for miR-148a (chr19:10,244,288-10,244,295). [C] The 8mer conserve site of 3'-UTR of DNMT1 in different vertebrates. The conserved region is highlighted in white.

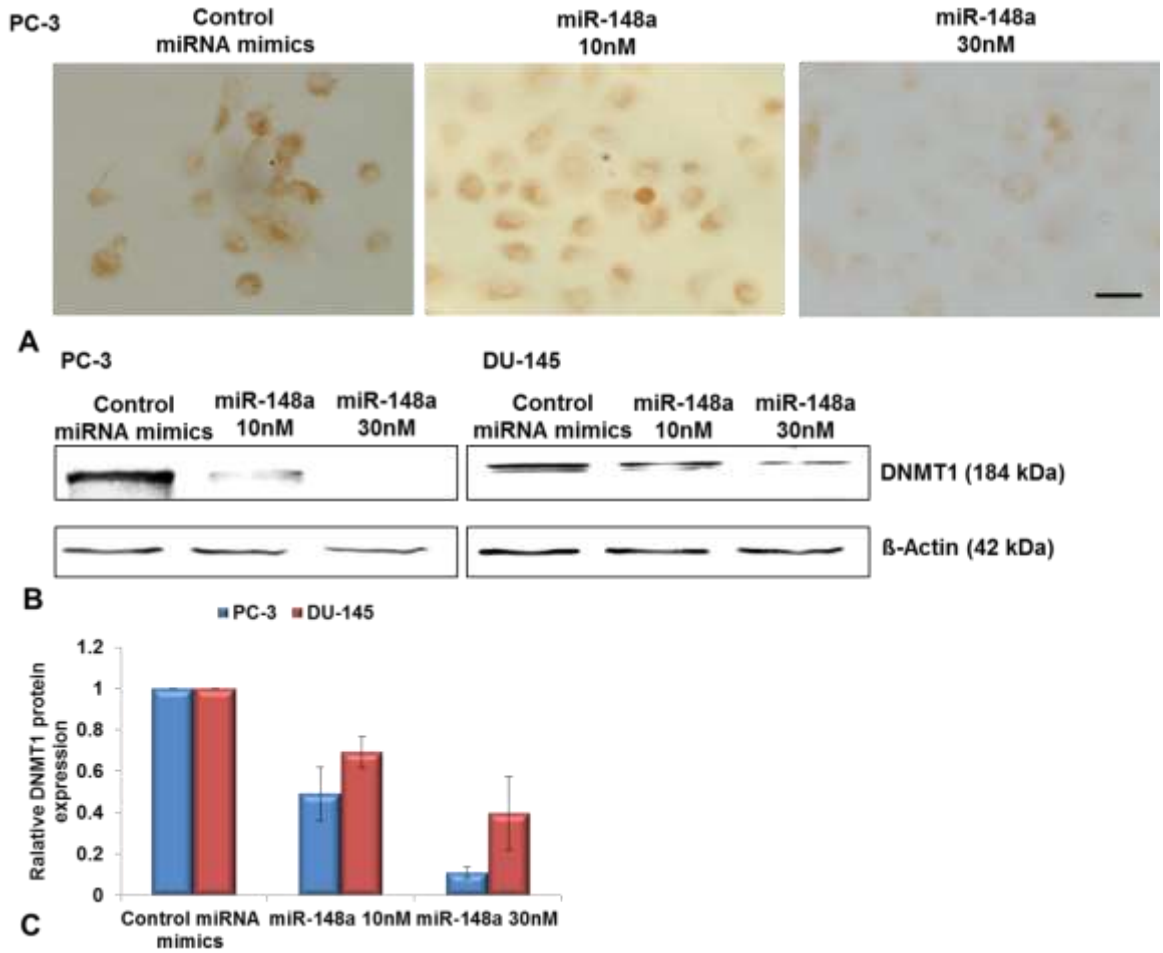

Figure 4.6: DNMT1 expression analysis:
[A] Immunocytochemistry analysis of DNMT1 expression in miR-148a transfected cells. Scale Bar = 40 µm. [B] DNMT1 protein expression and quantification were measured by Western blot in PC3 and DU-145 cells after 10nM and 30nM miR-148a mimic, control miRNA mimic transfection. [C] The band intensity was measured by ImageJ software. Beta-actin used as loading control. (n=3 independent experiment, mean±S.D.) $P < 0.05$

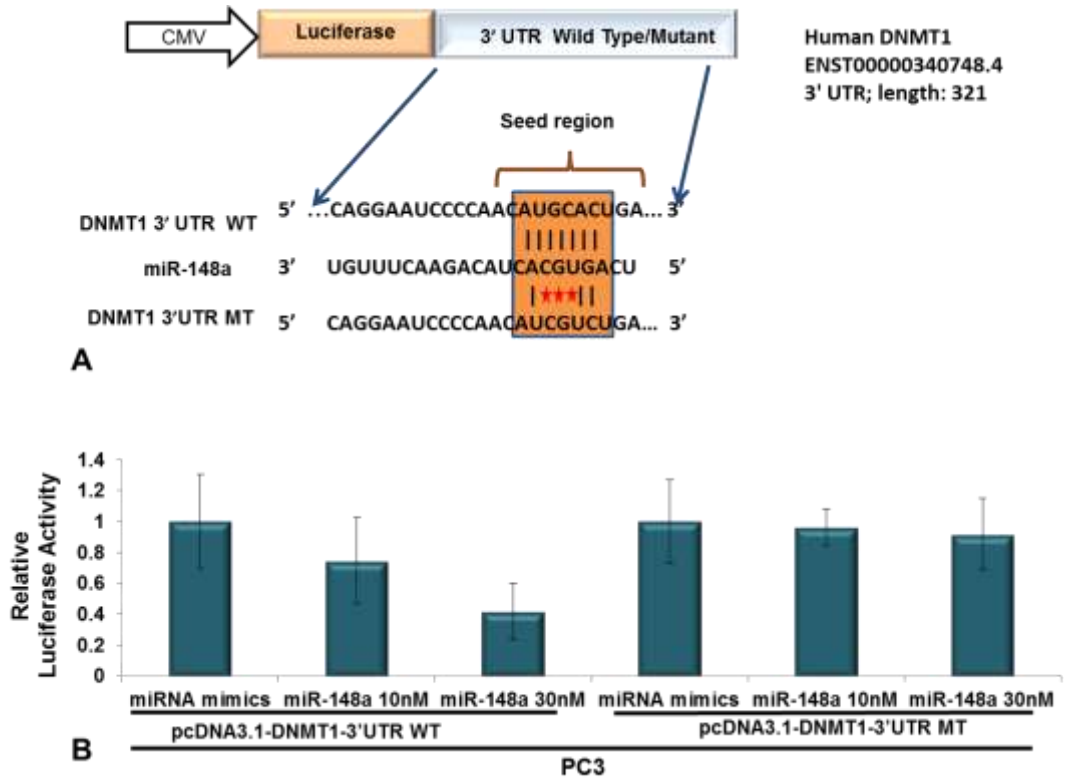

Figure 4.7: Functional analysis of the DNMT1-3' UTR and miR-148a binding:
[A] Schematic representation of DNMT1-3'-UTR, which was inserted downstream of Renilla luciferase gene in the pcDNA3.1 vector. Complementarity between miR-148a and the DNMT1 3'-UTR site target. The side directed mutation was located within the 'seed region' of the miR-148a binding site (red star). [B] Luciferase activity was measured in transfected PC3 cell. The luciferase activity of each construct was normalized against the negative control miRNA-transfected with control miRNA mimic. Renilla luciferase activity was normalized first to firefly luciferase activity and then to the values measured for the parental vector pcDNA. (n=3 independent experiments, mean±S.D.). $P < 0.05$.

4.3.6 MiR-148a overexpression increases the apoptosis in prostate cancer cell lines

On the basis of cell survival assay, it was hypothesized that overexpression of miR-148a can induce apoptosis in prostate cancer cells. For analysing the effect of miR-148a on DNA damage, the comet assay was done. Comet assay is a well-established and sensitive method for detecting DNA breaks and apoptotic nuclei [350]. The assay was done in both PC3 and DU-145 cell lines, whereby transfecting cells with miR-148a mimics with 10nM and 30nM shows DNA damage. The results were analysed in ImageJ software and found in control cells the DNA damage was lesser than 10nM and 30nM mimics transfected cells. The tail lengths were measured, and miR-148a induced DNA damage it shows in both the cell lines. In case of PC3, tail lengths were 15, 25 and 75 in case DU-145 tail lengths were 29,104 and 156 of control mimics, 10nM miR-148a mimics, and 30nM miR-148a mimics transfected cells respectively (Fig 4.8).

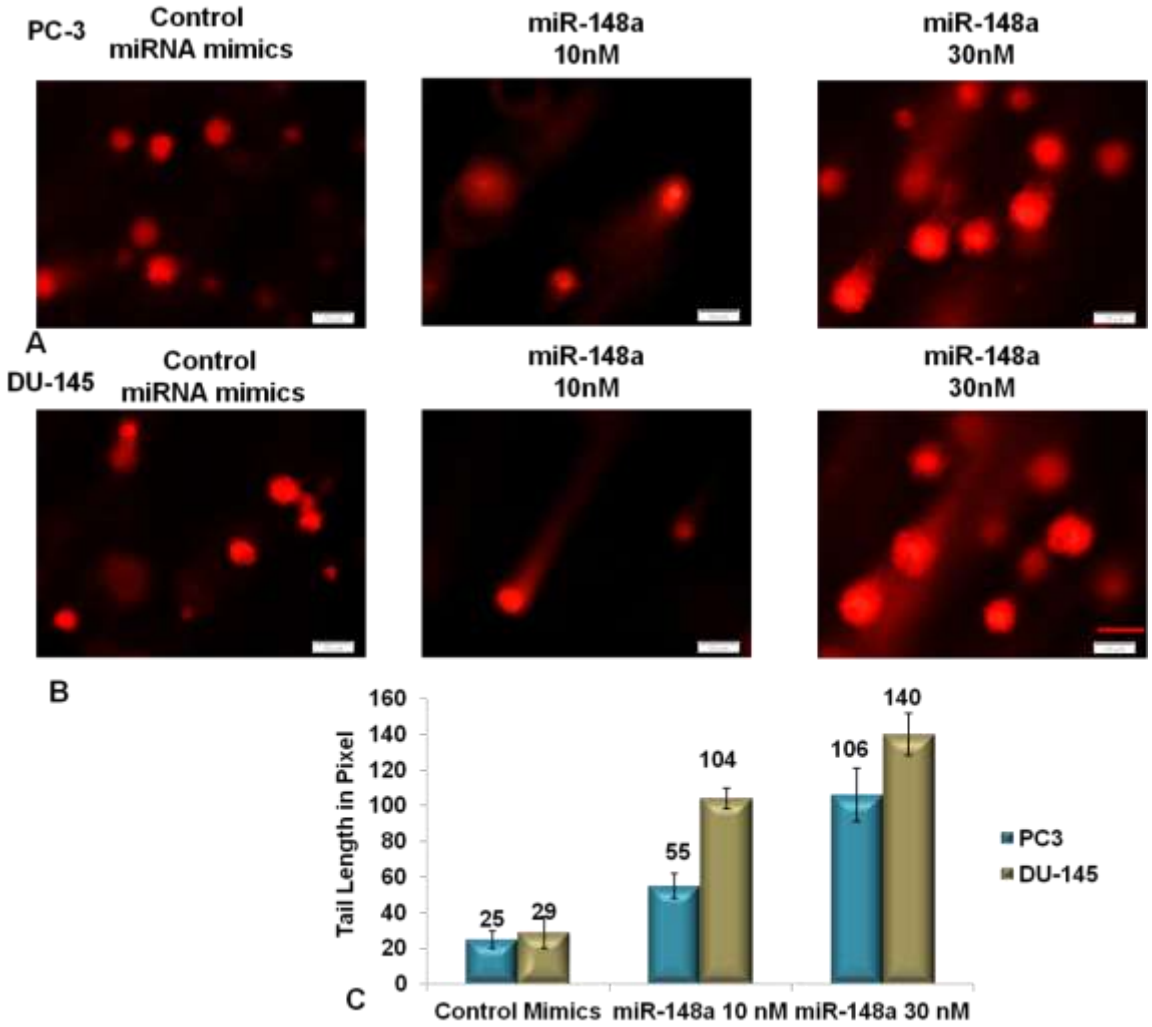

Figure 4.8: DNA damage analysis by comet assay:
DNA damage was studied by comet assay in [A] PC3 and [B] DU-145 after transfection with control miRNA and miR-148a mimic (10nM and 30nM). [C] Tail length in each experiment conditions were measured in 500 cells and represented graphically. Scale Bar = 40 µm. (n=3 independent experiment, mean±S.D.) $P < 0.05$. Tail length was increased with increase concentration of miR-148a mimic.

One of the unique properties of apoptosis was morphological changes and chromatin condensation which leads to the formation of crescent-shaped masses aggregating at the membrane. In parallel the nucleolus dissolves [351]. After the cells were transfected with miR-148a mimics the number of chromatin condensed cell were increased in both the cell lines. This clearly indicates miR-148a induced chromatin condensations, which eventually initiate cell apoptosis. For further confirmation cell cycle analysis were done (Fig 4.9).

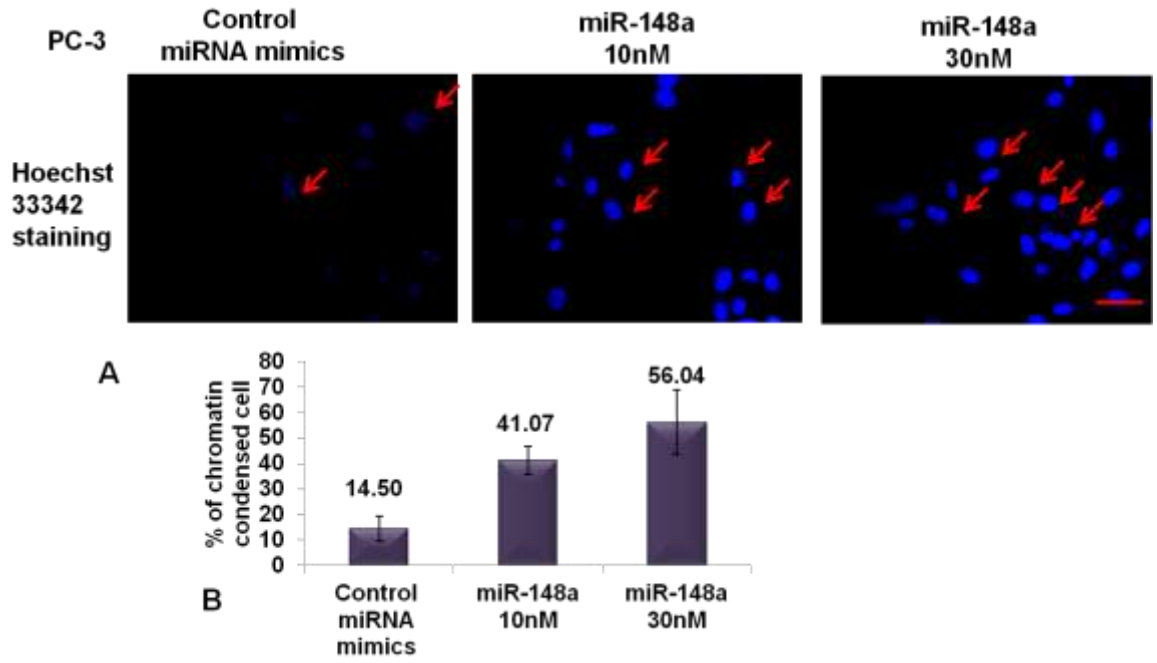

Figure 4.9: Chromatin condensation analysis:
[A] PC3 cells were stained with Hoechst 33342 after transfection with 10nM and 30nM of miR-148a mimic and control miRNA mimic. [B] Condensed nuclei containing cells represent graphically (n=3 independent experiment, mean±S.D.). $P < 0.05$. Scale Bar = 20 μm. The graph represented the induction of chromatin condensation after miR-148 overexpression.

