MicroRNA-mediated epigenetic regulation of HDAC6 and HDAC8: Functional significance in female-specific cervical and breast cancer

Thesis submitted to the University of Hyderabad for the award of Doctor of Philosophy in the Department of Animal Biology

By

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-- June 2024 --

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CERTIFICATE

This is to certify that this thesis entitled **"MicroRNA-mediated epigenetic regulation of HDAC6 and HDAC8: Functional significance in female-specific cervical and breast cancer"** is a record of bonafide work done by **Debasmita Naik**, a research scholar for Ph.D. program bearing registration number **19LAPH04** in the Department of Animal Biology, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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Further, the student has passed the coursework requirement for Ph.D. and the certificate has been attached.

7/6/24

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SEMESTER 1

COURSE NO	TITLE OF THE COURSE	CREDITS	RESULTS
SL801	RESEARCH METHODOLOGY/ANALYTICAL TECHNIQUES	4	PASS
SL802	RESEARCH ETHICS, BIOSAFETY, BIOSTATISTICS AND DATA	4	PASS
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DECLARATION

I, **Debasmita Naik**, hereby declare that this thesis entitled **"MicroRNA-mediated epigenetic regulation of HDAC6 and HDAC8: Functional significance in female-specific cervical and breast cancer"** submitted by me under the guidance and supervision of **Dr. Arunasree M.K.** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: 07/06/2024

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Debasmita Naik

DEDICATION

- To all my great teachers who taught me.
- To my family and friends, for their endless love and encouragement.
- And to all those who have inspired and guided me along the way.

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⁎⌘⊢ ABBREVIATIONS ⊣⌘⁎

ΔG	:	Gibbs free energy
µl	:	Microliter
µg	:	Microgram
µM	:	Micro molar
Ac	:	Acetylated
Ago	:	Argonaute
AML	:	Acute myeloid leukemia
ATAT1	:	α-tubulin acetyltransferase 1
BNIP3	:	Bcl-2 interacting protein 3
CCL4	:	C-C motif ligands 4
cDNA	:	Complementary DNA
ceRNAs	:	Competitive endogenous RNAs
CHD	:	Chromodomain helicase DNA-binding
CIP	:	Calf Intestinal Alkaline Phosphatase
CpG	:	Cytosine phosphate guanine
CREB	:	cAMP Response Element-Binding Protein
DAPI	:	4′,6-diamidino-2-phenylindole
DMEM	:	Dulbecco's Modified Eagle's Medium
DNA	:	Deoxy ribonucleic acid
DNMTs	:	DNA methyltransferases
dsRNA	:	Double-standard RNA-mediated
DTT	:	Dithiothreitol
E. coli	:	*Escherichia coli*
EDTA	:	Ethylene Diamine Tetra acetic Acid
EMT	:	Epithelial-mesenchymal transition
ERK	:	Extracellular signal-regulated kinase
ERRα	:	Estrogen Related Receptor Alpha
FBS	:	Fetal Bovine Serum
FFPE	:	Formalin-fixed paraffin-embedded
FP	:	Forward primer
GAPDH	:	Glyceraldehyde-3-Phosphate Dehydrogenase
GNATs	:	Gcn5 (general control non-derepressible 5)-related N-acetyltransferases

H2A	:	Histone 2A
H2B	:	Histone 2B
H3	:	Histone 3
H4	:	Histone 4
HATs	:	Histone acetyltransferases
HCC	:	Hepatocellular carcinoma
HCl	:	Hydrogen chloride
HDAC8	:	Histone deacetylase 8
HDACi	:	Histone deacetylase inhibitors
HDACs	:	Histone deacetylases
HEK 293T	:	Human Embryonic Kidney epithelial cells
HeLa	:	Henrietta lacks (Human cervical cancer cells)
HIF-1	:	Hypoxia-inducible factor 1
HIV	:	Human immunodeficiency virus
hmC	:	Hydroxymethylcytosine
HMTs	:	Histone methyl transferases
HPV	:	Human papillomavirus
Hr	:	Hour
Hsp90	:	Heat Shock Protein 90
IFNB1	:	Interferon Beta 1
INO80	:	Inositol- requiring 80
ISWI	:	Imitation of SWI
JAK2/STAT	:	Janus kinase 2/signal transducer and activator of transcription
KATs	:	Lysine acetyltransferases
KDACs	:	Lysine deacetylases
lncRNAs	:	long non-coding RNAs
M	:	Molar
MAPK	:	Mitogen-activated protein kinase
mC	:	methylcytosine
MCF-7	:	Human breast adenocarcinoma
min	:	Minutes
miRNA	:	microRNA
ml	:	Milliliter

MLN64	:	Metastatic lymph node protein 64
mM	:	Millimolar
MMP-9	:	Matrix metallopeptidase 9
mRNA	:	messenger RNA
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MUT	:	Mutant
NaCl	:	Sodium chloride
NaF	:	Sodium Fluoride
NC	:	Nitrocellulose
NCOAs	:	Nuclear receptor coactivators
ncRNAs	:	Non-coding RNAs
NF-κB	:	Nuclear factor kappa B
OncomiRs	:	Oncogenic microRNAs
p53	:	Tumor Protein 53
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate Buffered Saline
PBST	:	Phosphate Buffered Saline Tween 20
PCR	:	Polymerase Chain Reaction
PLAP	:	Placental alkaline phosphatase
Pre-miRNA	:	Precursor miRNA
Pri-miRNA	:	Primary miRNA
PTEN	:	Phosphatase and TENsin homolog
qRT	:	Quantitative Real-time
RIPA	:	Radio-Immunoprecipitation Assay
RISC	:	RNA-induced silence complex
RNA	:	Ribonucleic acid
RNA pol II	:	Ribonucleic acid Polymerase II
RNAi	:	RNA interference
RP	:	Reverse primer
RPMI 1640	:	Roswell Park Memorial Institute 1640
RT	:	Room Temperature
RUNX2	:	Runt-related transcription factor 2
SAM	:	S-adenosylmethionine

shRNA	:	Short hairpin RNA
siRNA	:	Small interfering RNA
SIRT	:	Sirtuin
SMC3	:	Structural maintenance of chromosomes 3
sncRNAs	:	Short non-coding RNAs
SOCS	:	Suppressor of cytokine signaling
SRC-1	:	Steroid receptor coactivator-1
SWI/SNF	:	Switching-defective/sucrose-non-fermenting
SWR1	:	SWI2/SNF2-Related 1
TCGA	:	The Cancer Genome Atlas
TET	:	Ten-eleven translocation
TGF-β	:	Transforming growth factor-β
TP53	:	Tumor protein 53
TSmiRs	:	Tumor suppressor microRNAs
UTR	:	Untranslated region
VCP	:	Valosin-containing protein
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization
WT	:	Wild-type

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Methods

Introduction

1.0 Introduction

1.1 Cancer

Cancer refers to a large group of diseases distinguished by their ability to alter cell phenotypes, resulting in uncontrolled growth of abnormal cells that grow beyond their usual boundaries. As a result, they invade adjoining parts (invasion) and spread to other organs (metastasis). It also disrupts normal body functions, thereby hampering human health and causing mortality, contributing to nearly 10 million deaths globally in 2020 (Ferlay et al., 2021). Cancer was discovered to be characterized by several biomarkers, such as unlocking phenotypic plasticity, deregulated cellular metabolism, resisting cell death, initiating or accessing vasculature, stimulating invasion and metastasis, evading immune destruction, and so on (Figure 1) (Hanahan, 2022; Hanahan & Weinberg, 2011).

Pathologically, a tumor can be distinguished as benign or malignant. A tumor that remains confined to its primary growing area and does not invade or metastasize to other body parts is called a benign tumor. These are non-cancerous and characterized by controlled cell growth mediating slow and orderly cell division. On the other hand, a tumor capable of invading surrounding tissues and metastasizing to different body organs via the lymphatic and circulatory systems is called a malignant tumor. These are cancerous and characterized by uncontrolled cell growth mediating fast cell division and can form secondary tumors in other parts of the body. As an impact on health, malignant tumors are life-threatening and more likely to recur even after treatment that involves surgery, chemotherapy, radiation therapy, immunotherapy, or a combination of these, whereas, benign tumors can removed surgically and rarely recur after removal.

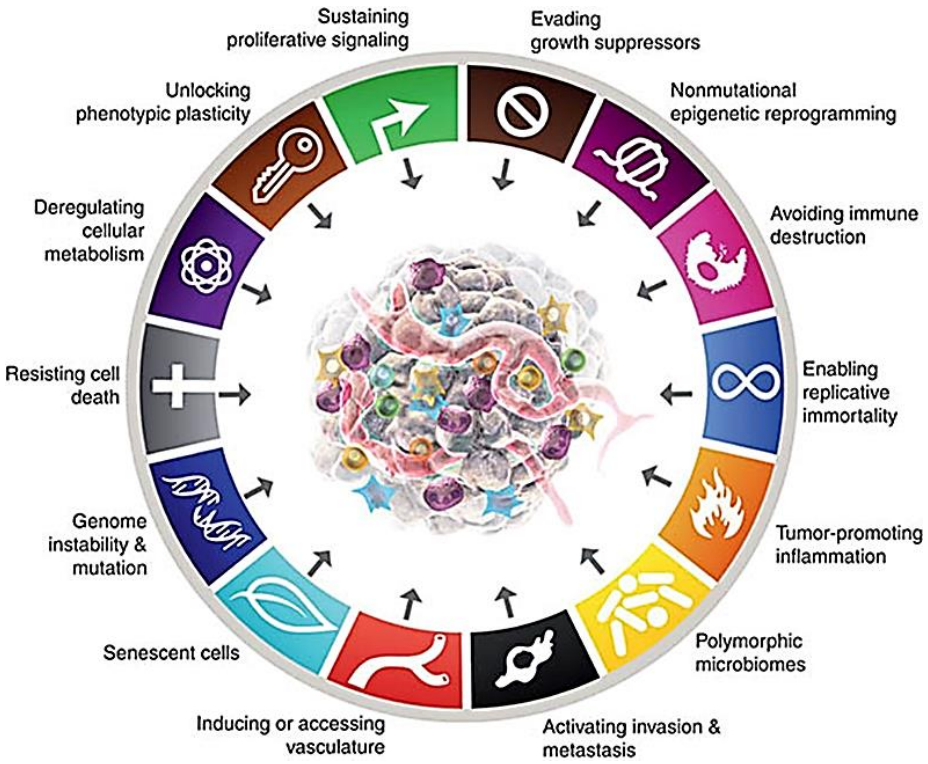


Figure 1: Hallmarks of Cancer. *An increasing research identified several potential characteristics to recognize cancer at its early stages, which includes resisting cell death, deregulating cellular metabolism, unlocking phenotypic plasticity, sustaining proliferative signaling, activating invasion & metastasis, senescent cells, inducing vasculature, and so on. Adopted from Hanahan D, 2022, doi: 10.1158/2159-8290.CD-21-1059.*