The cell cycle analysis shows that, cells transfected with miR-148a does not effect the S phase but it reduces the population in G_0 phase in both PC3 and DU-145 cells. In both the cell lines it induces apoptosis with increasing concentration of miR-148a (Fig 4.10).

Western blot analysis of cleaved PARP demonstrated that, cells transfected with miR-148a mimics activates apoptosis in both PC3 cell lines. Previously it was reported in pancreatic and colorectal cancer cells that, miR-148a induces apoptosis by targeting the anti-apoptotic oncogene BCL-2. However, it differs in case of bladder cancer where miR-148a doesn't affect the expression BCL-2. Moreover, in prostate cancer miR-148a decreases the protein expression of BCL-2. It also affected the expression of apoptotic gene BAX and increased its expression. To find if these results are related to DNMT1 downregulation DNMT1 siRNA was transfected in the PC3 cell line. The western blot analysis shows a similar effect on inducing apoptosis-like miR-148a. This suggests miR-148a induce apoptosis by downregulating DNMT1. These data suggested overexpression of miR-148a has a predominant role in activation of apoptosis (Fig 4.11).

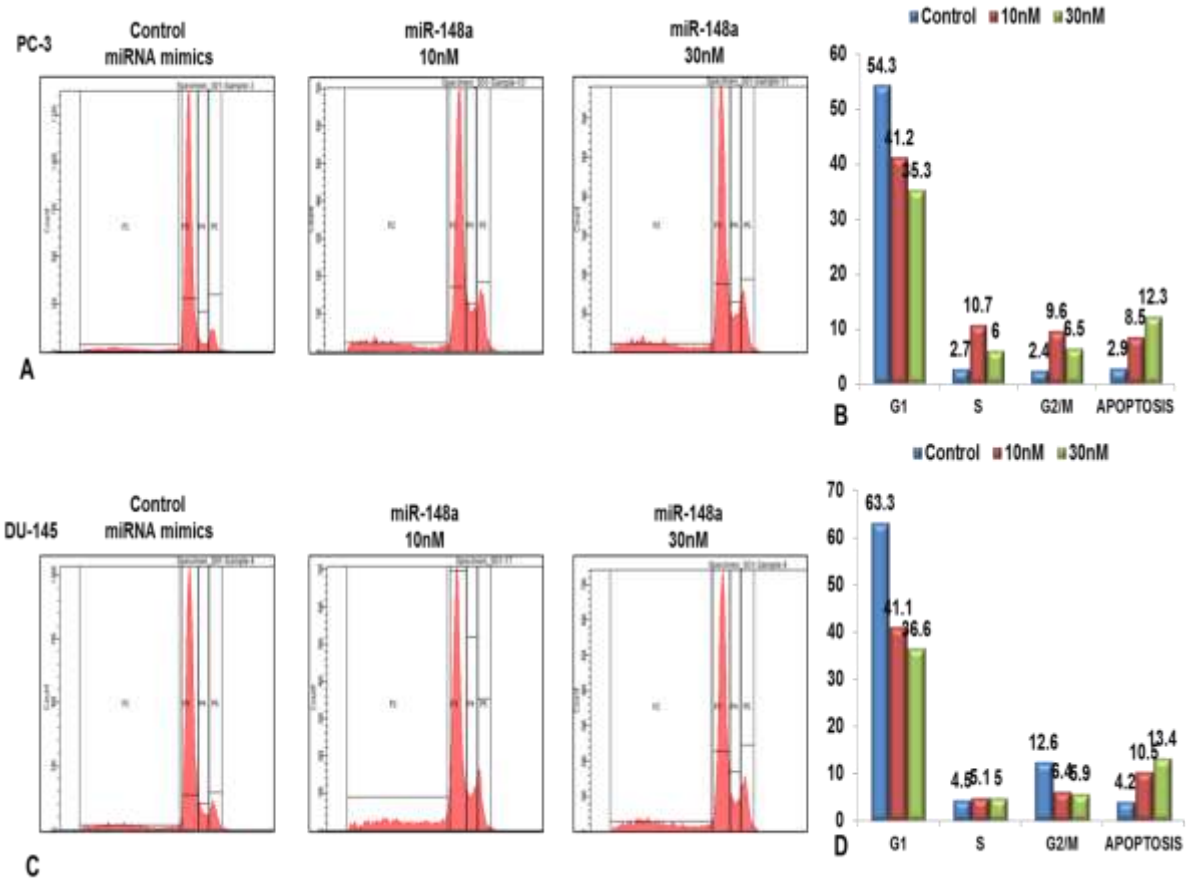

Figure 4.10: Apoptotic population and cell cycle analysis by flow cytometry:
miR-148a mimic transfection [A-B] PC3 and [C-D] DU-145 cell increase apoptotic population. [C-D] Graphical representation of apoptotic population and different cycle stage population in [C] PC3 and [D] DU-145 cells are represented. (n=3 independent experiment, mean±S.D.). $P < 0.05$

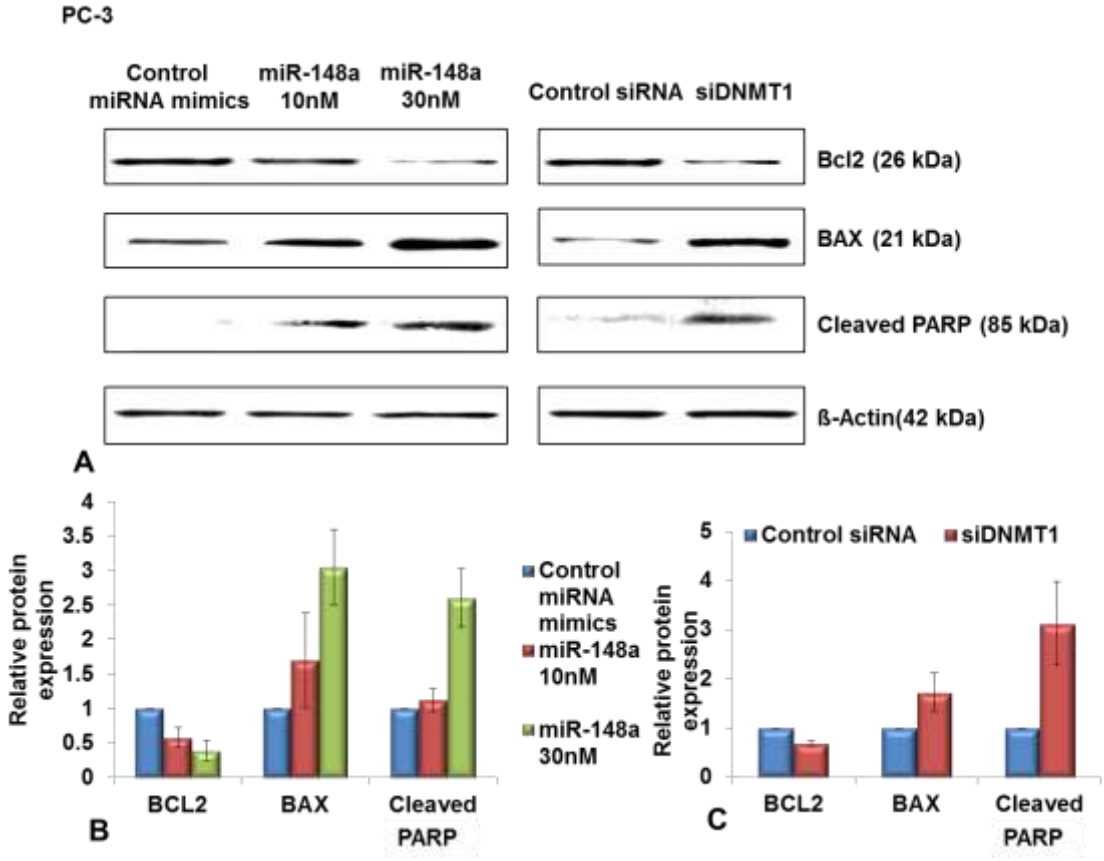

Figure 4.11: BCL2, BAX and cleaved PARP expression:
[A] BCL2, BAX and cleaved PARP protein expression and quantification were measured by Western blot in PC3. β-actin was used to confirm equal loading. The graphical representation of relative protein expression was measured by comparing with respective control. [B] A control miRNA transfected cells were used as a control for miR-148a transfected cells and [C] control siRNA transfected cells were used as a control for siDNMT1 transfected cells. Band intensity was measured by ImageJ software. (n=3 independent experiment, mean±S.D.). $P < 0.05$.

4.3.7 miR-148a expression is regulated by DNMT1 in prostate cancer

In pursuit,of the connection between downregulation of miR-148a and overexpression of DNMT1 in prostate cancer the promoter of miR-148a was analysed. It was previously stated that DNMT1 can silence any tumour suppressor by methylation of DNA and silence gene expression [29]. http://methhc.mbc.nctu.edu.tw/php/index.php database analysis confirms that promoter region of miR-148a is methylated in clinical prostate cancer samples. This database has stored information regarding the correlation between expression of genes and DNA methylation % at promoter region. Data mining from above database indicates a negative correlation value which confirms miR-148a expression is regulated by DNA methylation. To confirm the role of DNMT1 in the regulation of miR-148a in prostate cancer PC3 and DU-145 cells were transfected with DNMT1 siRNA and its help in the restoration of the miR-148a expression. Then to ascertain the role of DNMT1 in repression of miR-148a PC3 and DU-145 cells were

transfected with pcDNA3/MycDNMT1 overexpression vector shows repression of miR-148a (Fig 4.12).

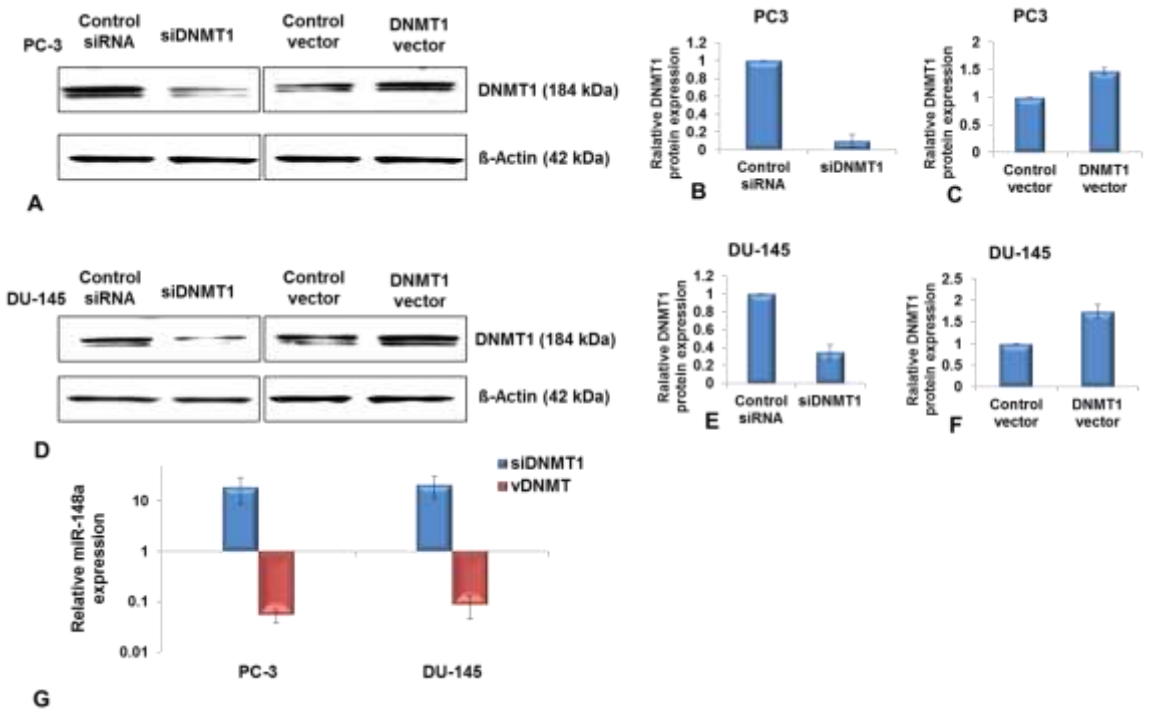

Figure 4.12: miR-148a expression analysis after DNMT1 overexpression and knockdown: DNMT1 protein level was measured by western blot after transfection with pcDNA3/Myc-DNMT1 vector and siDNMT1 in PC3 and DU-145 cells. DNMT1 protein expression and quantification were measured in [A-C] PC3 and [D-F] DU-145. β-actin was used to confirm equal loading. The graphical representation of relative protein expression of DNMT1 compare to respective control is represented in [B and C] for PC3 and [F and G] for DU-145. The band intensity was measured by ImageJ software. [G] Expression level of miR-148a in PC3 and DU-145-145 cells after transfection with siDNMT1 and vDNMT1 (n=3 independent experiment, mean±S.D.). $P < 0.05$

4.4 Discussion

The dual roles of miRNA as tumour suppressors or oncogenes create a great mystery about its nature and function in cancer. These phenomena of miRNAs were also applicable for prostate cancer [352-355]. Still, the large portion of miRNAs function in hormone-refractory prostate cancer is unknown. MiRNA expression profiling of prostate cancer patient and cell lines now has been documented [356-358]. Previous microarray data analysis reported by Mattie et al. [356] revealed miR-148a remain silenced in the advanced prostatic tumour (Gleason score 8) and prostatic lymph node metastasis than the normal adjacent tissues. Another group Porkka et al. [357] showed the expressional difference between prostate carcinoma samples and benign prostatic hyperplasia (BPH) where it was acknowledged that miR-148a remain downregulated in hormone-refractory carcinomas compared with BPH. In this chapter, it was shown that the miR-148a

expression was downregulated in PC3 and DU-145 cell lines than normal immortalized keratinocyte cell line HaCaT by RT- PCR. In order to identify the down-regulated miRNA that acts like tumour suppressor and can be a viable therapeutic target for hormone-refractory prostate cancer here, miR-148a has been studied. Previous studied already identified the downregulation of miR-148a is not due to a defect in p53 in PC3 cells [348]. In primary breast cancer tissue samples and metastasized lymph node derived cell line shows aberrant hypermethylation of the CpG island in the promoter causes the silencing of miR-148a [4, 359]. But the exact mode of silencing of miR-148a in prostate cancer is still not studied. Ectopic expression of miR-148a in head and neck cancer SIHN- 011B cells inhibits cell motility and proliferation. It also reduced the tumour size and inhibits metastasis in xenograft models [4]. In this study, it is demonstrated that ectopic expression of miR-148a induces apoptosis in part by downregulating DNMT1. This results suggested that dysregulation of miR-148a can initiate the metastatic potential of PC3. DNA methyltransferase plays a significant part in the aberrant gene expression related to tumour initiation and progression [360]. Previously it was reported miR-148a directly targets DNMT3b in MCF-7 and Hela cell line. In PC3 cells downregulation of DNMT3b inhibits cell migration and cell growth which suggest the gene responsible for the inhibition of migration and growth may be silenced by DNMT3b [361].

This study proves that ectopic expression of miR-148a leads to initiation of apoptosis in part by targeting DNMT1 the maintenance DNA methyltransferase. Variation of DNMT1 expression level modifies the global transcriptomes because downregulation of DNMT1 de-repress the silenced genes. The overall effect of the global change is cell context dependent. The previous study on bladder cancer shows DNMT1 expression was increased in stage-dependent manner [362]. Additionally, it also reported that it inhibit cell proliferation and induce apoptosis [363]. Dhawan et al. demonstrated in canine model targeting DNMT1 can be used a viable therapeutic model for future discoveries [364]. Here it was shown from the patient databases in prostate cancer DNMT1 expression was also dependent on the stages of cancer. The metastatic stages show a higher level of DNMT1 expression. The de-regulation of DNMT1 plays a dynamic role in prostate cancer progression, but the cause remains unknown. In this chapter, it was also shown that the survival of the patient depends on the DNMT1 expression. Repression of miR-148a plays an imperative protagonist in prostate cancer progression. Previously it was reported miR-148a increase the chemosensitivity of PC3 cells [348]. This study reveals targeting DNMT1 by miR-148a increased cellular apoptosis in cells. Previously in

colorectal cancer, it was reported miR-148a repress the expression of BCL2 [365]. This study reveals in prostate cancer cell line PC3 miR-148a inhibits BCL2 and induces apoptosis. It also increases the expression of BAX and cleaved PARP. Here it was also shown that the increasing level of miR-148a in prostate cancer helps in survival and delay cancer recurrence. miR-148a inhibits cell proliferation and inhibits cell growth was determined by cellular assays. It causes cell death by anoikis is also evidenced by this study. From this, it can be concluded that miR-148a inhibit metastasis by causing the death of cells by anoikis. This report suggests miR-148a target DNMT1 in a region which is conserved, and it was proved by luciferase assays. In cell cycle analysis the results indicate it affect the cellular apoptosis and reduce the cells in G_0 phase. The increasing rate of apoptosis was also observed when PC3 cells were transfected with DNMT1 siRNA. So the role of DNMT1 in prostate cancer can be defined as an oncogene. Then the cause of silencing of miR-148a was revealed by inhibiting and overexpressing DNMT1 by siRNA and vector. Targeting DNMT1 induces miR-148a expression whereas overexpression downregulated its expression. From this results it can be concluded miR-148a remain downregulated in PC3 and DU-145 by repression of miR-148a gene by DNMT1. These results demonstrate miR-148a has a potential as a novel therapeutic agent in the treatment of hormone-refractory prostate cancer. Not only targeting DNMT1 its role can be extended as an apoptosis inducer. MiRNA has the ability to target multiple mRNA targets simultaneously, and it gives it a cutting edge advantage to the fight with cancer. In conclusion here in this chapter the role of miR-148a has been elucidated for a novel therapeutic for prostate cancer.

Chapter 5
Objective 3

5. To decipher the role of miR-193a in regulation of histone modifier MLL1 and a comparison with DNA methylation in prostate cancer

5 .1 Introduction

Cancer is a group of diseases developed due to multiple genetic and epigenetic changes that cause aberrant gene expression and exhibit uncontrolled growth. Prostate cancer is one of the most common cancers in men and increasing significantly worldwide [366]. Androgen receptors are essential for initiation and progression of prostate cancers. So androgen –ablation therapies has been a primary treatment option for androgen –dependent prostate cancer. However, when prostate cancer progress to a late stage it become a fatal castration-resistant disease. Recent studies on epigenetic changes during prostate cancer opened a new avenue in understanding the mechanisms leading to the onset of prostate cancer and prognosis, diagnosis and treatment of the disease would be facilitated further. Epigenetic changes involve multiple chemical modifications of chromatin including DNA methylation, covalent modification of histone tails and small RNA interference. Histones are not only DNA packaging proteins; a large no of posttranslational modifications of histone including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization are associated with gene regulation. In a recent study, it was indicated that presence of bivalent domains (when histone marks are present at multiple positions and dictates apparently opposite functions) in prostate cancer cell line PC-3 in which the bivalent mark genes are activated [367] . Some studies show overexpression of H3K4me1 and H3K4me2 demethylase KDM1 significantly decreases AR binding [99] other study shows increased H3K4me3 in prostate cancer cells related to activation of genes involved in cell growth and survival (e.g., FGFR1 and BCL2) [100]. Since the increased active chromatin marks like H3K4 methylations in prostate cancer facilitate activation of proto-oncogene and other genes involved in cell growth and survival, it is possible that increased repressive histone marks in prostate cancer lead to tumour suppressor gene silencing. So in this study, the focus was on H3K4 methylation mark and its methyltransferase. Using *in-silico* tools miRNA were searched, which remains downregulated in prostate cancer and targets histone methyltransferase. Those searches identified that more than 25 miRNA remain downregulated in prostate cancer. Out of them, miR-193a have seed region in MLL1, which is the key methyltransferases for H3K4 trimethylation mark. Ectopic upregulation of miR-193a significantly decrease global H3K4me3 in prostate cancer cell

line. This study also found miR-193a plays a major role in cell death cellular migration, anchorage-independent growth of cancer cells. In this study, the changes in expression of miRNA and its effect on histone code in prostatic carcinogenesis have been showed.

5.2 Material and methods

5.2.1 Tissue samples and immunohistochemistry

25 Formalin-fixed paraffin-embedded (FFPE) prostate cancer tissue sample were collected from Drs.Tribedi & Roy Diagnostic Laboratory (Kolkata, India). FFPE specimens were sliced into 0.5μm and subjected to antigen retrieval with tris-EDTA buffer, an endogenous peroxidase blocking and rinsed with tris-buffered saline (TBS) containing 0.025% Triton X-100 (TBS-T). Mouse monoclonal antibody against MLL1, rabbit polyclonal antibody against H3K4me1, H3K4me2, and H3K4me3 were used as primary antibodies. The secondary antibodies used were Alexa flour 647 conjugated anti-mouse (Abcam) and FITC conjugated anti-rabbit antibody (Santa Cruz Biotech) solution for 1 h. Then the samples incubated with DAPI (1 mg/ml) for 10 min. Finally, cells were washed three times with TBS and slides were mounted with a mount with ProLong® Gold Antifade Mountant (Thermo fisher) and observed under Epifluorescent Microscope (Olympus IX71).

5.2.2 Cell culture

PC-3 and DU-145 prostate cancer cell lines and HaCaT immortal keratinocyte cell line were obtained from The National Centre for Cell Sciences, Pune (NCCS), India. PC-3 cells were grown in Ham's F-12 Nutrient Mix (Gibco Auckland, New Zealand,) and DU-145 and HaCaT cells were Dulbecco's modified Eagle's medium (Gibco Auckland, New Zealand,) supplemented with 10% fetal bovine serum (FBS) (16000–44; Gibco, Carlsbad, CA), and 100 U Penicillin & 0.1 mg Streptomycin in a humidified atmosphere of 5% CO_2 at 37^0C.

5.2.3 Transfection of cells with miR-193a vector

The effect of miR-193a on cell survival and apoptosis are analyzed by miR-193a vector which was obtained from Genecopoeia (HmiR0277-MR04). PEZX-MR04-hsa-miR-193a and pEZX-MR04 GFP vector were used to transfect PC3 cells along with Lipofectamine 2000 transfection reagent (Invitrogen) and optimem transfection media

(Invitrogen). Further cells were incubated at 37 °C, 5% CO_2 for 48 h to carry out different assays. As a control, CmiR0001-MR04 vectors from Genecopoeia were used. GFP expression was analyzed by ImageJ software. Corrected total cell fluorescence (CTCF) was calculated by this formula = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

5.2.4 RNA extraction and quantitative reverse-transcription (qRT)-PCR

Total cellular RNA was extracted with TriReagent (Sigma) according to the manufacturer's instructions. qRT-PCR was performed using cDNA prepared from 1 μg of total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentas) and SYBR® Green JumpStart™ TaqReadyMix in the Realplex4Eppendorf system. The PCR were carried out using standard protocols, and the cDNA was amplified under the following conditions: 95°C for 2 min, 40 cycles of 95°C for 30 s, 59.8°C for 30 s and 72°C for 30 s. The expression level of MLL1 was analyzed after the cells were transfected with miR-193a vector. For apoptosis analysis, BCL2 and BAX mRNA were also analyzed. The mRNA level was normalized to the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. To quantify the level of miR-193a in cell lines miRNA was isolated by using miRVana™ miRNA Isolation Kit, with phenol (Invitrogen) by following the protocol of the kit. From the isolated miRNA, cDNA of miRNA was prepared using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) followed by analysis using qRT PCR.

For qRT-PCR of miRNA, primers were designed by Invitrogen miRNA primer designing protocol. The qRT-PCR of miRNA was done by NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen). The cDNA of miRNA was amplified under the following the temperature cycle 50^{o}C for 2 min, 95^{o}C for 2 min 40 cycles of 95^{o}C for 15 s, 60^{o}C for 30 s. Then all the results were interpreted using Eppendorf qRT-PCR software. We analyze the expression of miR-193a, normalized to the expression of U6 small RNA. All the primers sequence was mentioned in Table 5.1

Table 5.1 Primer List

Gene	Forward Primer	Reverse Primer
MLL1	AATCCTAGCCGTTAGGCCG	TTGGGGCAGGTTTGGGTTA

BCL2	CCTGTGGATGACTGAGTACC	GAGACAGCCAGGAGAAATCA
BAX	TTCATCCAGGATCGAGCAG	CGCTCAGCTTCTTGGTGG
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAACGCA
miR-193a	CATGGGTCTTTGCGGGCGAGATGA	Universal Reverse Primer for miRNA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT

5.2.5 Western blotting

The cells were grown up to 80–85% confluence and then transfected with miR-193a. The cells were then harvested, washed with PBS and lysed using RIPA buffer (Sigma). Then the cells were kept in 4^{o}C for 10 min to complete the cell lysis. Then the lysate was centrifuged at 8000 g for 10 min at 4^{o}C. Same amount of protein was loaded in 10% SDS-PAGE for electrophoresis. Then separated proteins were transferred onto PVDF membrane. After blocking the membrane with 5% BSA in PBST solution, the membrane was incubated with specific primary antibody Mouse monoclonal antibody against MLL1, rabbit polyclonal antibody against BCL2; BAX cleaved PARP, H3K4me1, H3K4me2, and H3K4me3 were used as primary antibodies overnight at 4^{o}C. Subsequently, the membrane was washed three times; 10 min/each time with PBST buffer, and incubated again with an appropriate HRP conjugated secondary antibody [Rabbit anti-mouse IgG-HRP, Goat anti-rabbit IgG-HRP Santa Cruz Biotechnolog] at 37°C for 2 h. The membranes were washed with PBST buffer and were developed by Supersignal West Femto-chemiluminescent substrate (Thermo Scientific). β Actin protein levels were used as a control for adequacy of equal protein loading.

5.2.6 Luciferase miRNA target reporter assay

3′ UTR of MLL1 gene (1536 bp), containing predicted binding sites of miR-193a, were amplified by PCR HaCaT DNA and inserted into the pcDNA3.1luc immediately downstream from the stop codon of Firefly luciferase (pcDNA3.1- MLL1 3'-UTR WT). Deletion of the first 3 nucleotides corresponding miR-193a seed region complementary site was inserted in mutant constructs using, GeneArt® Site-Directed Mutagenesis System (Catalog number: A13282, Thermo fisher scientific) according to the manufacturer's protocol. (pcDNA3.1- MLL1 3'-UTR MUT). PC3 cells were cultured in 96-well formats and cotransfected with 100 ng of pcDNA3.1luc vector 20 ng of pRL-TK Renilla luciferase

control vector (Promega) and 600ng and 800ng per miR-193a vectors well with negative control miRNA using Lipofectamine 2000. Firefly and Renilla luciferase activities were measured consecutively using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection. All the experiments were done in triplicate and repeated at least twice on different days.

5.2.7 Analysing cell viability, morphology, and cytotoxicity

For analyzing cell viability, MTT assay was performed. Cells are seeded at a density of 5000 cells per well in 96 well plates to assess the minimum inhibitory concentration (IC50). After 24 hours of treatment, cells were washed with PBS and 100 µl of MTT was added in serum-free media at a concentration of 0.8 mg/mL prepared freshly from a stock of 5 mg/ml. After treatment with MTT, cells were incubated for 4 hours in the dark CO_2 chamber. The medium was then aspirated, and 100 µl of DMSO was added to each well and left again for a period of 15 minutes in dark at room temperature. The plates were mixed gently until a clear purple color appears. Optical density (OD) was measured at 595 nm using an ELISA plate reader, and the % of cell viability was calculated using the following formula: Inhibition rate (%) = (Average OD value of experimental group-Average OD value of the control group)/Average OD value of the control group x 100%. Morphological analysis of cells was performed in bright field microscopy using Epifluorescent Microscope (Olympus IX71).

5.2.8 Flow-cytometry analysis for cell cycle

For cell cycle analysis, 10^5–10^6 PC-3 cells were incubated and then trypsinized, followed by centrifugation (500 x g) for five minutes at 4°C. Next, cells are washed twice with PBS and fixed at -20°C in 90% ice cold methanol. After 1 h of incubation, cells were centrifuged and suspended in PBS with RNase A (500 U/mL) to digest the residual RNAs followed by PI (10 µg/mL) staining and incubation at 37 °C for 30 minutes. The analysis was performed on Becton-Dickinson fluorescence-activated cell sorter (FACS). Three independent samples of treated samples were analyzed, and descriptive statistics of the results are reported as mean ± SEM.

5.2.9 Analysis of Reactive oxygen species (ROS) production by flow cytometry

PC3 cells were (5×10^5) cultured in a 6 well culture plate up to 70% confluency after transfected with miR-193a vector for 24 h. Cells were washed with fresh medium twice

and resuspended in 1× binding buffer at a concentration of 1 × 105 cells/mL. The cell suspension (100 µL) was transferred to a 5 mL culture tube for analysis. For ROS detection, cells were treated with DCFH-DA (2′,7′-dichlorofluorescein-diacetate). Cells were analyzed in a Becton Dickinson FACScan flow cytometer using Ex/Em 490/525 nm for the oxidative stress. Negative controls included untreated cells and cells treated only with Lipofectamine and control vector.

5.2.10 Cell Viability Assays

Approximately 500 cells were seeded in 96 well plates for cell viability assay. After 24 hrs cells were transfected with miR-193a vectors in 1µg/ml, 2µg/ml, 4µg/ml, 6µg/ml and 8µg/ml concentration with lipofectamine and using optimem medium. After 6hrs transfecting media was removed and fresh media was added. To detect the cell viability MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] working solution was prepared by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS to the final concentration of 0.8mg/ml. 100 µl of MTT working solution was added to each well and incubated for 24 hrs in CO_2 incubator. After incubation, the media was removed carefully without disturbing formazan precipitate and dissolved in 100 µl of 100% DMSO. An incubation of 15 min was carried out in the dark, and the colorimetric estimation of formazan product was observed at 570nm in a microplate reader. The data was plotted against drug concentration, and non-linear regression curve fitting was performed using software to calculate the optimal growth inhibitory concentration (LC50) of the miR-193a vector.

5.2.11 Soft agar colony and invasion assays for the effect of miR-193a

For studying cellular transformation process in the presence of miRNA soft agar assay was performed as described previously [349]. In this assay first, a base agar/media mix was added to 6-well plates (final concentration of 0.5% agar) to coat the plate and allowed to polymerize. Then cell suspensions were made up of 100 cells/ml in media/agar mix (final concentration of 0.3% agar). On the top of the base agar, 1.5 ml of the cell suspension is added to each well and allowed to polymerize. After complete polymerization, 2 ml complete media were added on top of the agar once again, and the plates were put in 37°C, 5% CO_2, and overlay media was changed every 3–4 days. Cultures were grown until a few colonies were visible by eye and stained with crystal violet.

5.2.12 Colony Formation Assay

The colony-forming potential of adherent cells is demonstrated as previously described [346]. After the transfection of cells with miR-193a vector cells were re-seeded onto 12-well plates at 200 cells/well. After 2 weeks, colonies were fixed with 100% methanol for 15 min and stained with crystal violet for 20 min. After taking photographs, the number of colonies with a diameter more than 1.5 mm was counted. Only adherent cells were considered for the colony-forming potential experiment. Plating efficiency (PE) and the number of colonies that arise after treatment of cells, surviving fraction (SF) was measured by following formulas.

PE= (no. of colonies formed/no. of cells seeded) x 100

SF= [(no. of colonies formed after treatment/(no: of cells seeded x PE)] x 100

Three independent experiments were done with three replicas to calculate the P value and validate the results (mean±S.D, $P < 0.05$).

5.2.13 Chromatin condensation assay and nuclear staining with PI

After treatment with different concentration of the miR-193a vectors for the specific time, the cells were stained with Hoechst 33342 stain (1 mg/ml) and P1 (10µg/ml) and incubated for 10 min at 37 °C. Images were observed under ~460 nm emission for Hoechst 33342 dye and ~617nm emission for PI using Epifluorescent Microscope (Olympus IX71). Three independent experiments were done to calculate the P value and validate the results (mean±S.D, $P < 0.05$). In each condition, 5000 cells were counted to analyze the result.