Moreover, according to the origin of the tumor, there are several benign tumors such as adenoma which arises from glandular tissues, fibroma which arises from fibrous or connective tissues, hemangioma that occurs from blood vessels, neuroma that arises from nerve cells, osteoma that arises from bone tissue, chondroma that develops from cartilage and so on. Similarly, cancers or malignant tumors are categorized as carcinoma, sarcoma, lymphoma, leukemia, melanoma, and so on. Cancer which develops from epithelial cells that act as covering for external and internal body surfaces is called carcinoma such as adenocarcinoma and squamous cell carcinoma. Cancer which develops from several connective tissues (bones, muscles, and cartilage) is known as sarcoma such as osteosarcoma and liposarcoma. Lymphomas are named as per their origin from the lymphatic system which includes lymph nodes as well as lymphatic tissues. Cancer that originates from the tissues and cells involved in the formation of blood cells such as bone marrow is called as leukemia. These

categorizations of Cancer describe the nature and characteristics of tumors based on their cellular or tissue origins which help in advancement in cancer-specific diagnosis and treatment.

1.2 Cervical Cancer

Cervical cancer is the fourth most common cancer among women globally and has become a health burden for developing as well as industrialized countries. In accordance with the World Health Organisation (WHO) report, over 6 lakh new cases and more than 3 lakh deaths have been recorded in 2020 due to cervical Cancer (https://www.who.int/news-room/fact-sheets/detail/cervical-cancer?). Women living with HIV are known to be more susceptible to developing cervical cancer in comparison to the normal population, making up 5% of cases. In more than 90% of cervical cancer cases, it is known that Human papillomavirus (HPV) infection is the cause if left untreated. Typically, abnormal cells grow into cancer for 15-20 years; but, in the case of women with impaired immune systems, particularly those with untreatable HIV, this process can be hastened to 5-10 years. Cancer progression risk factors include the oncogenicity grade of the HPV type, immunological condition, presence of additional sexually conveyed diseases, number of births, young age pregnancy, hormonal contraceptive usage, and smoking. Heightening public awareness, cancer screening, earlier diagnosis, and treatment in addition to HPV vaccination are key to preventing and controlling cervical cancer. As of 2023, there are only six HPV vaccines obtainable across the globe. Being vaccinated between the ages of 9 and 14 is a precise efficient approach to prevent cervical cancer, and various HPV-related infections and malignancies. However, lack of awareness is one of the major reasons for increased mortality rates in cervical cancer.

1.3 Breast cancer

Breast cancer is the leading Cancer caused in humans and majorly affects women more than men (0.5-1% of breast cancer). In accordance with WHO, there were 2.3 million women receiving diagnoses of breast cancer and 685,000 mortalities worldwide in 2020 (https://www.who.int/news-room/fact-sheets/detail/breast-cancer?). As of the close of 2020, 7.8 million women had gotten a breast cancer diagnosis in the previous five years, making it the most conjoint disease worldwide. Certain factors increase breast cancer risk rate include advanced age, obesity, alcohol consumption, a family descent into breast cancer, exposition to radiation, and a reproductive history. The subtype of the illness and how far it has spread to lymph nodes (stages II or III) or other parts of the body influence the course of treatment for breast cancer (stage IV). To lower the chance of cancer returning, doctors employ a variety of techniques. These include radiation therapy to lower the risk of recurrence in the breast and surrounding tissues, surgery to remove the tumour, and medications like chemotherapy or targeted biologics to kill cancer cells and stop them from spreading. Although mammogram is used as a diagnostic method, early diagnosis of breast cancer is challenging and is associated with high rates of metastasis.

1.4 Mechanism of cancer development and progression

Cancer development is composed of a multistep process following which normal cells gradually become malignant through a progressive series of alterations. The mechanisms of development can vary among various cancers, however, the general steps include initiation, promotion, progression, and metastasis (Figure 2). However, at the cellular level there are several factors such as mutation, and epigenetic alterations play crucial roles in making changes in normal cells which in result progressively increase cell capacity for proliferation, survival, invasion, and metastasis (Figure 3).

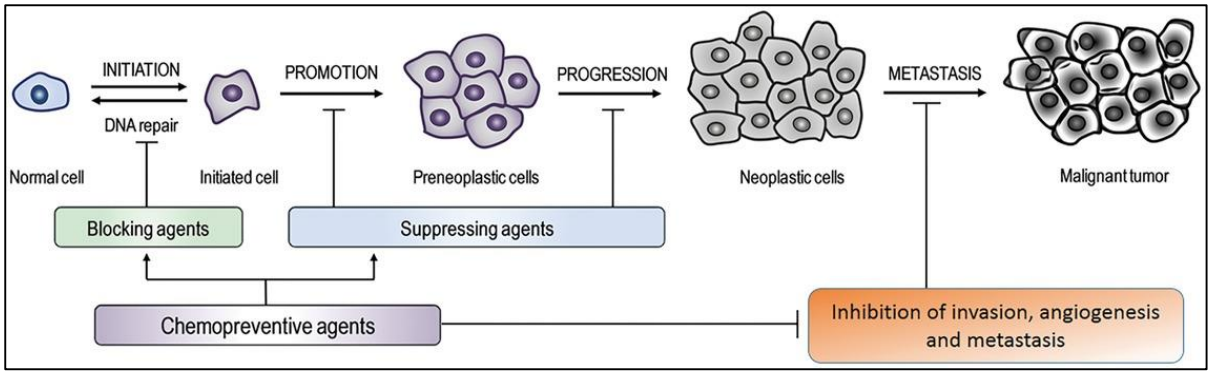


Figure 2: Mechanism of cancer development. *Cancer cells develop from normal cells through different stages, which include initiation, promotion, progression, and metastasis. These stages are activated by suppression of tumor suppressors specific to a particular stage. Adopted from Siddiqui et. al., 2015, doi: org/10.1111/nyas.12811.*

Initiation of cancer starts with genetic mutation in the DNA of a single cell which can be caused by exposure to carcinogens, radiation, and certain viruses in a result there is activation of proto-oncogenes like RAS and MYC into oncogenes which promote excessive cell growth and division. Cancer promotion is characterized by begin of uncontrolled and abnormal cell division which leads to the formation of a small cluster of cells called a preneoplastic lesion or tumor, however, several promoting factors such as environmental factors promote tumor growth. Further, the progression of cancer preneoplastic cells towards a malignant state by acquiring additional genetic mutations that might inactivate tumor suppressor genes like TP53. As a result, malignant cells produce vascular endothelial growth factor (VEGF) to form their own blood vessels, a process called as angiogenesis which supplies nutrients and oxygen to tumors to grow larger and cells gain the capability to invade surrounding soft tissue via entering the blood vessels or via the lymphatic system (invasion). At the final stage of cancer progression bloodstream or lymphatic system-invaded malignant cells disseminate to distant organs and tissues (metastasis) acquiring epithelial-mesenchymal transition (EMT) and forming secondary tumors which makes cancer more challenging to treat. Early detection and intervention play a key role in managing cancer effectively however,

understanding the changes inside malignant cells at a molecular level is crucial for developing targeted therapies and improving cancer treatment outcomes.

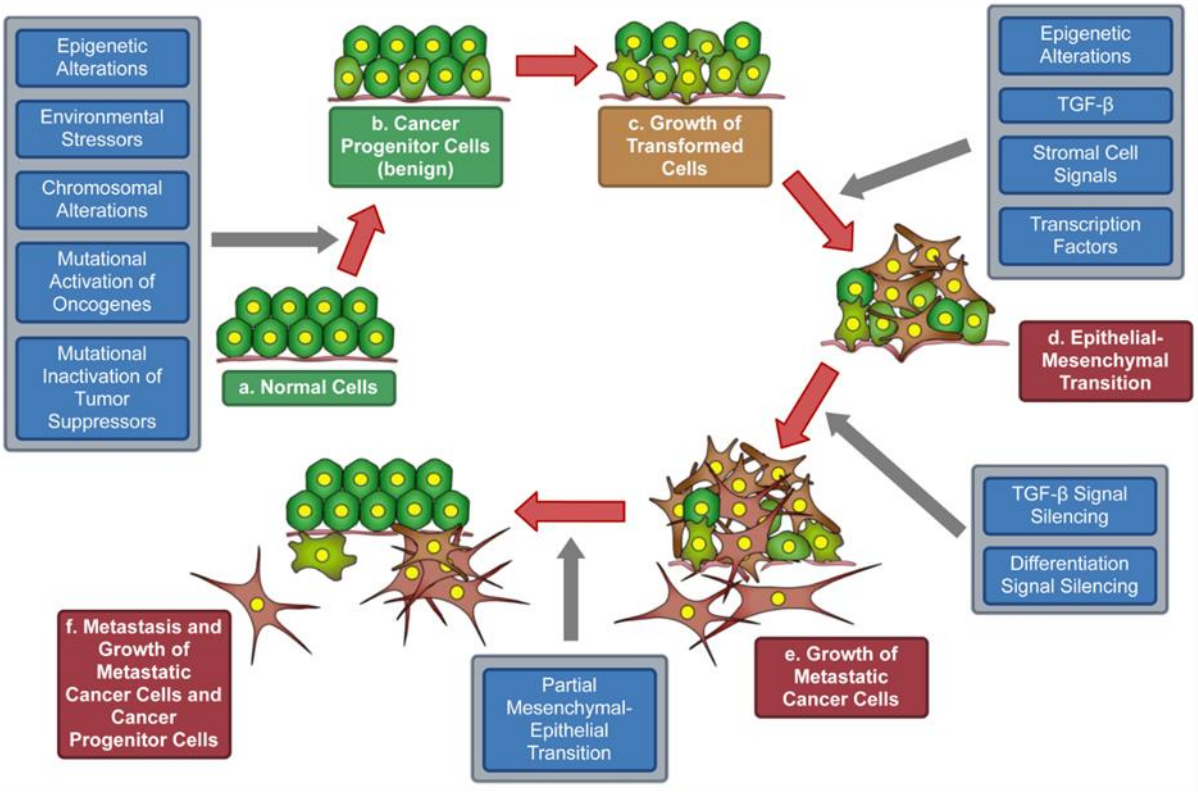


Figure 3: Factors affecting the development of Cancer. *Several changes at the molecular level in a cell are responsible for its advancement into a cancer cell such as epigenetic alterations, environmental stressors, chromosomal alterations, activation of oncogenes, inactivation of tumor suppressors, oncogenic signaling activation, and so on. Adopted from Sarkar et. al., 2013, doi: 10.3390/ijms141021087.*

Deregulation of several signaling pathways regulating different cellular cancer hallmarks such as cell growth, survival, differentiation, and apoptosis directly or indirectly are intricate in the cancer progression at the molecular level (Vogelstein & Kinzler, 2004). Some of the primary signaling pathways that are deregulated in some of the cancers are listed below.