5.2.14 Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using the Student's t- test by SPSS software. Values of $P < 0.05$ were considered as significant value.

5.2.15 Ethical Approvals

This study was deemed exempt from ethics approval from the National Institute of Technology and consent was not required due to use of cell lines.

5.3 Results

5.3.1 Analysis of survival potential based on miR-193a expression in clinical samples and cell lines

To find out the effect of miR-193a on prostate cancer patients survival Kaplan-Meier plot of prostate cancer clinical samples were drawn. The survival curve, retrieved from Kaplan- Meier plot analysis show that higher expression of miR-193a is associated with the increased survival of the patients with a significant P-value (Fig. 5.1 A).

The expression level of miR-193a in prostate cancer cell line was analyzed, using PhenomiR [368] database. It was observed that in PC3, DU-145, PPC-1, LNCaP and Tsu-Pr1 cell lines miR-193a remain downregulated. For validating, the data acquired from the dataset miR-193a expression level was analyzed by RT-PCR analysis in PC3, DU-145 cell lines where it shows miR-193a expression was downregulated in PC3, DU-145 cell lines 3.81, 4.67 fold respectively when compared HaCaT cell line (Fig. 5.1 B).

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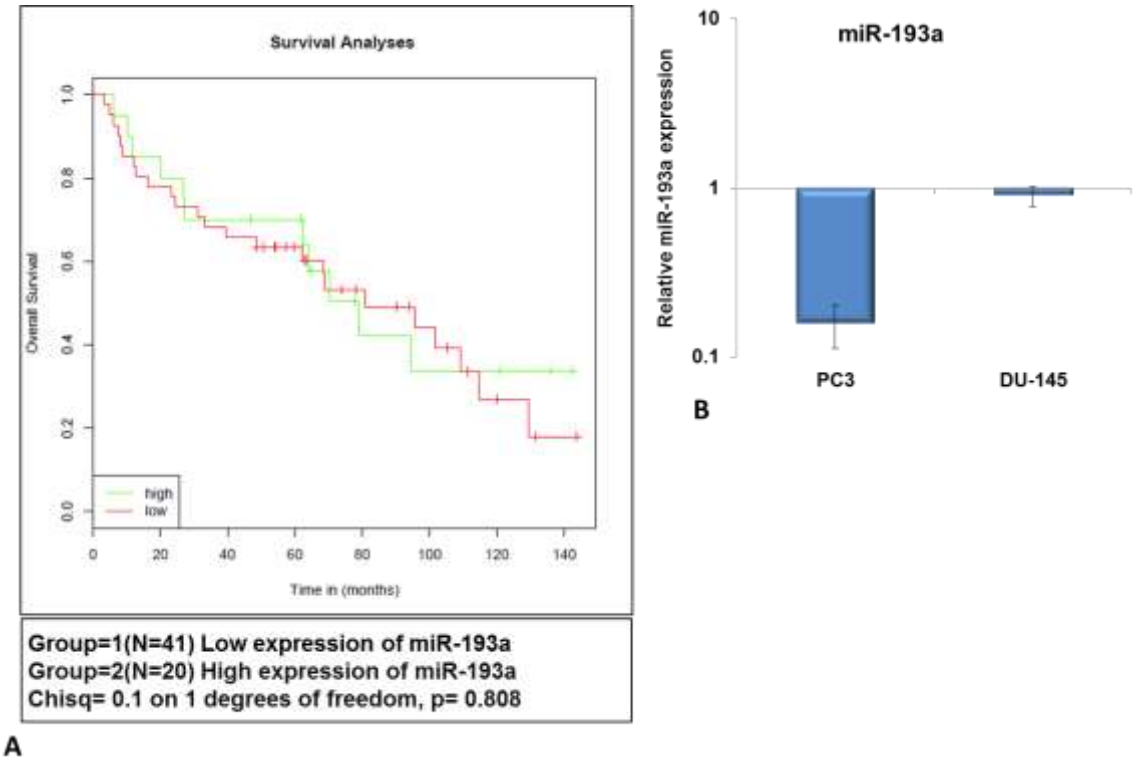

Figure 5.1: Kaplan–Meier analysis plot and mRNA expression of miR-193a:
[A] Overall patient survival associated with miR-193a was analyzed by Kaplan–Meier plot. Total 61 numbers of samples were analyzed from miRumiR GEO database. P- Value is [p= 0.0963]. [B] RT-PCR analysis of mRNA level expression of miR-193a expression in PC3 and DU-145 cell lines.

5.3.2 MLL1/KMT2A overexpression is associated with prostate cancer progression

Database analysis shows that unlike miR-193a, MLL1 overexpression is associated with prostate cancer patient death. mRNA expression level analysis by cBioPortal database shows the expression level of MLL1 is very high in prostate cancer patients population (Fig. 5.2 A). Overall patient survival decreased with increase in MLL1 expression. GEO dataset of 61 patients was analyzed by Kaplan- Meier survival plot from prostate cancer clinical samples. The survival curve shows an antagonistic role of MLL1 expression in patient survival (Fig. 5.2 B). Moreover, MLL1 expression is inversely related to prostate cancer recurrence. The cancer recurrence plot demonstrates that MLL1 expression positively regulates the prostate cancer recurrence. Although the P-value is not very highly significant in recurrence, curve but it gives a view towards the correlation of MLL1 expression and prostate cancer recurrence (Fig. 5.2 C). Altogether, the cancer supportive role of MLL1 is crystal clear.

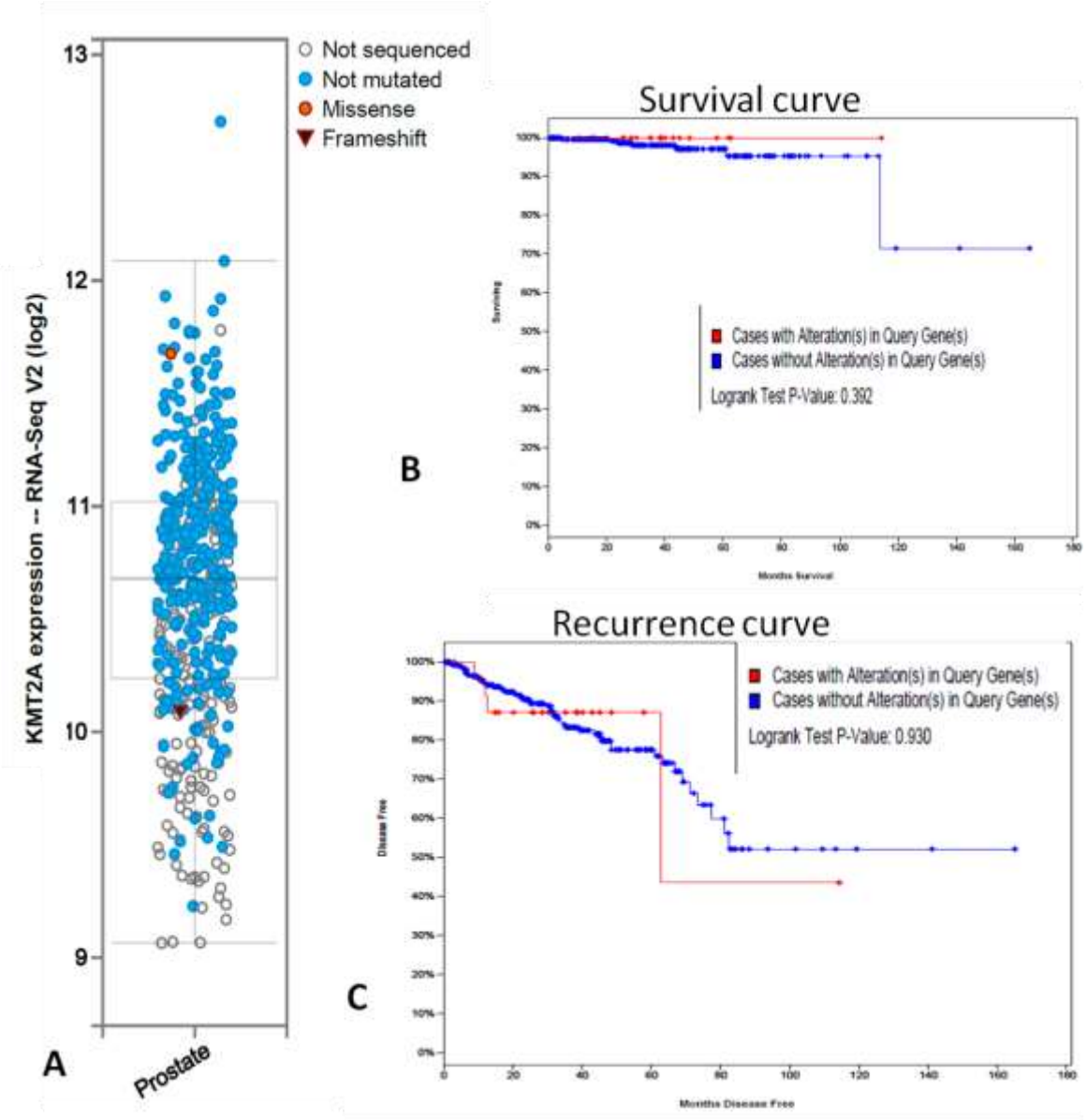

Figure 5.2: MLL1 expression and patient survival analysis:
MLL1/KMT2A mRNA expression plot in prostate cancer patients. [B] Overall patient survival was analyzed by Kaplan–Meier plot and the P-value is 0.392. [C] The cancer recurrence plot basis on the MLL1 expression in the prostate cancer patients and the P-value is 0.930.

5.3.3 Identification of conserved miR-193a target sites within the 3'-UTR of MLL1

Above results confirm that miR-193a is downregulated in prostate cancer. Still, the miR-193a mediated regulatory mechanism was unknown in the prostate. Previously several groups reported that MLL1 overexpression is associated with different type of cancer development [369]. MLL1 is not only acting as H3K4 methyltransferase but also connected with other genes expression. Using String database, the connections of MLL1 with other genes have been shown (Fig. 5.3). Nevertheless, none explains the relation between miRNA-mediated regulations of MLL1 in cancer development. First time in this

study the regulation of MLL1 was connected to miR-193a expression. UCSC genome browse search shows the binding region of miR-193a to the 3'UTR of MLL1 (Fig. 5.4 A). The 7mer-8m conserve site of 3'-UTR of MLL1 for miR-193a is also conserve among different vertebrates (Fig. 5.4 B). On the basis of this information it was considered that the 3'-UTR of MLL1 gene is regulated by miR-193a during transcription. To evaluate this hypothesis future experiments were conducted.

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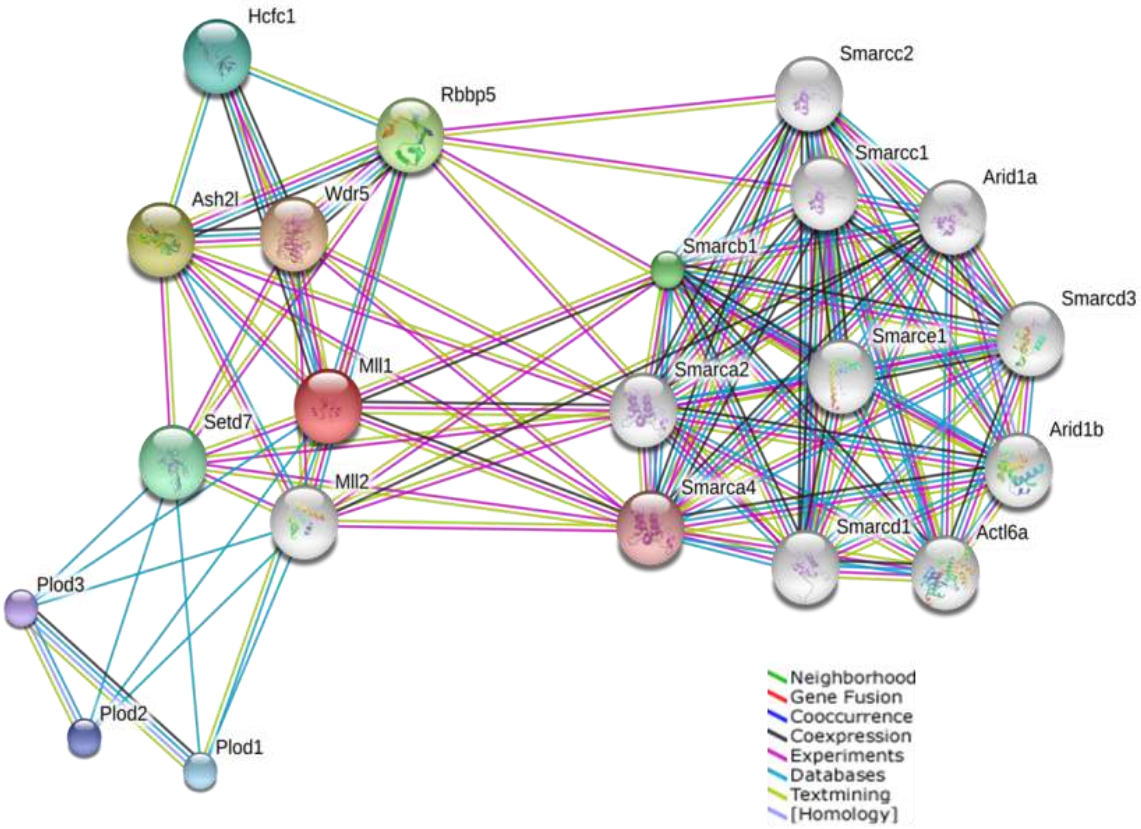

Figure 5.3: String databases analysis:
Correlation of MLL1 with another protein is shown. From this database, it shows MLL1 forms a network with other chromatin modifier enzymes like MLL2, smarca4, smarcd1 etc. This analysis shows the importance of MLL1 in cellular gene expression.

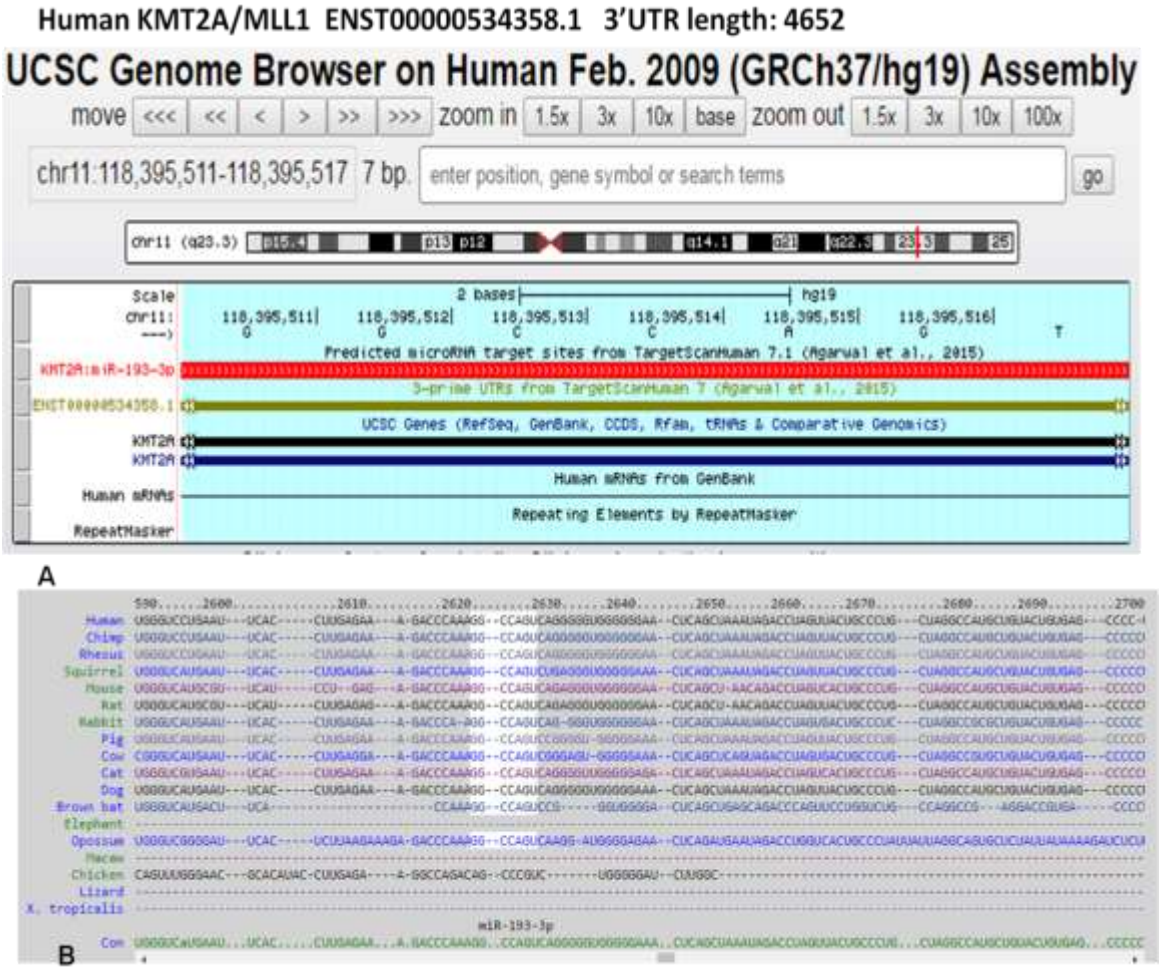

Figure 5.4: Characterization and binding site of the MLL1-3'-UTR with miR-193a:
[A] UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly view of 3' UTR of MLL1 or KAT2A. The highlighted area shows the binding site of miR-193a with 3' UTR of MLL1. The 3'UTR has a 7mer-8m binding site for miR-193a (chr11:118,395,511-118,395,517). [C] The 7mer-8m conserve site of 3'-UTR of MLL1 in different vertebrates. The conserved region is highlighted in white.

5.3.4 Validation of MLL1 as a direct target of miR-193a

To validate the correlation between miR-193a-3p and MLL1 expression was analyzed in PC3 cells after miR-193a overexpression. In these experiments, overexpression of miR-193a was achieved by transfecting cells with pEZX-MR04-hsa-miR-193a vector with relative to CmiR0001-MR04 transfecting cells. The efficient overexpression of miR-193a in PC3 cells are shown in Fig. 5.6. Increase GFP intensity with increase vector concentration proves the successful transfection of miR-193a vector (Fig. 5.5 A and B). Increased vector concentration simultaneously increases the miR-193a expression which was analyzed by RT-PCR. Cellular miR-193a levels were increased approximately 20-fold when PC3 cells were transfected with vectors (Fig. 5.5 C)

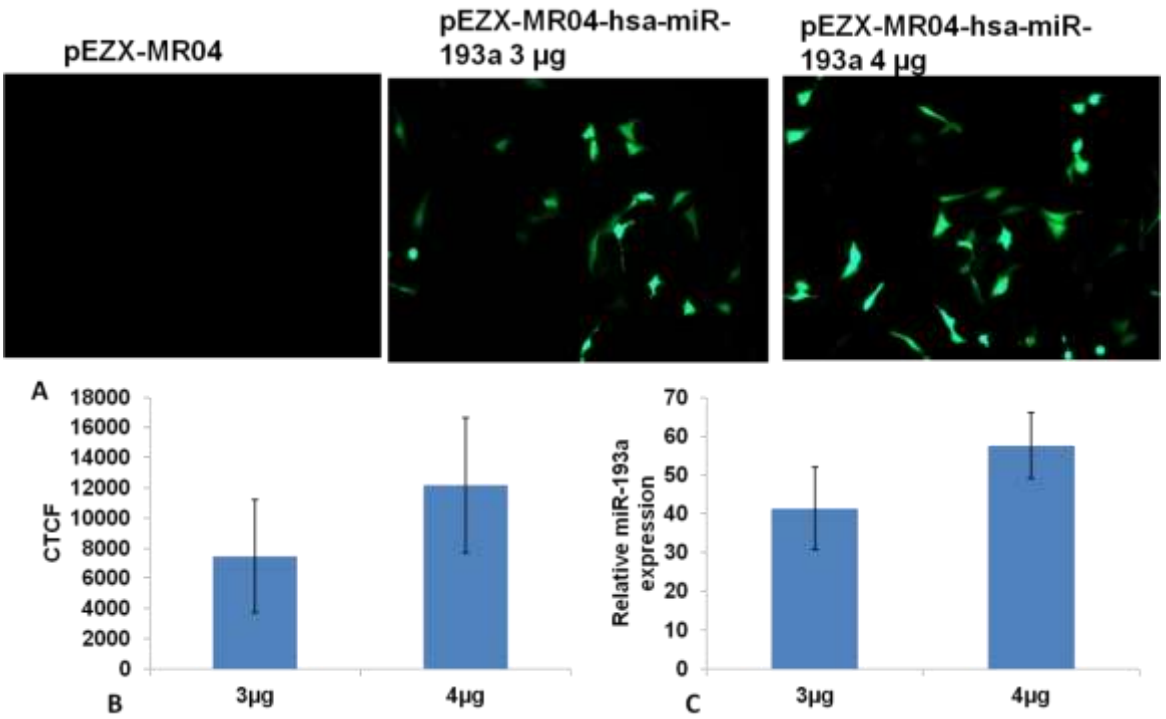

Figure 5.5: pEZX-MR04-hsa-miR-193a transfection and miR-193a overexpression:
[A] GFP expression after transfection with GFP vector pEZX-MR04-hsa-miR-193a (3µg and 4 µg) and pEZX-MR04 transfected cells. [B] The fluorescence intensity is represented in terms of CTCF. [C] The miR-193a expression was analysed by RT-PCR after transfection. miR-193a level in pEZX-MR04 transfected cell is normalized to 1 (n=3 independent experiment, mean±S.D.) $P < 0.05$.

5.3.5 miR-193a binds to 3' UTR of MLL1 to regulate the MLL1 expression

To confirm that, miR-193a targets the 3' UTR of MLL1 the luciferase reporter assays was done using 3' UTR of MLL1. Wild type (WT) and mutated (MT) 3' UTR of MLL1 was cloned separately into luciferase vector pCDNA3.1-LUC (Fig. 5.6 A and B). Cells were co-transfect the pCDNA3.1-LUC- MLL1-3'UTR WT or pcDNA3.1-LUC- MLL1 3'-UTR MUT and pEZX-MR04-hsa-miR-193a (3µg and 4µg) with Renilla luciferase vector and as control co-transfect luciferease-3' UTR-MLL1 and CmiR0001-MR04 (4µg) in PC3 cells. To confirm miR-193a binds to the predicted seed region of MLL1 3' UTR, the mutated pCDNA3.1-LUC-3'UTR-MLL1MUT was also transfected with pEZX-MR04-hsa-miR-193a. After measuring the Firefly: Renilla luciferase ratio it was found, that increasing concentration of miR-193a lowers the luciferase expression in the case of pCDNA3.1-LUC-3' UTR-MLL1WT transfected cells respective to control. But in the case of pCDNA3.1-LUC- MLL1-3' UTR MT transfected cells does not show any significant changes (Fig. 5.6 C). It confirms that miR-193a regulates MLL1 expression by binding in the 3' UTR region of the mRNA.

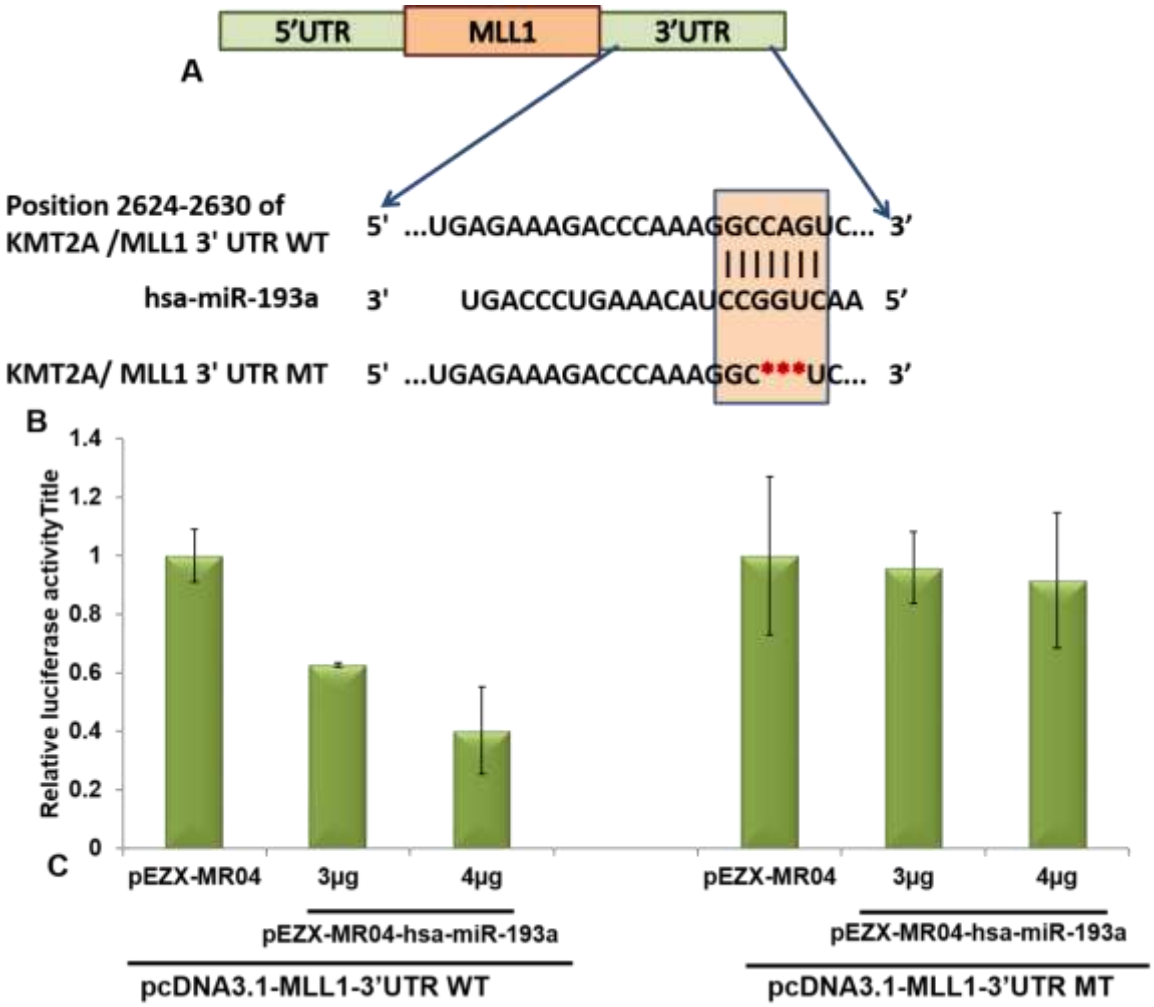

Figure 5.6: Functional analysis of the MLL1-3' UTR and miR-193a binding:
[A] Schematic representation of MLL1-3'-UTR, which was inserted downstream of Renilla luciferase gene in the pcDNA3.1 vector. [B] Complementarities between miR-193a and the MLL1 3'-UTR site target is shown. The side directed mutation was located within the 'MRE' region of the miR-193a binding site (red star). (C) The luciferase activity measured by luciferase assay. Renilla luciferase activity was normalized first to firefly luciferase activity and then to the values measured for the parental vector pcDNA. (n=3 independent experiments, mean±S.D.). $P < 0.05$.

5.3.6 miR-193a regulates global H3K4 methylation by targeting MLL1

To determine the regulatory level at which miR-193a affected MLL1 expression, we examined the expression of MLL1 mRNA in pEZX-MR04-hsa-miR-193a and CmiR0001-MR04 cells. It is found that overexpression of miR-193a affects the MLL1 expression at mRNA levels in PC3 cells (Fig. 5.7). As anticipated, overexpressing miR-193a significantly decreased the MLL1 protein levels in PC3 cells.

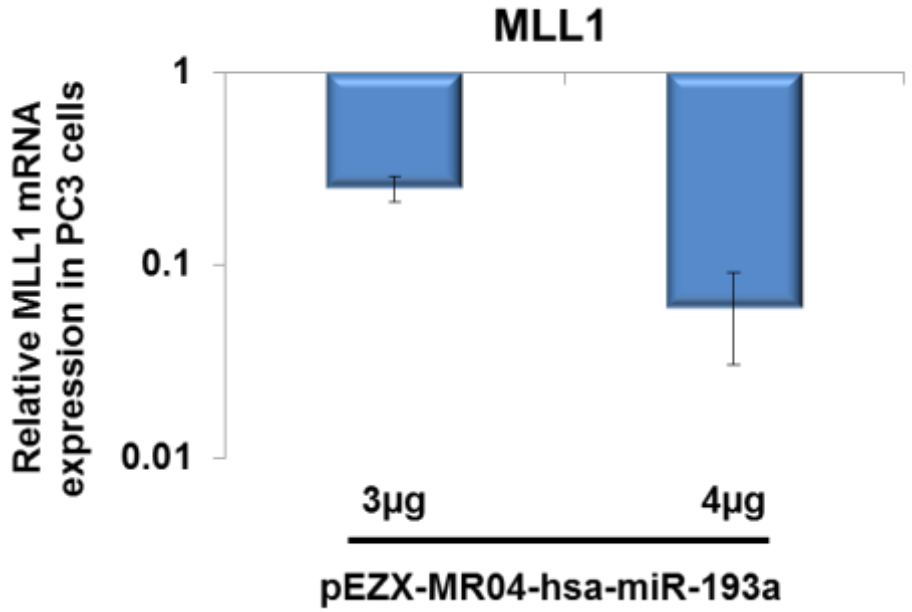

Figure 5.7: MLL1 expression after pEZX-MR04-hsa-miR-193a transfection:
3µg and 4 µg pEZX-MR04-hsa-miR-193a transfected cells were subjected to RT-PCR analysis for MLL1 mRNA level study. MLL level in pEZX-MR04 transfected cells is normalized to 1 (n=3 independent experiment, mean±S.D.) $P < 0.05$.

Above results thus demonstrated that miR-193a downregulate the MLL1 expression in transcriptional level. Protein level analysis confirms that miR-193a regulates MLL1 expression at the both transcriptional and translational level. The graphical representation of western blot shown that relative MLL1 and MLL2 protein expression decrease after miR-193a overexpression. MLL1 protein level decreases up to 1.34 and 14.28 fold in 3µg and 4 µg pEZX-MR04-hsa-miR-193a transfected cells respectively with compared to control cells. It was also observed when miR-193a targets MLL1 simultaneously downregulated MLL2 expression at both mRNA and protein level. MLL2 does not have any seed region for miR-193a but its expression gets affected by MLL1 downregulation (Fig. 5.8 A and B).

Histone methyltransferase activity of MLL1 is specifically related to H3K4 methylation. H3K4 mono-, di- and trimethylation (H3K4me1, H3K4me2, and H3K4me3, respectively) are mostly connected with euchromatin formation and active gene expression [370]. Global H3K4 methylation pattern was elucidated after finding that miR-193a targets MLL1 and repress its expression. To understand the alteration in the global H3K4 methylation level PC3 cells were transfected with miR-193a vector. The protein level analysis of global H3K4 methylation shows that H3K4me3 and H3K4me2 level has been decreased in 3µg and 4 µg miR-193a clone transfected cells compare to control cells.

But surprisingly global H3K4me1 level has been increased. Previously it was studied that increased H3K4me3 in prostate cancer cells compared with normal prostate cells is correlated with activation of genes like FGFR1 and BCL2 which contributed to cell growth and survival [100]. These findings suggest miR-193a regulates the global H3K4 methylation mark by targeting MLL1 (Fig. 5.8 C and D).