- RAS-RAF-MEK-ERK pathway: Activation of RAS or RAF by gene mutations leads to activation of MAPK signaling that results in enhancement of cell proliferation and viability of the tumor cells in lungs, colorectal, and pancreas (Wan et al., 2004).

- PI3K-AKT-mTOR pathway: Activated by mutations in PIK3CA or impairment of functional mutation in PTEN leads to the enhancement in cell survival, growth, and metabolism in breast, ovarian, and prostate cancer (Noorolyai, Shajari, Baghbani, Sadreddini, & Baradaran, 2019).
- Wnt/β-catenin pathway: This pathway is convoluted in cell proliferation and survival with mutational activation of APC or β-catenin protein in colorectal cancer and hepatocellular carcinoma (Krishnamurthy & Kurzrock, 2018).
- Other pathways: Signalling pathways mediated by gene alterations in TGF-β, NF-κB, hedgehog, notch, and HIF-1α also promote tumor growth (Bierie & Moses, 2006; Dolcet, Llobet, Pallares, & Matias-Guiu, 2005; Rashid et al., 2021; Skoda et al., 2018).

Numerous targeted drugs are designed to disrupt specific nodes in these pathways to impede the growth and survival of cancer cells. Ongoing research is continuously uncovering new therapeutic targets. Hence, developing targeted therapies for cancer treatment requires a comprehensive understanding of the deregulation of signaling pathways. Nowadays apart from genetic regulation such as mutation of genes, epigenetic gene regulation mediated by Histone modifications, DNA methylation, and RNA interference (RNAi) also play a crucial role and are well-defined in the advancement of cancer (Dawson & Kouzarides, 2012; Lengauer, Kinzler, & Vogelstein, 1998; Luo, Huang, Wei, Sun, & Gong, 2023; Zaib, Rana, & Khan, 2022).

1.5 Epigenetics

Epigenetics, an intriguing branch of genetics, delves into modifications in genetic expression and inheritable characteristics that transpire without any changes to the DNA sequence. Derived from the Greek prefix "epi," meaning "above" or "on top of," the term "epigenetics" denotes that these alterations occur beyond the realm of the genetic code. It is

characterized as various covalent modifications that occur with histone proteins and DNA which regulate the expression of several genes, and function making genes turn ON and OFF. This epigenetic regulation is functionally crucial for normal cellular functions (Morris, Willcox, & Donlon, 2019) and the progression of several diseases like Cancer. There are many epigenetic modifications, of which the methylation of DNA, chromatin remodeling, modifications of histone, and RNA-mediated regulations are important mechanisms (Figure 4).

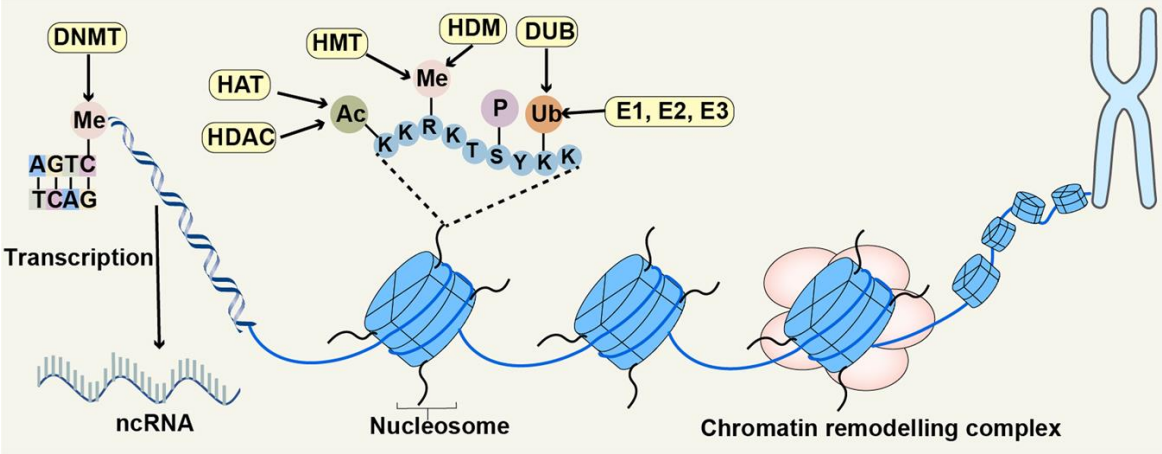


Figure 4: Different types of epigenetic modifications. *Several epigenetic modifications happen in the human cell at the posttranscriptional and post-translational level of a gene including methylation, acetylation, phosphorylation, and ubiquitination. Abbreviations: DNMT, DNA Methyltransferase: HDAC, Histone deacetylase; HAT, Histone acetyltransferase; HMT, Histamine N-methyltransferase; HDM, histone demethylase; DUB, Deubiquiting enzyme; Ac, acetyl; Me, methyl; P, phosphate; Ub, ubiquitin. Adopted from Wu et. al., 2023, doi: 10.1038/s41392-023-01333-7.*

1.5.1 DNA methylation

A well-studied universal chemical modification process that comprises addition of methyl groups to the DNA thereby making gene silencing is DNA methylation. It majorly occurs at the cytosine phosphate guanine (CpG) islands (Bird, 1986) which are majorly located within centromeres, telomeres, repeat sequences, and inactive X- chromosomes. Functionally, it is associated with different biological processes like gene expression regulation, genome stability, and genomic imprinting (Dawson & Kouzarides, 2012; Nishiyama & Nakanishi, 2021). Of several forms of DNA methylation (like 5mC, 5caC, 5fC, and 5hmC), 5mC is

commonly found in the human genome and is well-studied (Kriaucionis & Heintz, 2009; Maiti & Drohat, 2011; Tahiliani et al., 2009).

DNA methyltransferases (DNMTs) the enzymes which catalyse DNA methylation by transferring methyl group at the 5' site of the cytosine ring from the S-adenosylmethionine (SAM) of the target DNA. There are five DNMTs discovered in the human genome namely DNMT1, DNMT2, and DNMT3A/B/L classified into two major groups, maintenance DNMTs and de novo DNMTs. Functionally, DNMT1 is a maintenance DNMT and is involved in maintaining established methylation, whereas, DNMT3A/3B are de novo DNMTs involve in establishment of a new methylation pattern. However, DNMT2 and DNMT3L do not have catalytic activity. Since the patterns of methylation are constantly changing and responding to stimuli, DNA demethylation may take place passively in growing cells or as a proactive event in non-dividing cells which involves ten-eleven translocation (TET) enzymes mediated oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Chen & Zhang, 2020; Moore, Le, & Fan, 2013) (Figure 5).

DNMTs have been well studied and are known to perform a crucial role in various human diseases such as cancers, autoimmune diseases (like rheumatoid arthritis), (Liu et al., 2013), systemic lupus erythematosus (Chung et al., 2015), type-II diabetes (Dayeh et al., 2014), metabolic disorders, neurological disorders, and aging (Jin & Liu, 2018), breast cancer (McCann et al., 1996), osteosarcoma (Ulaner et al., 2003), colorectal Cancer (Cui et al., 2002), and ovarian cancer (Murphy et al., 2006).

1.5.2 Chromatin remodeling

It refers to the dynamic rearrangement of chromatin architecture which plays vital role in the transcription of condensed genomic DNA allowing its accession to different transcriptional regulators. These modifications are carried out with involvement of chromatin remodelers and modifiers thus Providing accessibility to the underlying DNA for chromatin

assembly, repair, and other processes(Petty & Pillus, 2013). Chromatin remodelers usually remodel the DNA mediating the formation of ATP-dependent chromatin complexes whereas chromatin modifiers remodel DNA by covalent histone modification.

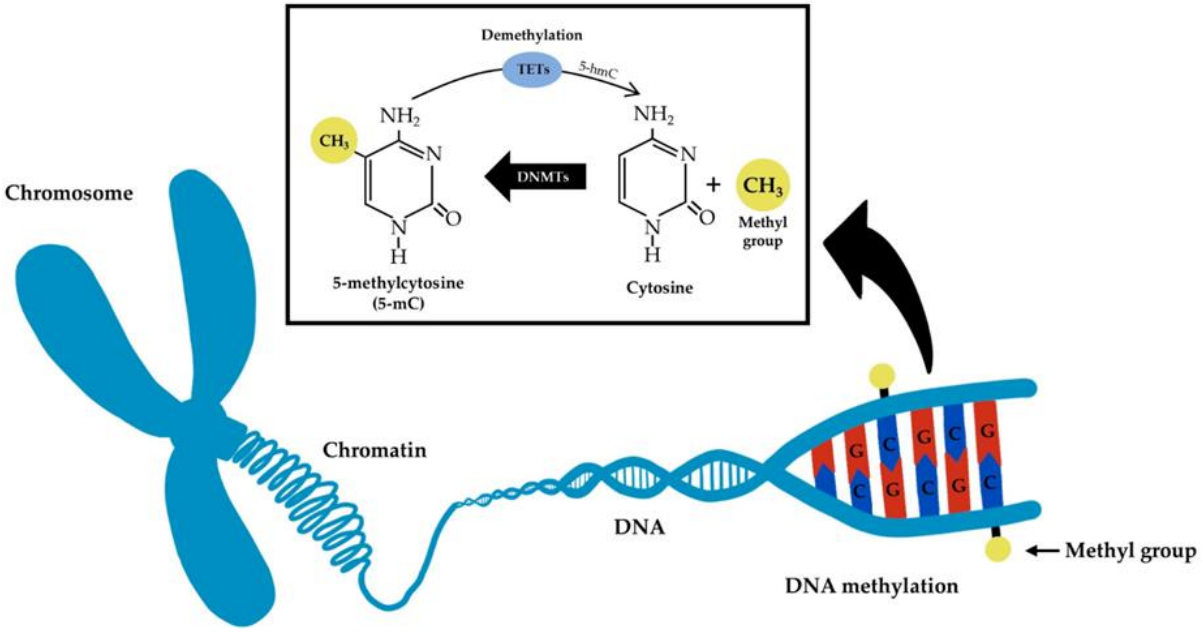


Figure 5: Mechanism of DNA methylation. *DNA methyltransferases catalyze DNA methylation by transferring methyl group at the 5' site of the cytosine ring from the S-adenosylmethionine of the target DNA, whereas TET enzymes catalyze DNA demethylation. Abbreviations: TETs, ten-eleven translocations; DNMTs, DNA methyltransferases. Adopted from Valente et. al., 2023, doi: 10.3390/nano13121880.*