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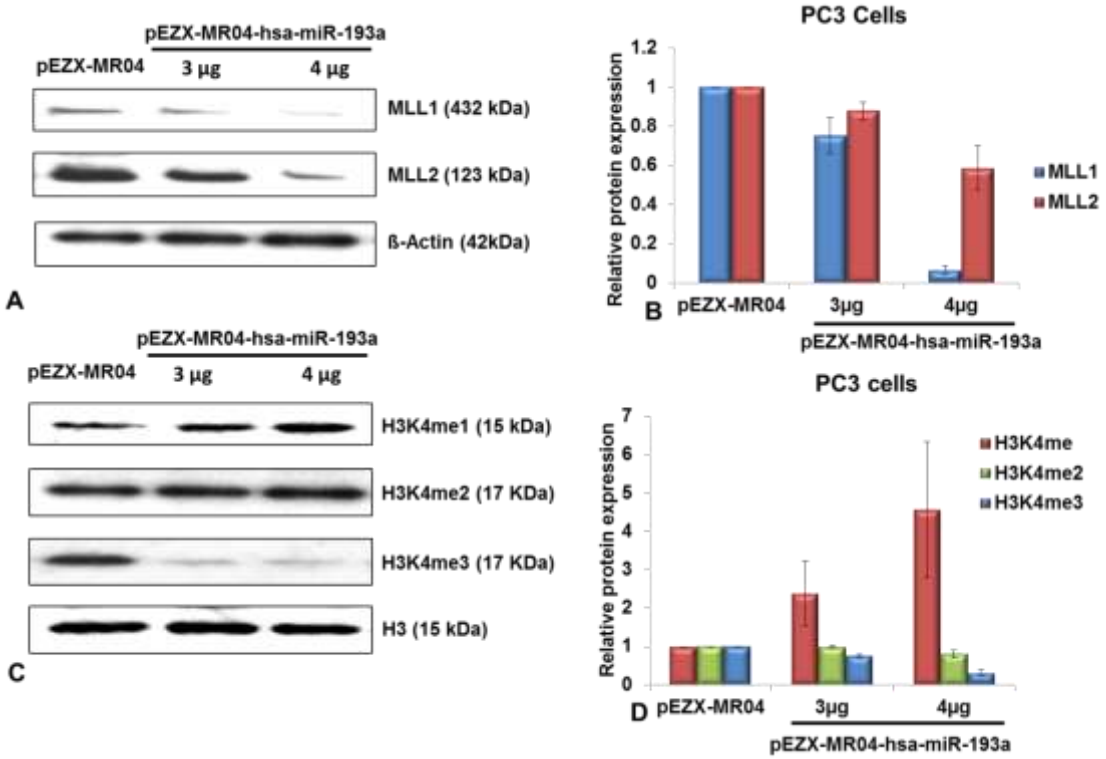

Figure 5.8: MLL1 and global histone modification analysis:
[A] MLL1 protein expression and quantification were measured by Western blot in PC3 cells after transfecting with 3µg and 4 µg pEZX-MR04-hsa-miR-193a. [B] The band intensity was measured by ImageJ software. β-actin was used as loading control. (n=3 independent experiment, mean±S.D.) $P < 0.05$. [C] Global H3K4me1, H3K4me2, and H3K4me3 marks were analyzed by western blotting in the PC3 cell line. [D] Protein quantification analyszed by ImageJ. Histone H3 was used as loading control. (n=3 independent experiment, mean±S.D.) $P < 0.05$.

5.3.7 Expression of MLL1 and global H3K4 methylation marks in prostate cancer tissue samples

During tumour development and cancer progression, modifications of lysine at various positions in histone 3 are predominant. To provide a rational view of what happens during physiological (in vivo) cancer development, 25 FFPE prostate cancer tissue samples were analyzed. H3K4 mono-, di- and trimethylation and MLL1 expression were evaluated by immunofluorescence staining. Among all the FFPE samples expression level of MLL1, H3K4me2, and H3K4me3 was high, and the percentage was 84%, 60%,

and 92% respectively whereas H3K4me1 level was low in prostate cancer samples, 36% (Table 5.2 and Fig. 5.9).

Table 5.2: Global protein expression in prostate cancer samples

Name	Number of Clinical prostate cancer sample	Protein expression (%)	Absence of protein expression (%)
MLL1	25	84% (21 sample)	16% (4 sample)
H3K4me1	25	36 % (9 sample)	64% (11 sample)
H3K4me2	25	60% (15 sample)	40% (10 sample)
H3K4me3	25	92% (23 sample)	8% (2 sample)

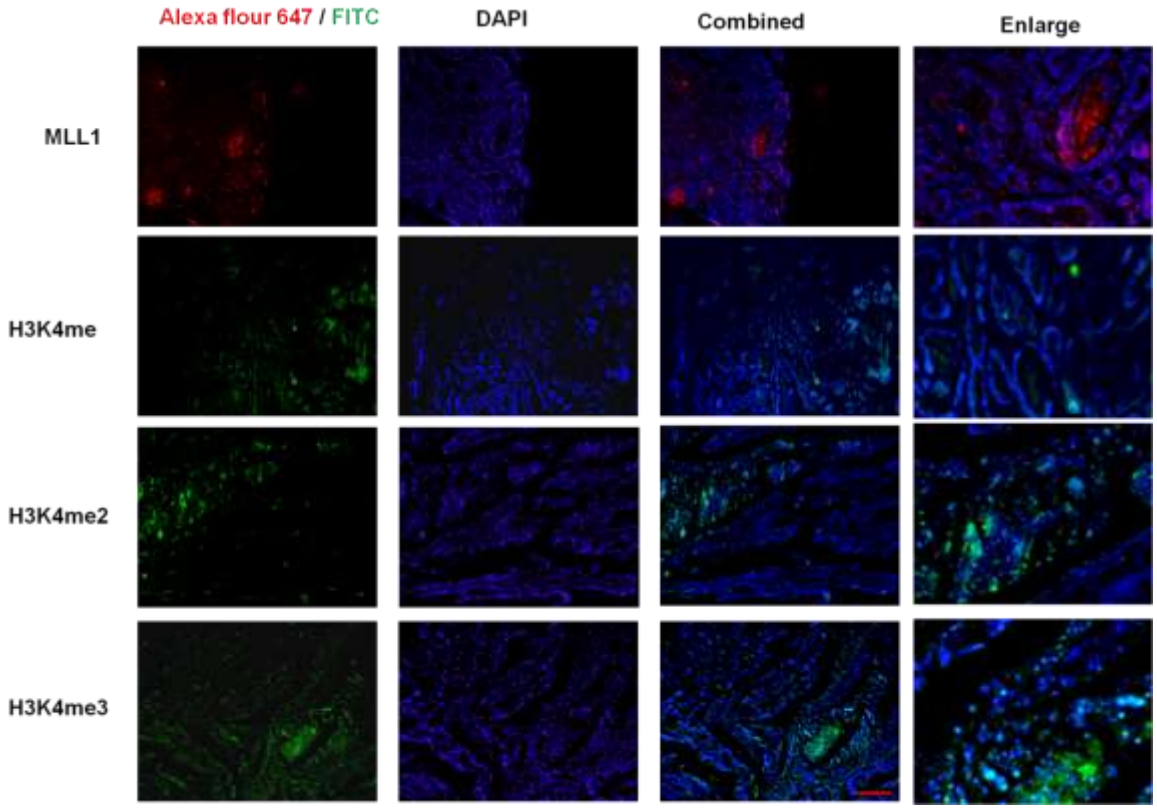

Figure 5.9: Fluorescent immunohistochemistry analyses of prostate cancer tissue samples: MLL1 (red), H3K4me1 (green), H3K4me2 (green), and H3K4me3 (green) expression was analyzed. Scale Bar = 10 μm

5.3.8 Overexpression of miR-193a suppress anchorage-independent growth and induces apoptosis in prostate cancer cells

After confirming that, miR-193a is targeting MLL1 and changing the global histone H3K4 methylation we evaluate whether miR-193a would regulate MLL1 to

modulate cell proliferation, Anchorage-independent growth, and apoptosis in prostate cancer cells.

Anchorage-independent growth is the ability of transformed cells to grow independently of a solid surface and is a hallmark of carcinogenesis. pEZX-MR04-hsa-miR-193a and CmiR0001-MR04 transfected PC3 cells were subjected to soft agar assay. Observation reveals the inhibitory effect of miR-193a on anchorage-independent growth. 3 µg and 4 µg pEZX-MR04-hsa-miR-193a cell exhibit lower colony formations than control cells (CmiR0001-MR04 cells) (Fig. 5.10 A). Graphical representation shows the increasing concentration of miR-193a gradually decrease the colony number. In 3 µg and 4 µg transfected cells exhibits 330 and 108.33 number of colonies whereas in control condition 544.67 colonies were found. This result clearly indicates towards an antagonistic role of miR-193a on anchorage-independent growth in prostate cancer (Fig. 5.10 B).

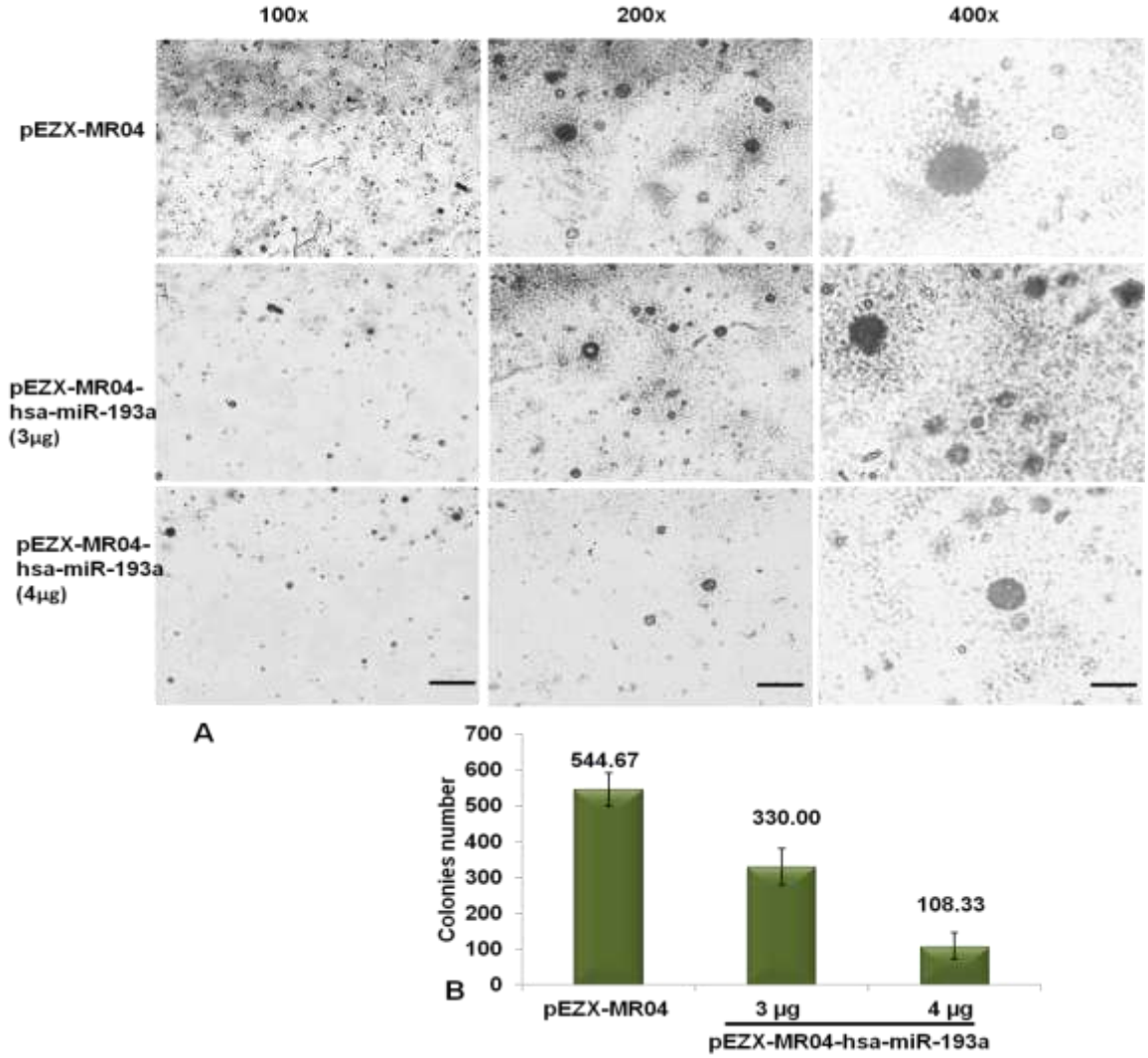

Figure 5.10: Anchorage-independent survival analysis:
[A] Colony formation of PC3 cell lines on semisolid soft agar plates were examined after 3 weeks culture in 3µg and 4 µg of pEZX-MR04-hsa-miR-193a and pEZX-MR04 transfected cells. Then

colonies were stained and visualized microscopically. A representative view of each condition with different magnification is shown. Scale Bar = 10 µm in 100x, Scale Bar = 20 µm in 200x and Scale Bar = 40 µm in 400x. (D) Quantification of colony formation data derived from colonies was counted in a colony counter. Results from one representative experiment are shown. (n=3 independent experiment, mean±S.D.). $P < 0.05$.

To assess the role of miR-193a cellular proliferation MTT assay has been done by using PC3 cells. As we thought, transfection of PC3 cells with a higher concentration of pEZX-MR04-hsa-miR-193a reduces cellular proliferation compare to CmiR0001-MR04 transfected PC3 cells. This data clearly showed the cytotoxicity effect of miR-193a on PC3 cells (Fig. 5.11).

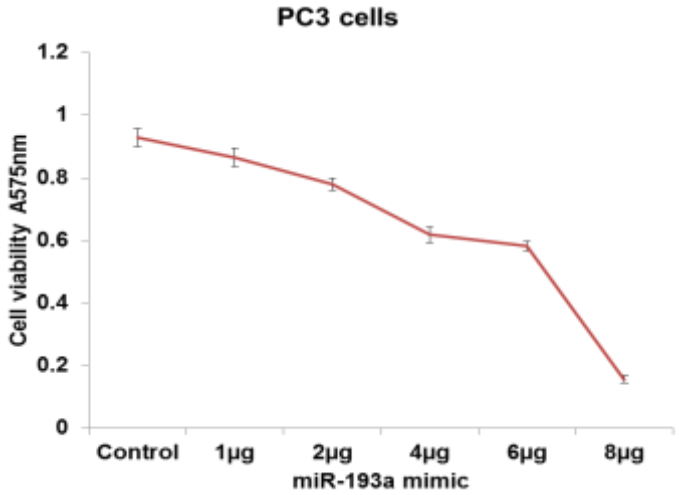

Figure 5.11: Concentration-dependent effect of miR-193a on PC3 cell viability:
Cells were transfected with various concentrations of pEZX-MR04-hsa-miR-193a for which cell viability was measured using MTT assay. As control pEZX-MR04 transfected cells were used. (n=3 independent experiment with three replica, mean±S.D.) $P < 0.05$. Cell viability is represented in term of absorbency.

Finally, cell cycle and apoptotic population was investigated using flow cytometry analysis. The results showed that the percentage of apoptotic cells was significantly higher in PC3 cells transfected with pEZX-MR04-hsa-miR-193a vector compared to control cells. In control cell, there were only 2.5% apoptotic cells but after transfection with pEZX-MR04-hsa-miR-193a vector, it increases with increasing concentration of vector 6.5% to 7.7% in average respectively (Fig. 5.12 A and B).