Chromatin remodelers functionally make DNA mobilization around nucleosomes with the help of ATP hydrolysis as an energy source which result in sliding of nucleosomes, eviction, and replacement of canonical histones. In eukaryotes, remodelers are clustered into four families, named, SWI/SNF (Switching-defective/sucrose-non-fermenting), ISWI (Imitation of SWI), CHD (Chromodomain helicase DNA-binding), and INO80 (Inositol-requiring 80) based on shared domains associated with the enzymatic core. Remodelers of the SWI/SNF family are functionally involved in the eviction and sliding of nucleosomes. The ISWI family contributes to nucleosome assembly and spacing in chromatin structure at higher levels. CHD remodelers are responsible for nucleosome slipping, expulsion, separation, and

gathering. The INO80 family is also specialized for nucleosome remodeling, which involves the family member of SWR1 (SWI2/SNF2-Related 1) which accomplishes so via substitution of H2A.Z and H2B histone variants dimers in place of H2A-H2B dimers (Petty & Pillus, 2013) (Figure 6).

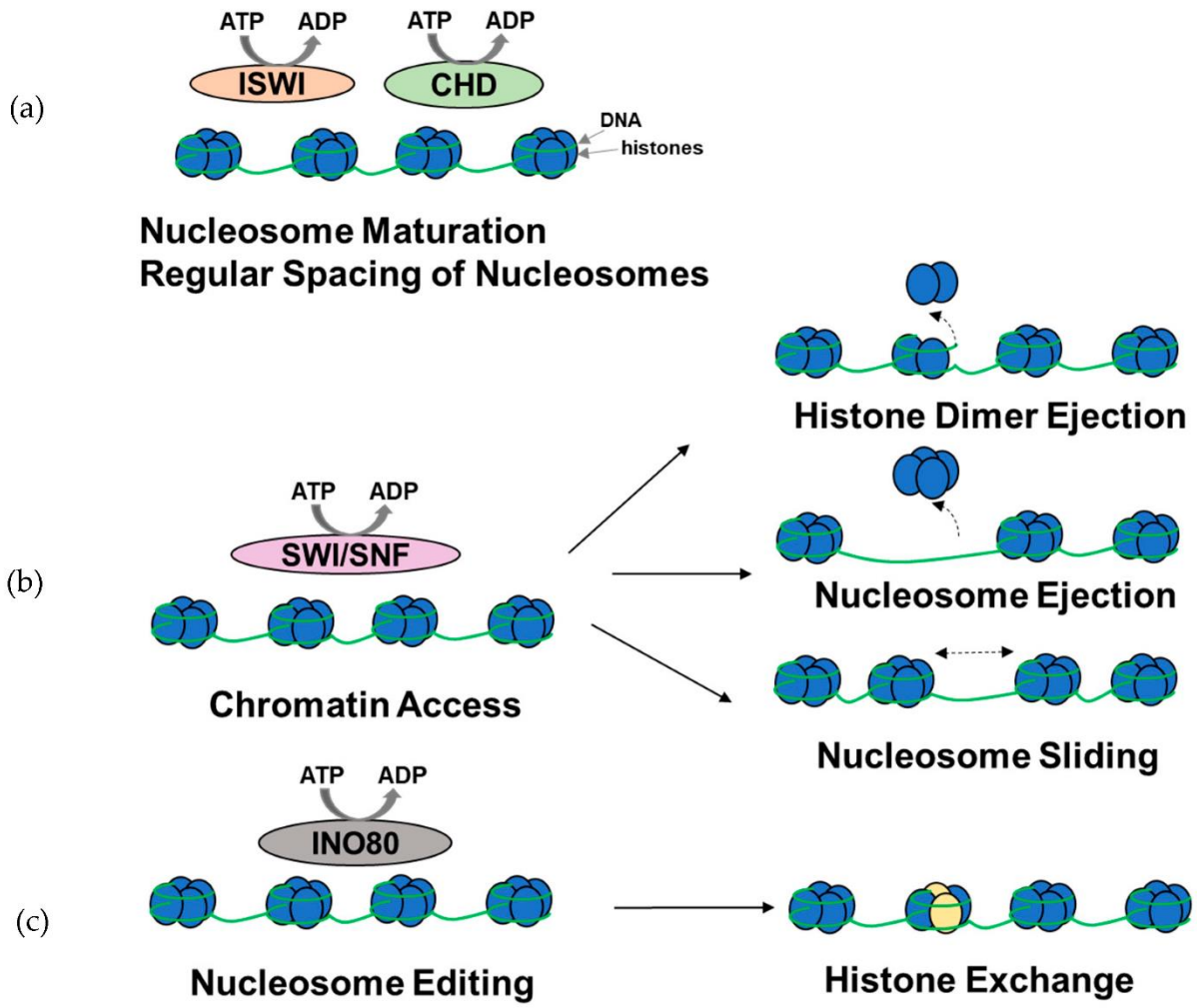


Figure 6: Mechanism of chromatin remodeling. *In eukaryotes, chromatin remodelers involved in chromatin remodeling are SWI/SNF, ISWI, CHD, and INO80, which results in nucleosome maturation, histone dimer ejection, nucleosome ejection, nucleosome slide, and histone exchange. Abbreviations: ATP, Adeno triphosphate; ADP, Adeno diphosphate; SWI/SNF, Switching-defective/sucrose-non-fermenting; ISWI, Imitation of SWI; CHD, Chromodomain helicase DNA-binding; INO80, Inositol- requiring 80. Adopted from Hasan and Ahuja, 2019, doi: 10.3390/cancers11121859.*

1.5.3 Histone modification

Structurally to make nucleosome, the chromatin building block, DNA (around 146 base pairs) is wrapped around a histone protein core that is made up of octamer (eight) of histones i.e., reproduce copies of each, H2A/2B/3/4 (Figure 7).

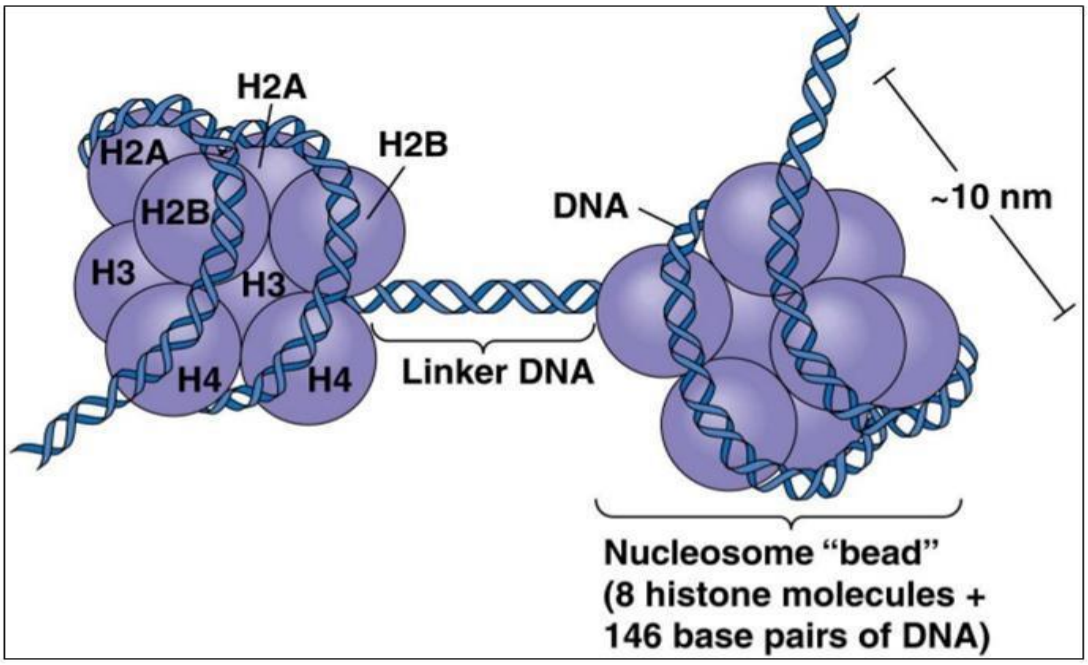


Figure 7: Schematic diagram representing the structure of a nucleosome. *The nucleosome core is composed of a histone octamer [(H2AH2B)×2, (H3-H4)×2]. The DNA double helix is wrapped around (~1.7 times) the histone octamer. With nuclease digestion, 146 bps of DNA are tightly associated with the nucleosome but ~200 bps of DNA in total are associated with the nucleosome. Source; http://www.mun.ca/biology/desmid/brian/BIOL2060/BIOL2060-18/18_21.jpg.*

Chromatin modifiers covalently modify DNA by adding and removing modifications post-translationally associated with principal histones as well as supplementary chromatin proteins. These enzymatic post-translational adaptations involve reversible accumulation of several chemical structures such as in processes like methylation, acetylation, ubiquitination, phosphorylation, etc., on histone N-terminal tails of histones H1-H3 (histone modifications). These alterations affect the binding affinity of DNA to that of histone proteins,

thereby leading to changes in chromatin organization or nucleosome stability and correlate with specific transcription rates.

Among many histone modifications, histone acetylation or deacetylation are very well studied. The current study focuses mainly on the histone deacetylation mechanisms involved in female-specific cancers such as cervical and breast.

1.5.3.1 Histone acetylation & deacetylation

Acetylation is one of the well-considered modification types of histone that happens principally on the residues of lysine and is promoted by enzymes, histone acetyltransferase (HATs). Acetylation of tails of histone neutralises lysine's partial electric charge, which opens the chromatin structure facilitating gene transcription (Figure 8). Acetylation of histone 3 on lysine 9 (H3K9) has a significant undesirable effect on the development of chromatin fibers of length 30 nm and higher-imperative structures (Shogren-Knaak et al., 2006). The acetyl group on lysine residues is removed by another family of proteins called histone deacetylases (HDACs) Removal of the acetyl group increases the positive charge on the histones leading to tight condensation of the DNA and thus gene repression. Although initially the acetylation and deacetylation were identified on histone proteins, now it is very well established that many other cellular proteins' function is also regulated by lysine acetylation and deacetylation. Therefore, HATs and HDACs are sometimes referred to as KATs and KDACs where K is the single letter designation of lysine amino acid.

1.5.3.1.1 HATs/KATs

HATs, also called writers of acetylation, functionally add acetyl chemical structures to lysine residues present in the ε-amino position from a coenzyme Acetyl-CoA thereby regulating the protein function by neutralizing the positive charge and increasing the hydrophobicity. In general, HATs are grouped into two classes, one is type A which is

predominantly positioned in the nuclei and functionally regulates nucleosomal histones, and another is type B which is located in cytoplasm and associated with acetylation of cytoplasmic proteins as well as synthesis of new histones (Brownell & Allis, 1996). Moreover, based on homology in catalytic domains HATs are classified majorly into three categories (Sterner & Berger, 2000) namely, GNAT or Gcn5-related N- acetyltransferase superfamily (Neuwald & Landsman, 1997), MYST family which is named after its members which consist of MOZ, Ybf2/Sas3, Sas2, and Tip60 (Borrow et al., 1996), and NCOAs (Nuclear receptor coactivators) for example, steroid receptor coactivator-1 (SRC-1) (Figure 9).

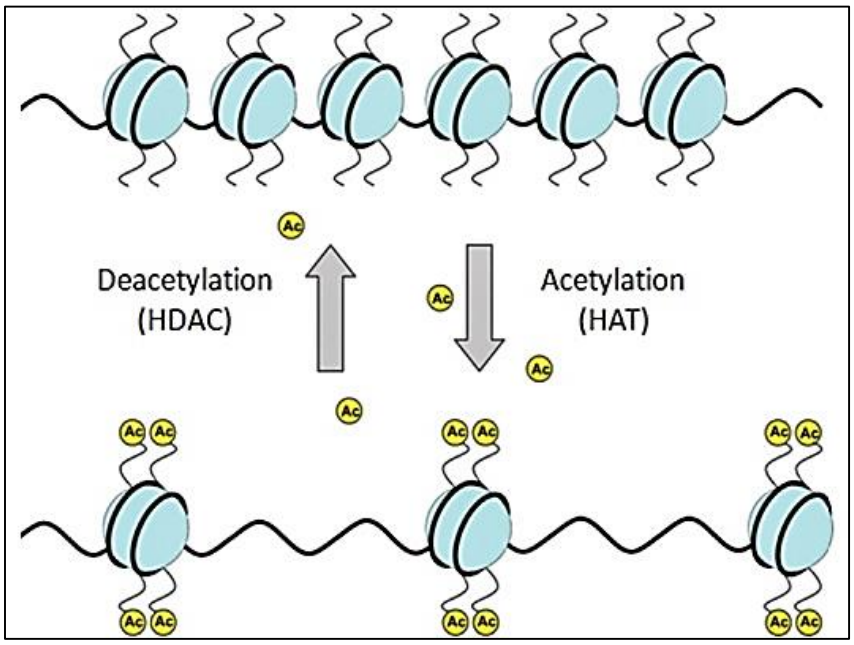


Figure 8: Histone acetylation and deacetylation mechanism. *Histone acetylation is mediated by HATs whereas, Histone deacetylation is mediated by HDACs respectively. Abbreviations: HDAC, Histone deacetylase; HAT, Histone acetyltransferase; Ac, Acetyl. Adopted from Pons et. al., 2009, doi: 10.1093/eurheartj/ehn603.*

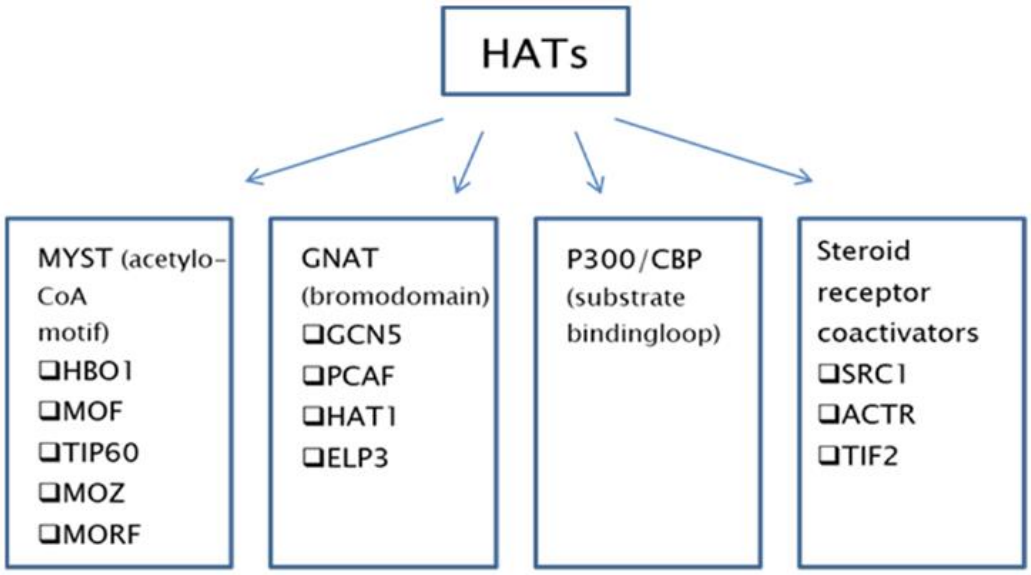


Figure 9: Classification of HATs. Differentiation of histone acetyltransferase subtypes based on the presence of catalytic domains. *Abbreviations: HATs, Histone acetyltransferases; GNAT, Gcn5-related N- acetyltransferase; SRC-1, steroid receptor coactivator-1; CBP, CREB-binding protein. Adopted from Kopytko et. al., 2021, doi: 10.3390/ijms22062828.*

1.5.3.1.1 HDACs/KDACs

HDACs, also called erasers of acetylation, functionally reverse lysine acetylation thereby regulating the protein function. In mammals, there are eighteen different HDACs Traditionally, based on homology to HDACs of yeast they are divided into four separate classes (Figure 10). Class I which comprises HDAC1,2,3, and 8 has homogeneity to the yeast protein Rpd3. Class II which comprises HDAC4,5,6,7,9, and 10 has homogeneity to the yeast protein Hda1. Class III comprises Sirtuins namely SIRT1,2,3,4,5,6, and 7 that have homogeneity to the yeast protein Sir2. Class IV comprises HDAC11 which shares homology to the yeast Rpd3 and Hda1 proteins. HDACs modify protein function by removing protein acetyl groups from ε-amino lysines, and they also enhance the creation or deletion of alternate posttranslational modifications of lysine residues like as methylation, ubiquitination, and sumoylation (Seto & Yoshida, 2014).

HDACs are well-studied to play a critical role in several signaling networks and are linked with development, physiology, and numerous disease conditions (Haberland, Montgomery, & Olson, 2009; Krämer, Göttlicher, & Heinzel, 2001; Mahlknecht & Hoelzer, 2000) such as inflammatory lung diseases (Barnes, Adcock, & Ito, 2005), pulmonary diseases (Barnes, 2005), central nervous system diseases (Langley, Gensert, Beal, & Ratan, 2005; Shukla & Tekwani, 2020), and various cancers (Brancolini, Gagliano, & Minisini, 2022; Hai et al., 2022; Y. Li & Seto, 2016; Liang et al., 2023). Additionally, HDACs are revealed as a potential drug target for many diseases.

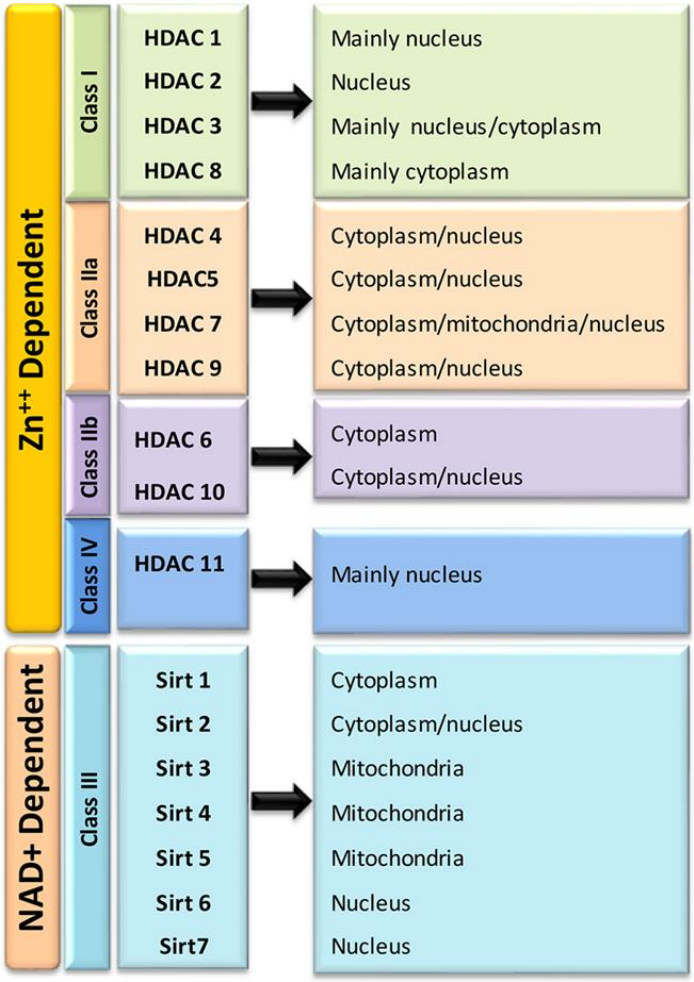


Figure 10: Classification of HDACs and their cellular localization. *Depending on homology to Yeast there are 18 HDACs studied in humans, which are divided into four classes. Abbreviations: HDAC, Histone deacetylase; Sirt, Sirtuin. Adopted from Shukla and Tekwani, 2020, doi: 10.3389/fphar.2020.00537.*