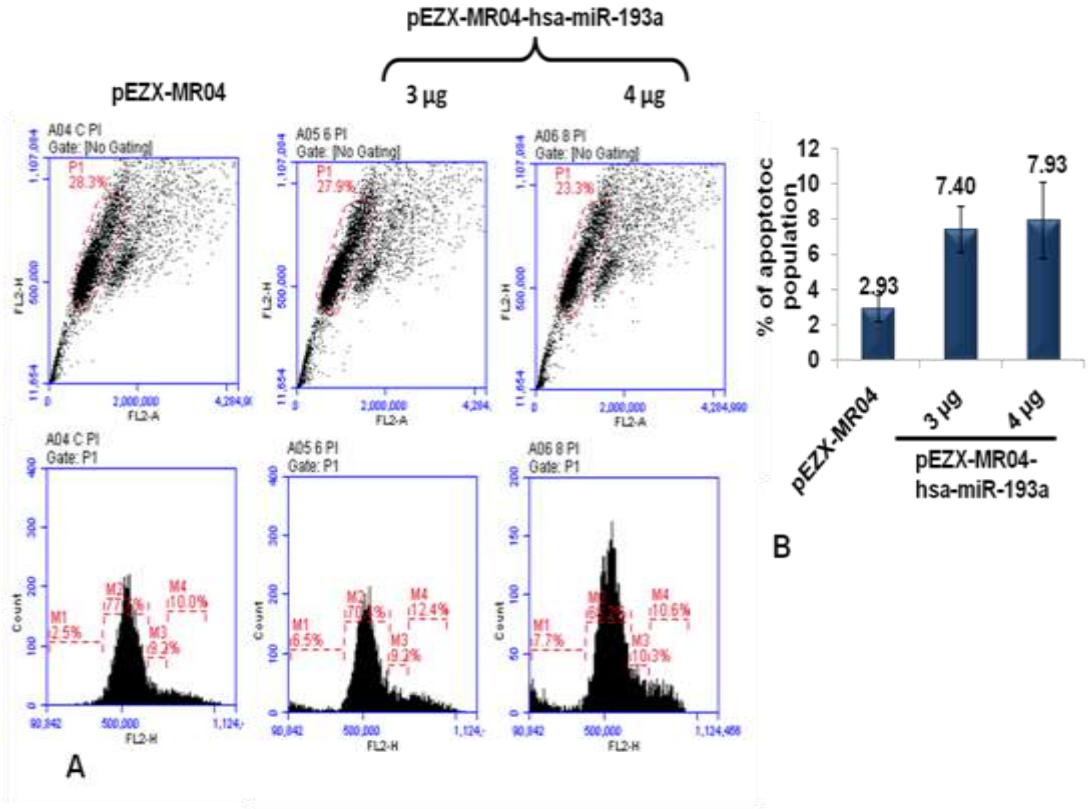

Figure 5.12: Apoptotic population analysis by flow cytometry:
[A] FACS analysis of 3µg and 4 µg of pEZX-MR04-hsa-miR-193a vector transfected cells. Above row shows the total population of gated cells. The lower row is the histogram of the gate population and percentage of different cell cycle stage and apoptotic cell population [B] Graphical representation of the apoptotic population in PC3 is represented. (n=3 independent experiment, mean±S.D.). $P < 0.05$

To confirm that, miR-193a inducing apoptosis cells were stained with propidium iodide (PI) and ROS production was analyzed of miR-193a transfected PC3 cells. Nuclear changes of PC3-miR193a cells demonstrated by PI staining. Within 36 hours after pEZX-MR04-hsa-miR-193a and CmiR0001-MR04 transfection, a large proportion of PC3-pEZX-MR04-hsa-miR-193a cells displayed nuclear fragmentation compared to PC3-CmiR0001-MR04 transfected cells (Fig 5.13 A) which clearly shows the loss of nuclear DNA content. DNA degradation was increased with increased miR-193a expression. The number of apoptotic cells were 39.20 and 48.89 in 3µg and 4 µg of pEZX-MR04-hsa-miR-193a transfected cells respectively where as in control cells it was 1.91 (Fig 5.13 B). Along with chromatin damage, production of ROS was also measured by FACS to observe the effect of miR-193a on cells. During apoptosis, the ROS level increased, and it also induces further apoptosis. Higher levels of ROS-induced DNA damage and inhibit cell proliferation by Ca^{2+} potential [371]. Overexpression of miR-193a induced higher ROS production in the PC3 cells. The cells were transfected with 3µg and 4 µg of MR04-

hsa-miR-193a and pEZX-MR04 as control. It was found in control cells that, the level of ROS was lower than the treated cells (Fig.5.13 C). The ROS production in terms of fluorescence intensity was significantly higher in miR-193a over expressing cell than control cells (Fig. 5.13 D).

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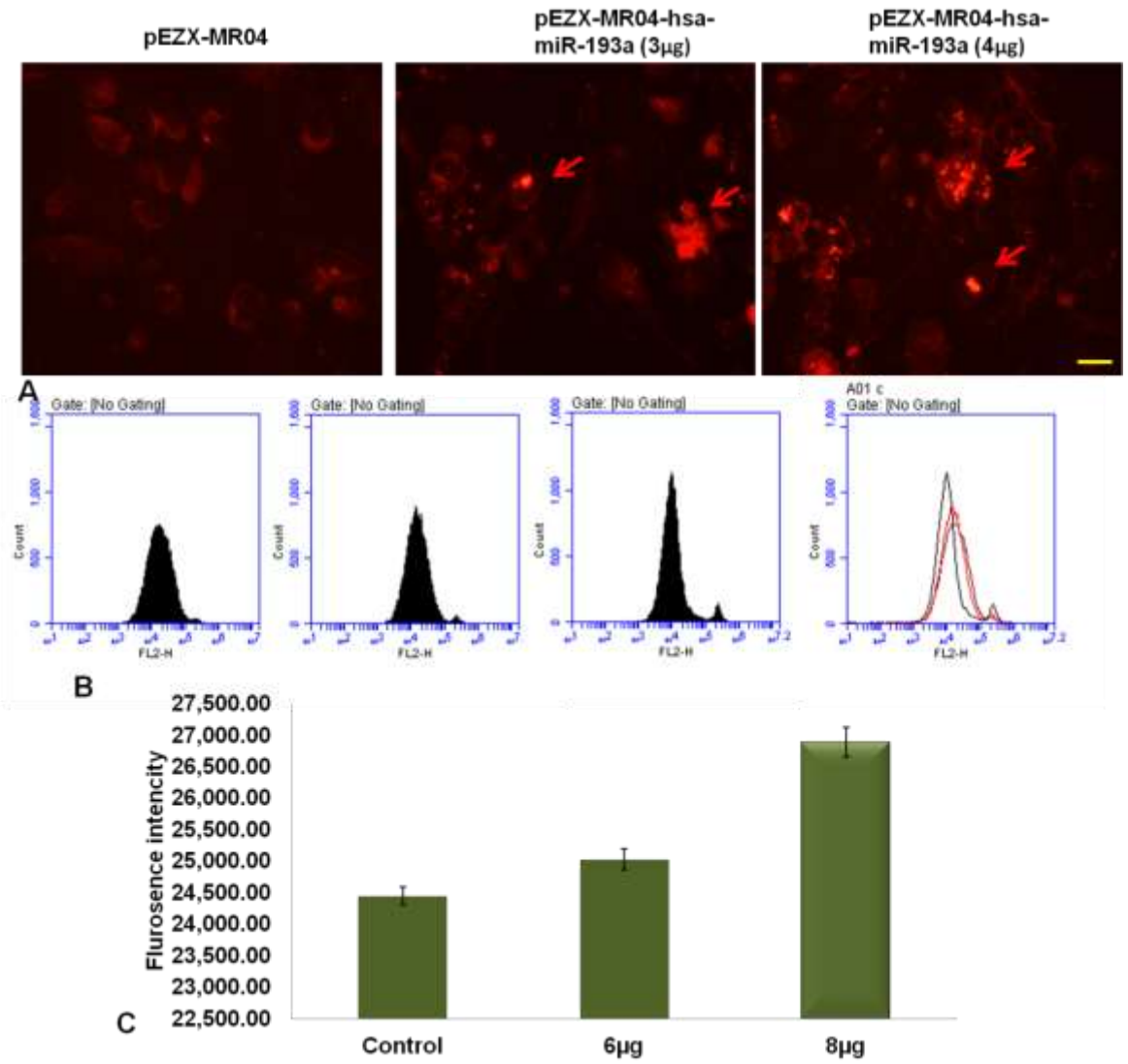

Figure 5.13: chromatin damage and ROS production analysis:
[A] PC3 cells were stained with PI in pEZX-MR04-hsa-miR-193a (3µg and 4 µg) and pEZX-MR04 transfected cells. Bright red dots indicated the fragmented DNA during apoptosis Scale Bar = 40 µm. [B] Cells were stained with DCFDA after transfection with 3µg/ml and 4 µg/ml of pEZX-MR04-hsa-miR-193a and pEZX-MR04 and the fluorescence intensity was measured by FACS. Here vector was treated with 2 ml of media, so the concentration of vector shows double. (n=3 independent experiment, mean±S.D.). $P < 0.05$.

To confirm that cells death is apoptotic in nature the apoptotic markers were analyzed. Well established apoptotic markers, BCL2, BAX, and cleaved PARP were analyzed by qRT-PCR and western blotting. The mRNA level of pre-apoptotic molecule

BAX was increased up to 4.47 and 8.51 fold in 3 µg and 4 µg pEZX-MR04-hsa-miR-193a cells respectively whereas BCL2; anti-apoptotic molecule level was decreased (Fig. 5.14 A). Not only transcriptional level but also in translational level BAX increased 3.05 and 3.95 fold in 3 µg and 4 µg pEZX-MR04-hsa-miR-193a cells respectively. BCL2 decreased by 0.74 and 0.29 in 3 µg and 4 µg pEZX-MR04-hsa-miR-193a cells respectively. Additionally, cleaved PARP also significantly increases after miR-193a overexpression in PC3 cells. This above results together established that miR-193a induced apoptosis in PC3 cells (Fig. 5.14 B and C).

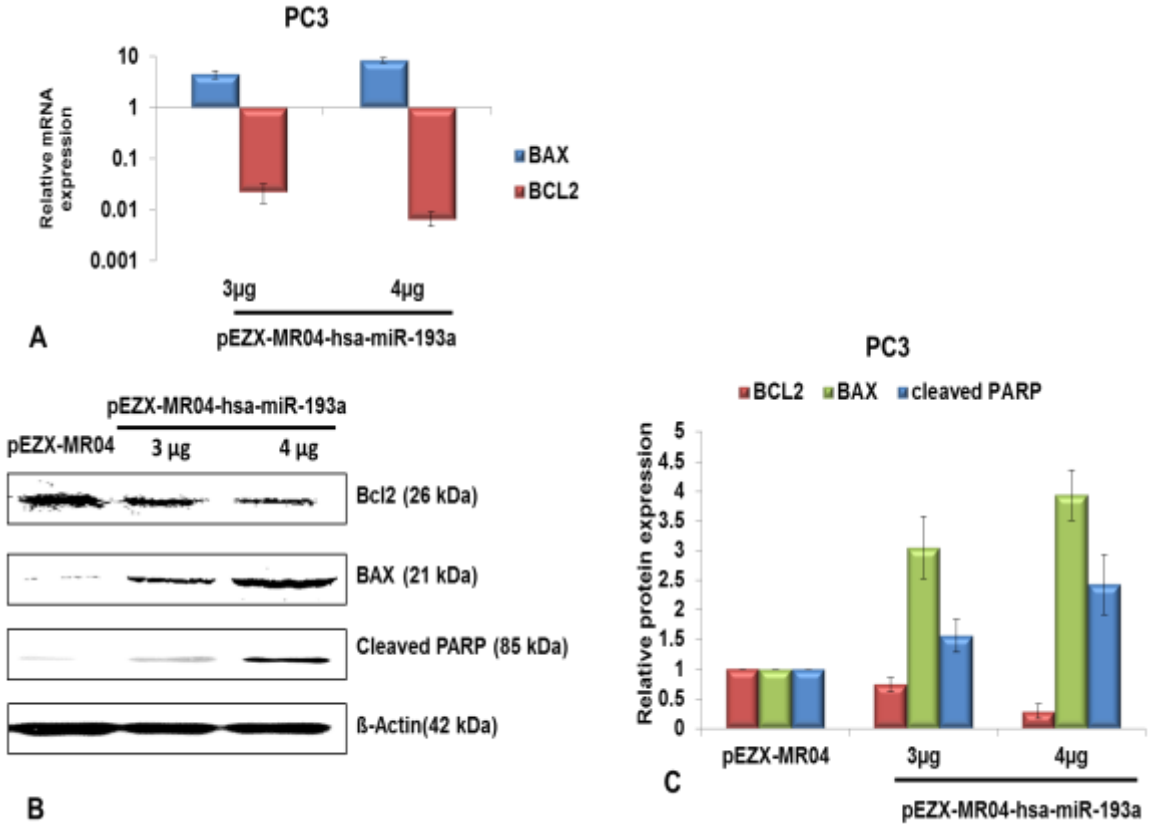

Figure 5.14: BCL2, BAX and cleaved PARP expression:
[A] mRNA level of BCL2 and BAX was analysed by RT-PCR after transfected with pEZX-MR04 and (3µg and 4 µg) of pEZX-MR04-hsa-miR-193a. [B] BCL2, BAX and cleaved PARP protein expression and quantification were measured by Western blot in PC3. β-actin was used to confirm equal loading. [C] The graphical representation of relative protein expression was measured by comparing with respective control. pEZX-MR04 transfected cells were used as a control for miR-13a transfected cells. Band intensity was measured by ImageJ software. (n=3 independent experiment, mean±S.D.). $P < 0.05$.

5.4 Discussion

In the family of six mixed-lineages leukaemia (MLL) of histone methyltransferase (HMTs), MLL1 is one the most important in mammals [372]. It methylates H3K4 through

evolutionarily conserved SET domain. Both MLL1 and H3K4me are spread through the genes promoter and transcriptional start sites and initiate transcription of target genes [373]. From the cBioportal database, MLL1 duplication and amplification was observed in the prostate cancer patient. miR-193a plays a tumour suppressor role in different cancer. Previous studies in ovarian cancer indicated that miR-193a regulates cell cycle and apoptosis by controlling different genes including ARHGAP19, CCND1, ERBB4, KRAS, MCL1, expression [374]. In prostate cancer also, miR-193a inhibits KRAS gene that acts as an oncogene. Although different studies implicated the role of miR-193a during cancer progression; but there is no data is available on miR-193a mediated regulation of MLL1 and/or histone H3 marks. For the first time, this study clearly demonstrated the relation between miR-193a and MLL1 mediated H3K4 methylation.

Here, observations from database analysis and experiments implicated that miR-193a downregulation in prostate cancer cell lines as well as in prostate cancer tissue samples is directly proportional to prostate cancer patient survival (Fig. 5.1). On the other hand, MLL1 expression was increased in prostate cancer samples, and the total patient survival decreased with increase in MLL1 expression. Moreover, prostate cancer recurrence is also facilitated by MLL1 overexpression (Fig. 5.2).

In-silico target findings show MLL1 gene has an MRE (miRNA recognition element) region for miR-193a in 3'UTR (Fig. 5.4). For analysis the effects of this microRNA, miR-193a overexpressing transient PC3 cell line were prepared. Co-transfection of pEZX-MR04-hsa-miR-193a overexpressing vector and pcDNA3.1-MLL1 3'-UTR WT or pcDNA3.1- MLL1 3'-UTR MT confirm that miR-193a bind to the MLL1 3'-UTR region and decrease MLL1 level (Fig. 5.6). To find the effect of miR-193a on MLL1 mRNA expression, MLL1 mRNA (Fig. 5.7) and protein content was analyzed wherein downregulation in MLL1 mRNA and protein level was observed (Fig. 5.8). Protein-protein interaction database shows that MLL1 makes a complex with MLL2, hence when MLL1 has been downregulated by miR-193a, MLL2 was also downregulated (Fig. 5.8 A and B). These results create inquisitiveness about the effect of miR-193a on global H3K4 methylation pattern (Fig. 5.8 C and D). Apart from western blot analyses, immunohistochemical staining of FFPE samples confirmed that MLL1 overexpression is associated with prostate cancer progression (Fig. 5.9).

In FFPE samples as well as in prostate cancer cell line, it was observed that H3K4me3 expression is higher than both H3K4me and H3K4me2. However, ectopic overexpression of miR-193a does not have much effect on H3K4me2 but H3K4me3 was significantly

decreased, and H3K4me was increased. It was known H3K4me3 mark act as an activation mark of the genes. This mark is mostly present on the proliferating genes during cancer progression.