1.5.4 RNA interference

RNA interference (RNAi), is another epigenetic regulation process that broadly involves double-standard RNA-mediated (dsRNA) sequence-specific suppression of gene expression (Figure 11) (Agrawal et al., 2003; Barik, Sahay, Behera, Naik, & Kalita, 2021). This RNA silencing mechanism limits the gene at the transcript level either by hindering translation or activating RNA degradation. The so-called non-coding RNAs (ncRNAs), the transcriptional junk, emerged as key players in RNA interference. There are several classes of ncRNAs, each of which plays a distinct functional role in gene regulation epigenetically. Despite their numerous possible activities, they are loosely allocated into two primary categories according to their length: short non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs), which are shorter and longer than 200 nucleotides respectively in length. However, this categorization is not connected to their biogeny, activities, and cellular localization (Kapranov et al., 2007). SncRNAs include siRNA (Small interfering RNA), shRNA (Short hairpin RNA), and miRNA (MicroRNA), of which miRNAs are the subclass of ncRNAs that are most extensively studied and one of the interests showing involvement in the regulation of more than 60% of protein-coding genes in eukaryotes (Bourassa & Ratan, 2014). NcRNAs form a complex regulation layer at transcriptional as well as posttranscriptional level, that is heavily influenced by an epigenetic landscape controlled by these molecules. Hence, they make interaction with histone-modifying complexes, as well as affect the function of DNA methyltransferases, hence modulating transcriptional activity covering the entire genome (Peschansky & Wahlestedt, 2014).

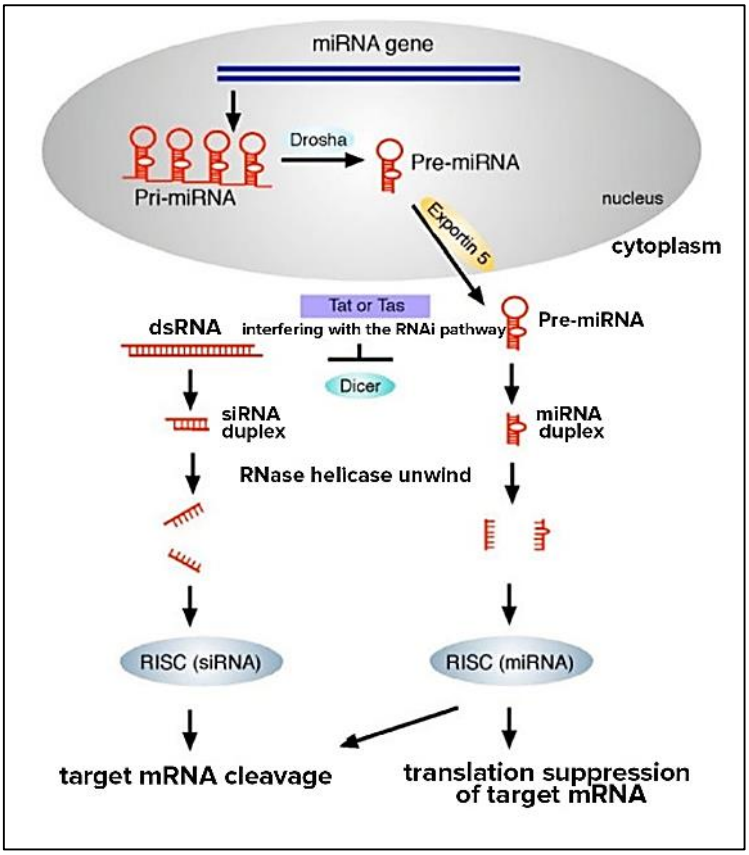


Figure 11: Gene regulation mediated by RNA interference. *The biogenesis of RNA silencing transcripts can be derived from either the host cell nucleus mRNA pathway to yield miRNA or the cytoplasmic double strand RNA to yield siRNA. Abbreviations: RISC, RNA-induced silencing complex; TRBP, Transactivation response element RNA-binding protein; DGCR8, DiGeorge syndrome critical region 8; miRNA, microRNA; siRNA, Small interfering RNA; mRNA, messenger RNA; dsRNA, double stranded RNA; Pre-miRNA, Precursor miRNA. Adopted from Mak, 2005, doi: 10.1186/1742-4690-2-35.*

1.5.4.1 MicroRNA (MiRNA)

MiRNAs are short ncRNAs of about ~22 nucleotides and functionally regulate gene expression by targeting messenger RNA (mRNA). Inside the nucleus, miRNAs are transcribed from primary miRNA (pri-miRNA) which consists of a hairpin loop by the enzyme RNA polymerase (RNA Pol) II (Figure 12). The pri-miRNA is excised and becomes pre-miRNA (~70 nucleotides) with the help of endoribonuclease enzyme named, Drosha and DGCR8, a RNA binding protein. The pre-miRNA further shipped to the cytoplasm from nucleus by a protein, exportin 5 in association with another protein called Ran-GTP. Inside the

cytoplasm, the pre-miRNA interacts with another endoribonuclease enzyme called Dicer resulting in double-stranded miRNA. The miRNA forms RNA-induced silence complex (RISC) in association with argonaute (Ago) protein, where it becomes functionally active and pairs with the 3' untranslated region (3'UTR) of destined mRNA thereby hindering its translation or leading to its degradation (Bartel, 2009). Interestingly, only 2-6 nucleotides of the miRNA appear to be critical for targeting mRNA (Lewis, Burge, & Bartel, 2005; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003), and a perfect complementarity with mRNA will lead to its degradation, whereas, imperfect complementarity results in repression in mRNA translation (Goodall, Heath, Bandmann, Kirby, & Shaw, 2013).

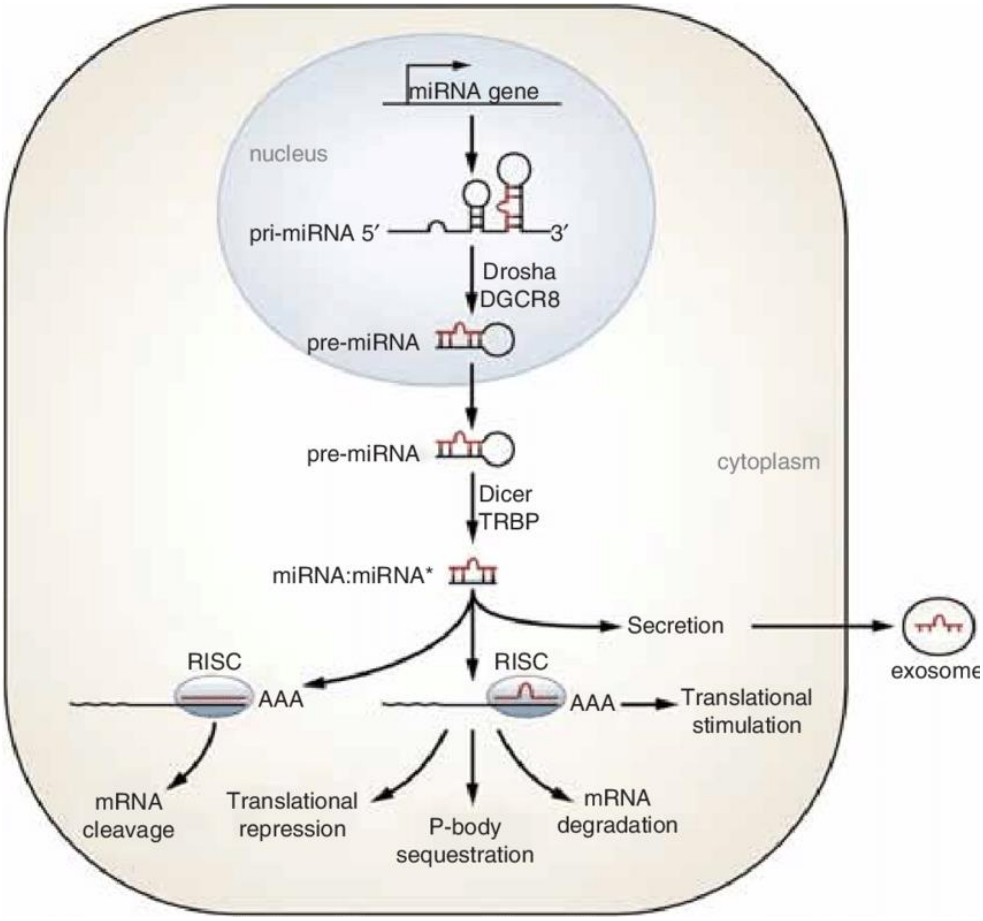


Figure 12: Biogenesis and functional activity of microRNAs. *In general, it is transcribed as a normal gene and undergoes enzymatic cleaves by several nuclear and cytoplasmic enzymes to become mature microRNA. Whereas, regulates its target mRNA showing complete or partial binding. Abbreviations: RISC, RNA-induced silencing complex; TRBP, Transactivation response element RNA-binding protein; DGCR8, DiGeorge syndrome critical region 8. Adopted from Tardito et. al., 2013, doi: 10.1517/13543784.2013.749237.*

1.5.4.1.1 Role of miRNAs in Cancer development

MicroRNAs have been confirmed as critical molecular elements within the cell in physiological and pathological conditions such as cancer (Ebert & Sharp, 2012). They play an essential role in cancer biology by controlling their target mRNA expression facilitating cancer development and progression. It has been well documented that they regulate all cancer hallmarks such as supporting proliferative signaling, shirking growth suppressors, inducing angiogenesis, and so on (Figure 13) (Hanahan, 2022; Stahlhut & Slack, 2013).

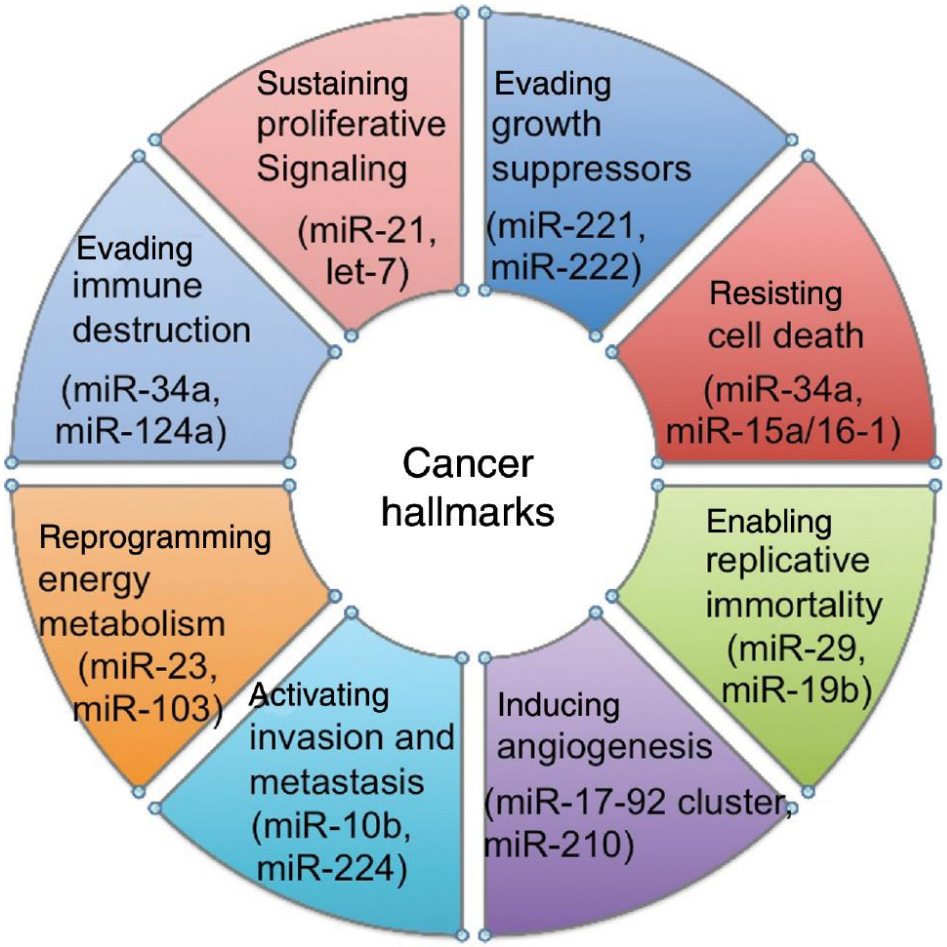


Figure 13: microRNAs involved in the regulation of different cancer hallmarks. *microRNAs are functionally associated with all aspects of cancer hallmarks. Typical examples of oncogenic or tumor suppressor microRNAs for each hallmark are shown. Adopted from Ling et. al., 2017, doi: 10.1515/cclm-2016-0740.*

In cancer advancement, miRNAs can function as oncogenes (OncomiRs) and tumor suppressors (TSmiRs), which functionally enhance cancer progression by targeting tumor suppressor mRNAs and suppress cancer progression activating of tumor suppressor target mRNAs respectively (Figure 14). The roles of microRNAs as tumour suppressors and oncogenes should not be stereotyped. The same microRNA is able to act like an oncogene in one type of tumor but stop another depending on the environment of the cell (Ling, Girnita, Buda, & Calin, 2017).

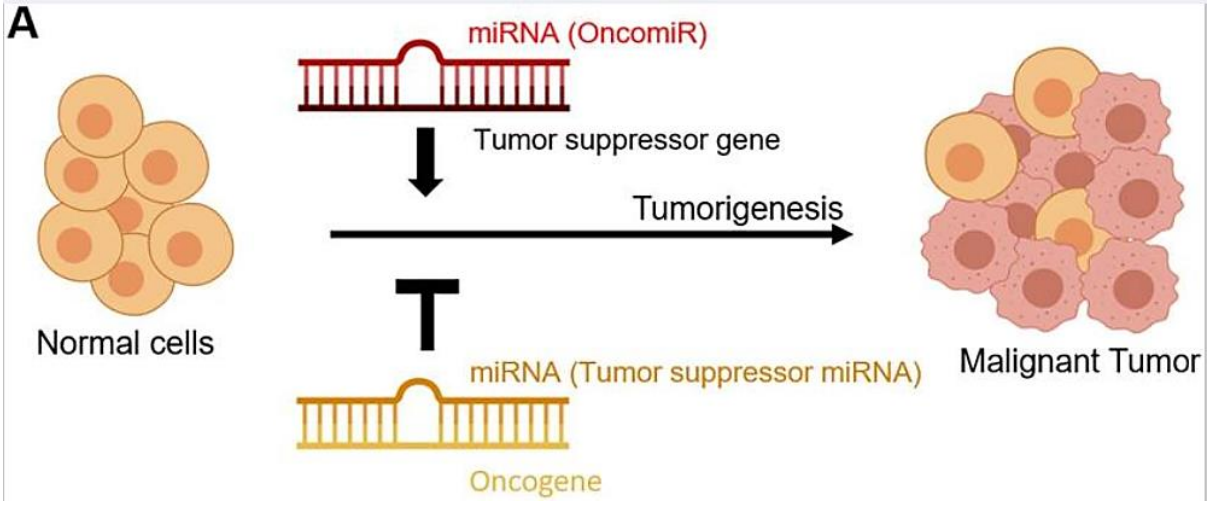


Figure 14: Schematic representation of miRNA-mediated regulation of cancer development. *Depending on the target gene, miRNAs suppress or stimulate cancer-related genes acting as oncogenic or tumor suppressor miRNAs. Adopted from Lee et. al., 2019, doi: 10.1016/j.jconrel.2019.10.007.*

MicroRNAs show aberrant expression in human cancer types, which are the result of several underlying mechanisms. First, as confirmed initially, microRNAs found in continually transformed genomic areas may lose their expression owing to chromosomal ablation or enhance their expression as a consequence of chromosomal enrichment (Calin et al., 2004). Epigenetic processes, which include CpG island methylation, play a key role in controlling microRNA expression. For example, miR-124 is suppressed by hypermethylation of its promoter CpG island in human's cancer (Lujambio et al., 2007). Additionally,

abnormalities in microRNA-processing machinery may drastically affect the expression levels of microRNAs with maturation. Indeed, reduced expression or mutations in genes involving in microRNA-processing (such as Dicer, TRBP, and Exportin) have been identified in many advanced cancer, and they influence extensive variety expression of miRNA (Ohtsuka, Ling, Doki, Mori, & Calin, 2015). According to the ceRNA theory, competitive endogenous RNAs that serve as sponge of microRNA because of their microRNA binding locations may de-repress the microRNA's target genes (Salmena, Poliseno, Tay, Kats, & Pandolfi, 2011).

Additionally, microRNAs are also well studied as potential biomarkers for cancer diagnosis due to various reasons. First, microRNAs can endure severe temperatures, prolonged storage, strong basic or acidic environments, and several freeze-thaw cycles. This capability allows for the identification of expression of microRNA in samples in formalin-fixed paraffin-embedded (FFPE) conditions, plasma, serum, urine, and other bodily fluids. Secondly, mature microRNA may be detected either by hybridization or PCR methods. Thirdly, microRNAs are good cancer biomarkers due to their tissue-specific expression patterns (Cortez et al., 2011). Circulating microRNAs are also valuable indicators for prognosis and treatment response. MicroRNA therapy offers the capacity to target several protein-coding genes of the equivalent pathways in various stages, thereby averting compensatory mechanisms like modification in the targeted oncogenes, which results to resistance thus providing therapeutic advantages (Ling et al., 2017).

1.6 Microtubule and Cancer

Microtubules, the main constituents of a cell's cytoskeleton, are long, filamentous, and tube-like protein polymers consist of alpha-tubulin and beta-tubulin heterodimers. They are critical in all eukaryotic cells playing a vital role in the development and cell shape maintenance, in the intracellular transports, in cell signaling, cell division, and migration (Desai & Mitchison, 1997; Gundersen & Cook, 1999; Prosser & Pelletier, 2017; Singh et al., 2018;

Waterman-Storer & Salmon, 1997). Notably, in the cancer scenario, the dynamic instabilities of microtubules are associated with activities that include the proliferation, invasion, and migration of cells (Jordan & Wilson, 2004; X. Li & Wang, 2020; Parker, Teo, McCarroll, & Kavallaris, 2017; Rowinsky, Donehower, Jones, & Tucker, 1988). Paclitaxel is known to inhibit this tubulin polymerization thereby stabilizing the microtubules thus leading to cell death (Jordan et al., 1996; Milas et al., 1995). Paclitaxel is the first significant microtubule-stabilizing agent identified and used as a front-line therapy for cervical and breast Cancer (Alqahtani, Aleanizy, El Tahir, Alkahtani, & AlQuadeib, 2019; Jordan & Wilson, 2004; M. Kim & Rejniak, 2014; Rodriguez et al., 1995; Schiff & Horwitz, 1980; Weaver, 2014; Wiseman & Spencer, 1998). However, paclitaxel not only inhibits tubulin polymerization in cancer cells but also inhibits in all normal dividing cells causing adverse effects such as anemia, hair loss, gastric ulcers, etc. (Jordan, Toso, Thrower, & Wilson, 1993; Schiff & Horwitz, 1980).

1.7 Epigenetic regulation of tubulin

In eukaryotic cells, tubulin is majorly regulated epigenetically by the processes called acetylation and deacetylation apart from other processes like detyrosination, polyglutamylation, and polyglycylation resulting in increased microtubule stability (Figure 15) (Schulze, Asai, Bulinski, & Kirschner, 1987).

1.7.1 Acetylation of Tubulin

The dynamic acetylation and deacetylation of α-tubulin at the conserved lysine residue (K40) plays a very important role in polymerization and microtubule stability. Acetylation of α-tubulin is done by HAT named α-tubulin acetyltransferase 1 (ATAT1) of the GNAT family (Figure 16) (Kalebic et al., 2013). Acetylated tubulin notably functions as a marker for microtubule stability by making them resistant to depolarization (Cambray-Deakin & Burgoyne, 1987; Palazzo, Ackerman, & Gundersen, 2003), regulating cell polarity,

involving in subcellular transport, playing a role in viral immune responses, and regulating stress-mediated signaling pathways. Hyperacetylation of tubulin enhances chemotactic cell motility (Y. Zhang et al., 2008). Alteration in tubulin acetylation is profoundly associated with different human diseases and acts as the target in therapeutic interventions (L. Li & Yang, 2015).