It has been previously reported that presence of H3K4me3 in the promoter region of anti-apoptotic Bcl-2 implicated that miR-193a may affect cellular apoptosis. Therefore, to gain a comprehensive insight into the regulation of apoptosis by miR-193a, further experiments were executed. Result elucidates that over-expression of miR-193a negatively regulate anchorage-independent survival which is a key cellular transformation during cancer progression (Fig. 5.10). Moreover, it was observed from cell cycle analysis that overexpression of miR-193a ignites apoptosis population in PC3 cells (Fig. 5.12). It was confirmed by overproduction of ROS in miR-193a overexpressed cells as compared with control. Chromatin condensation and nuclear staining show a higher level of DNA damage in miR-193a overexpressed cells. The colony formation assay and MTT assay shows miR-193a plays a role in the inhibition of cellular proliferation (Fig. 5.13). To confirm the role of miR-193a in apoptosis, western blotting was done of apoptotic and anti-apoptotic markers. These results show miR-193a downregulates anti-apoptotic gene BCL2, which was overexpressed in cancer cells, whereas it upregulates apoptotic gene BAX and cleaved PARP (Fig. 5.14). From the above results, it can be concluded that miR-193a regulates MLL1 gene in prostate cancer which eventually changes the global H3K4 methylation pattern and initiate apoptosis.

Chapter 6
General discussion
And
Conclusions

6. General discussion and Conclusions

In this thesis, an attempt to find out the difference between epigenetic modifications in the healthy state compared to disease state is sought for. The cellular mechanisms are very tightly regulated, and every modulation is strictly monitored. Still, sometimes there exist certain lacunae in cellular signalling mechanism causing disease. Here we studied the epigenetic mechanisms from DNA methylation to histone modification and miRNA regulation. Primarily it was thought that the gene which is methylated remained silenced, and it disrupts the whole pathway. DNA methylation is the key regulator of the expression of a number of genes. DNMT1 maintain the methylation pattern from the mother to daughter cells. But during disease state DNMT1 overexpressed and broke the pattern and hypermethylated several other genes. DNMT1 not only methylate other genes but binds to the methylated region with MBD proteins and form a complex, which stop the gene expression. This study demonstrated how miRNA can downregulate DNMT1 and again restores the gene expression which was silenced by DNMT1. It was previously discovered that, during cancer progression TS-gene got methylated, and its expression is blocked. Different drugs were used to overcome the methylation but due to toxic effects, other healthy cells got affected. Here miRNAs were used to target DNMT1 and suppress its expression. miRNAs are cells last line of defense to prevent unwanted and aberrant gene. However, during cancer miRNAs expressions are reported to be suppressed by other epigenetic modulators. Cancer cell first corrupts the methyltransferase which eventually silenced miRNA expression. MiR-152 is a tumour suppressor miRNA which targets different growth factor stimulating and proliferative genes. In this study, it reported that DNMT1 is the culprit enzyme which suppresses miR-152 expression in cancer cells. The presence of H3K4me3, an active histone mark in the miR-152 promoter inhibits expression of miR-152. This finding helps to understand the complexity of the epigenetic regulation. From this, it can be concluded that the expression of genes sometimes depends on both the epigenetic factors. Then using miRNA mimics miR-152 was overexpressed in breast cancer cell line to find out the fate of DNMT1. It was observed that higher concentrations of miR-152 mimics can successfully downregulate DNMT1 expression. Besides, miR-152 is observed to be repressing migration of cancer cells. Cellular migration is one of the characteristics of the metastatic cancer cell. Therefore, the genes responsible for the inhibition of migration were studied. From the database analysis, it was found that, CDH1 gene remain downregulated during cancer progression. It also depicted that, DNA methylation is the reason for the

downregulation of CDH1. Thus, when DNMT1 got downregulated by miR-152, downstream genes start to express. To conclusively prove this theory, cells were transfected with DNMT1 siRNA and DNMT1 overexpression vector along with miR-152 mimics. Results showed, when DNMT1 got downregulated by siRNA, the similar results like miR-152 mimics obtained. But the overexpression vector further silenced the gene. Thus, it was concluded that CDH1 gene was remain downregulated by DNMT1, and when miR-152 inhibits DNMT1 expression, CDH1 gene starts to express again. Therefore, by targeting DNMT1, other genes which are actively repressed by DNMT1 can be re-expressed.

Thereafter, another miRNA responsible for the repression of DNMT1 in prostate cancer was investigated. From the *in-silico* study, it was predicted that miR-148a can also target DNMT1. The effect of miR-148a on DNMT1 was investigated in prostate cancer cells. It is reported miR-148a remains downregulated in prostate cancer cells. The cell line in which this study was done was highly metastatic and AR-negative. For analysis of the effect of miR-148a, cells were transfected with miR-148a mimics. The ectopic expression of miR-148a shows a complete suppression of DNMT1. miR-148a suppresses DNMT1 in both in mRNA and protein level. From this study, it is also proved miR-148a remain downregulated in prostate cancer cells by DNMT1. Upon downregulating DNMT1 by siRNA improves the expression of miR-148a simultaneously overexpression of DNMT1 downregulates its expression. Then the effects of ectopic expression of miR-148a were studied where it shows induction of apoptosis of the cancer cells. This conclusion was obtained by different apoptotic assays along with an analysis of the expression of apoptotic protein markers. Hence, it can be concluded that miR-148a has a tumour suppressor role in prostate cancer, and restoration of miR-148a can be used for a therapeutic purpose.

After studying the effects of miRNAs which targets DNMT1, our next goal was to study how miRNA affect histone marks. MLL1 gene was selected for this study because of its H3K4 methyltransferase activity. Histone methylation study is a fascinating field of epigenetics. H3K4 methylation has 3 states of methylations mono-, di-, and tri-methylation. H3K4me was found basically in the activator sites of the genes, and H3K4me3 is located in the promoter regions. From the in-silico analysis, it was found miR-193a has a “seed” region on the 3' UTR of MLL1 gene. miR-193a reported to remain downregulated in prostate cancer cells. miR-193a overexpression vector was used for ectopic expression of miR-193a. Previously it was analyzed that in prostate cancer,

H3K4me3 mark is present in different oncogene and anti-apoptotic gene promoters. When miR-193a was ectopically expressed, it targets MLL1 and downregulates its expression. miR-193a not only directly downregulate MLL1, but it also indirectly downregulates MLL2; another histone methyltransferase which works in a complex with MLL1. These results excited us further to study its effect on global H3K4 methylation pattern. Analysis of the protein level of the H3K4 mark, confirmed that overexpression of miR-193a downregulates H3K4me3 mark and simultaneously upregulates H3K4me mark, but does not affect H3K4me2 marks. Thus, it was confirmed that MLL1 only methylate H3K4me to H3K4me3 but does not affect H3K4me2. Then the role of miR-193a in cellular apoptosis was analyzed, and it shows a tremendous promising role to induce apoptosis. Besides, miR-193a was found to initiate apoptosis by DNA fragmentations.

The link between miRNA functions and chromatin modifying enzymes governing cellular physiology and function are established here, at least in part. This is an important collection of miRNA regulation of DNMT1 and MLL1 gene transcripts with experimental evidence. This thesis resolves the boundaries between epigenetic modifier genes DNMT1, MLL1, and miRNAs like miR-152, miR-148a, and miR-193a. In addition, DNMT1 regulates the expression of these miRNAs to nullify their functional effects. On the other hand, miR-193a regulates histone methyltransferase MLL1, which eventually regulates H3K4 di and tri-methylation an active gene mark. These interactions prove that cells always have a backup plan for gene regulation. But in the diseased state, these regulations work against each other and create chaos.

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374. Nakano, H., et al., *Gain-of-function microRNA screens identify miR-193a regulating proliferation and apoptosis in epithelial ovarian cancer cells.* Int J Oncol, 2013. **42**(6): p. 1875-82.

Curriculum vitae

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  National Institute of Technology, Rourkela (2011-2016)
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- **Master of Science (M.Sc) in Microbiology & Microbial Technology**
  University of Kalyani, Kalyani, West Bengal, 2010
  Percentage obtained: 70.14 %, Division: 1st

- **Bachelor Science (B.Sc-Hons.) in Microbiology**
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- **Secondary Education (10)**
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RESEARCH EXPERIENCE

Ph.D Research Scholar

Title of Thesis: **Regulation of chromatin modifier genes by microRNA vis-à-vis regulation of microRNA by DNA methylation and histone modifications in human cancer**
Supervised by: Dr. Samir Kumar Patra, Associate Professor Department of Life Science, National Institute of Technology, Rourkela.
Project done at: Department of Life Science, National Institute of Technology, Rourkela.
Duration of project: **January 2011 to July 2016**

Summer Trainee

Title: **"Isolation of *Vibrio cholerae* & vibriophages and molecular analysis of vibriophages"**

Supervised by: Dr. B.L.Sarkar., Deputy Director,
Vibrio phage reference laboratory,
National Institute of Cholera and Enteric Diseases, (I.C.M.R), Kolkata

Project Completed at: National Institute of Cholera and Enteric Diseases, (I.C.M.R), Kolkata

Duration of project: **2 months (May 2009-June 2009)**

Industrial Trainee

Title: **"Brewing and Quality Control"**

Key learning:

- Testing procedures (chemical and bacteriological) of raw materials and finished products used commercially.
- Different stages of brewing and fermentation procedure.
- Coordinating with relevant departmental staffs to achieve the testing of the same.

Supervised by: Dr. T. K. Dutta, Quality Assurance Manager, UBL, Kalyani unit.

Project Completed at: United Breweries Ltd., Kalyani unit

Duration of project: 24 days

SEMINARS/WORKSHOP ATTENDED

- 100th Indian Science Congress Session, Kolkata, India, 3rd - 7th January, 2013.
- 8th International "MicroRNAs Europe 2013" Meeting on 'MicroRNAs: Biology to Development & Disease' Peterhouse College, University of Cambridge, Cambridge, United Kingdom on November 4 - 5, 2013.
- International Seminar on Emerging Trends In Cell & Molecular Biology, Jadavpur University, Kolkata, India, 14th December, 2012.
- 2nd Global Cancer Genomics Consortium TMC Symposium, ACTREC, Mumbai, India, 19th – 21st November, 2012.
- Participated in summer school of UGC-NRCBS at Madurai Kamaraj University on 'Human Cellular Functional Assays' (27 June –10 July 2011).

PUBLICATIONS IN PEER-REVIEWED JOURNAL

1. **Sengupta D,** Deb M, Rath SK, Kar S, Parbin S, Pradhan N, Patra SK. DNA methylation and not H3K4 trimethylation dictates the expression status of miR-152 gene which inhibits migration of breast cancer cells via DNMT1/CDH1 loop. Exp Cell Res (2016**). In press.**
2. **Sengupta D**, Deb M, Rath SK, Kar S, Parbin S, Pradhan N and Patra SK. MiR-193a regulates H3K4 trimethylation by regulating MLL1 and induces apoptosis in prostate cancer. (Manuscript submitted).
3. **Sengupta D**, Deb M, Rath SK, Kar S, Parbin S, Pradhan N and Patra SK. miR-148a targets DNMT1 and induce apoptosis inhibit prostate cancer progression. (Manuscript submitted).
4. Kar S*, **Sengupta D***, Deb M, Shilpi A, Parbin S, Rath SK, Pradhan N, Rakshit M, Patra SK. (2014) Expression profiling of DNA methylation-mediated epigenetic gene-silencing factors in breast cancer. Clin Epigenetics. 6(1):20.
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5. **Sengupta D**, Deb M, Kar S, Shilpi A, Parbin S, Mallick B and Patra SK. (2013). Epigenetic microRNA regulation of chromatin function and signaling pathways: A perspective in cancer. Epigenetic Diagnosis & Therapy. 1:81 – 90.
6. Rath SK, Deb M, **Sengupta D**, Kari V, Kar S, Pradhan N, Parbin S, Patra SK. (2016) Silencing of ZRF1 impedes survival of estrogen receptor positive MCF-7 cells and potentiates the effect of curcumin. Tumor Biology. In press.
7. Deb M, **Sengupta D**, Kar S, Shilpi A, Parbin S, Rath SK, Roy S, Das G and Patra SK (2016) Epigenetic drift towards histone modifications regulates CAV1 gene expression in colon cancer. Gene. 581(1):75-84.
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9. Parbin S, Shilpi A, Kar S, Pradhan N, **Sengupta D**, Deb M, Rath SK, Patra SK. (2015) Insights into the molecular interactions of thymoquinone with histone deacetylase: evaluation of the therapeutic intervention potential against breast cancer. Mol Biosyst. 12(1):4858.
10. Shilpi A, Parbin S, **Sengupta D**, Kar S, Deb M, Rath SK, Rakshit M and Patra SK (2015) Mechanisms of DNA methyltransferaseinhibitor interactions: Procyanidin B2 shows new promise for therapeutic intervention of cancer. Chem Biol Interact. 233:12238.
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12. Deb M, Kar S, **Sengupta D**, Shilpi A, Parbin S, Rath SK, Londhe V and Patra SK (2014) Chromatin dynamics: H3K4 methylation and H3 variants replacement in development and cancer. Cellular and molecular life sciences, 71:3439-3463.
13. Parbin S, Kar S, Shilpi A, **Sengupta D**, Deb M, Rath, SK and Patra SK (2013) Histone deacetylases: A saga of perturbed acetylation homeostasis in cancer. J Histochem & Cytochem, 62(1):11-33.
14. Kar S, Parbin S, Deb M, Shilpi A, **Sengupta D**, Rath SK, Rakshit M, Patra A and Patra SK (2013) Epigenetic Choreography of Stem Cells: the DNA demethylation episode of development. Cell Mol Life Sci., 71(6):1017-32.
15. Deb M, **Sengupta D** and Patra SK (2012) Integrin-Epigenetics: A system with imperative impact on cancer. Cancer Metast. Rev., 31:221–234.
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17. Kar S, Deb M, **Sengupta D**, Shilpi A, Bhutia SK and Patra SK (2012) Intricacies of Hedgehog Signaling Pathways: A perspective in tumorigenesis. Exp Cell Res., 318: 1959-1972.

CONFERENCE PROCEEDINGS

- **Sengupta D**, Deb M, Kar S, Rath S K, Shilpi A, Parbin S, Patra SK Modulation of miR-152 by epigenetic modulator in breast cancer.
  In: 8th International "MicroRNAs Europe 2013" Meeting on 'MicroRNAs: Biology to Development & Disease' Peterhouse College of the University of Cambridge, Cambridge, United Kingdom on November 4 - 5, 2013.

- **Sengupta D**, Deb M, Kar S, Shilpi A, Parbin S, Patra SK. Expression profiling of DNA Methyltransferases 1 in breast cancer.
  In: 100th Indian Science Congress Session, Kolkata, India, 3rd - 7th January, 2013.
- **Sengupta D**, Deb M, Kar S, Shilpi A, Parbin S, Patra SK. Expression of DNMT1 and its modulation by epigenetic modifiers in breast cancer cells.
  In: International Seminar on Emerging Trends In Cell & Molecular Biology, Jadavpur University, Kolkata, India, 14th December, 2012
- Deb M, **Sengupta D**, Kar S, Shilpi A, Parbin S, Patra SK. Caveolin and Clusterin expression and functional co-relation with human breast cancer.
  In: 100th Indian Science Congress Session, Kolkata, India, 3rd - 7th January, 2013.
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  In: 100th Indian Science Congress Session, Kolkata, India, 3rd - 7th January, 2013.
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  DNA methylation as a prognostic marker: Diagnostic and therapeutic implications in cancer research.
  In: 1st Global Cancer Genomics Consortium TMC Symposium, ACTREC, Mumbai, India, 10th - 12th November, 2011.

LIST OF AWARDS AND GRANTS

- Ph.D. Scholarship from MHRD at NIT Rourkela, India.
- GATE-2010 and GATE-2012 Examination Qualified, India.
- ARS NET 2016 Qualified, India
- Newton International Fellow 2016

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DECLARATION

I hereby declare that all the above information is true in best of my knowledge and belief.

Date: 28.11.2016
Place : Rourkela, Odisha, India

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