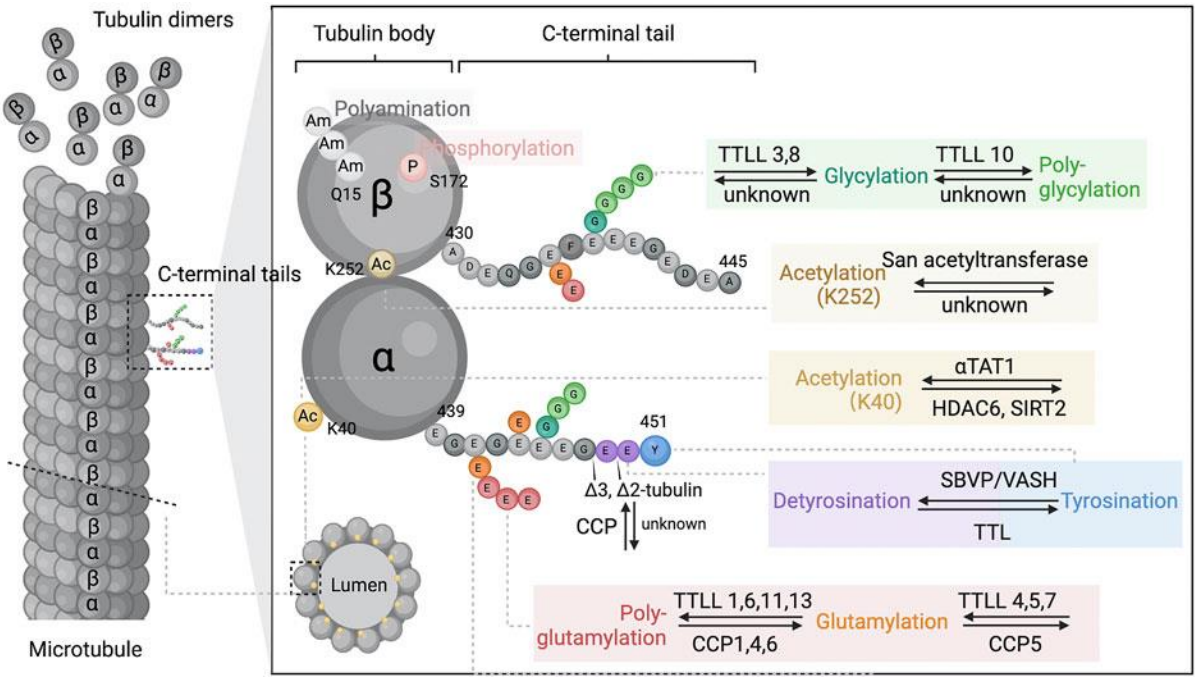


Figure 15: Epigenetic regulation of microtubule. *Tubulin post-translational modifications occur either at specified sites of the tubulin body (Ac, acetylation; P, phosphorylation; Am, polyamination), or within the C-terminal tails (detyrosination and detyrosination, glutamylation, glycylation, Δ2-tubulin and Δ3-tubulin prodeced by glutamate residue removals). These modifications are often dynamic reversible and catalysed by a range of enzymes from multiple families. During the process, the functional properties of microtubules are changed by single residue alternations (acetylation, phosphorylation, detyrosination) or modulating the non-binary signals by elongation of the side chains (polyamination, polyglutamylation, polyglycylation). Abbreviations: TTLL, tubulin–tyrosine ligase-like family; αTAT1, a-tubulin N-acetyltransferase 1; HDAC, tubulin-lysine deacetylase; SIRT, sirtuin; SVBP, vasohibin binding protein; VASHs, vasohibins; TTL, tubulin-tyrosine ligase-like family; CCP, cytosolic carboxypeptidase-like protein; K40, lysine 40. Adopted from Liu et. al., 2022, doi: 10.3389/fcell.2022.872058.*

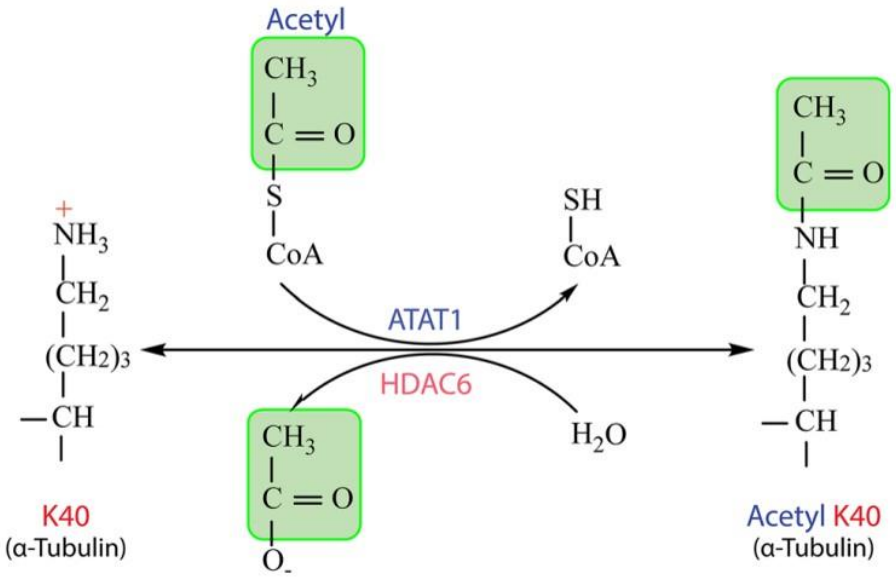


Figure 16: Acetylation and deacetylation of α-tubulin. *α-Tubulin acetylation refers to the transfer of the acetyl group from CoA to Lys-40. This modification is catalyzed by ATAT1 in mammals. The modification is reversible and its reverse reaction is catalyzed by HDAC6. Abbreviations: CoA, acetyl-coenzyme A; ATAT1, alpha-tubulin N-acetyltransferase 1; HDAC6, histone deacetylase 6. Adopted from Li and Yang, 2015, doi: 10.1007/s00018-015-2000-5.*

1.7.2 Deacetylation of tubulin by HDAC6 & HDAC8

The acetylated α-tubulin is deacetylated by HDACs. Tubulin is found as the first substrate for HDAC6 (Hubbert et al., 2002; Y. Zhang et al., 2003) and hence HDAC6 is likewise known as tubulin deacetylase (Figure 16). HDAC6, a class IIb HDAC, is located on human chromosome X at the p11.23 position and predominantly localized in the cytoplasm. With amino acid residues of 1215, it is the largest family member of HDACs and the only HDAC that contains two catalytic domains, situated at the N-terminal and middle portions of the protein (Figure 17) (Y. Li, Shin, & Kwon, 2013). However, both domains are independently functional and regulate the overall activity of HDAC6 (Grozinger, Hassig, & Schreiber, 1999; Kawaguchi et al., 2003; Y. Zhang, Gilquin, Khochbin, & Matthias, 2006). HDAC6 also has a ubiquitin-binding domain in the C-terminus (Seigneurin-Berny et al., 2001) and a domain for dynein binding (Kawaguchi et al., 2003).

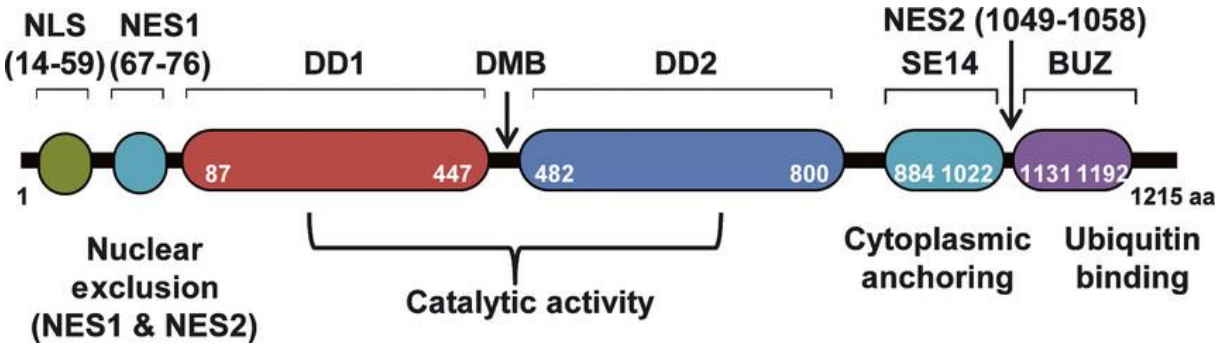


Figure 17: Structural representation and fuctional domains present in HDAC6. *HDAC6 is the only HDAC with two tandem deacetylase domains (DD1 and DD2) including catalytic activity. A nuclear export signal (NES) prevents the accumulation of the protein in the nucleus and the Ser-Glu-containing tetrapeptide (SE14) region ensures stable anchorage of the enzyme in the cytoplasm. The nuclear localization signal (NLS) translocates HDAC6 into nucleus. The linker (dynein motor binding, DMB) between both CATs can bind to dynein and the high affinity ubiquitin-binding zinc finger domain (BUZ). aa, amino acid. Adopted from Li et. al., 2013, doi: 10.1111/febs.12079.*

Moreover, it also interacts with other regulatory proteins associated with cell survivability (Ku70, Bax, CYLD, PP1, surviving), inflammation (Foxp3, NF-κB), cell signaling (GRK2, PCKα), protein degradation (VCP, PLAP, HSP90, HIF1α), angiogenesis (EB1, HSP90, HIF1α), and cell motility (Tubulin, cortactin, dynein) (Figure 18) (Hubbert et al., 2002; Y. Li et al., 2013; X. Zhang et al., 2007; Y. Zhang et al., 2003).

Apart from HDAC6, our lab has previously validated that HDAC8 also deacetylates α-tubulin when overexpressed in cells of cervical cancer (Vanaja, Ramulu, & Kalle, 2018). HDAC8, a member of the Class I HDAC family with 377 amino acid residues, is located on chromosome X at locus q13.1 and the one well-studied both structurally and functionally (Buggy et al., 2000; Hu et al., 2000; Van den Wyngaert et al., 2000). Initially, it was localized in the nucleus however, later recent studies found its distribution in both the nucleus and cytoplasm (Ahn, 2018; Huang et al., 2022; Nakagawa et al., 2007). Moreover, HDAC8 is the very first HDAC whose structure in three dimensions is established via X-ray crystallography (Figure 19) (Somoza et al., 2004).

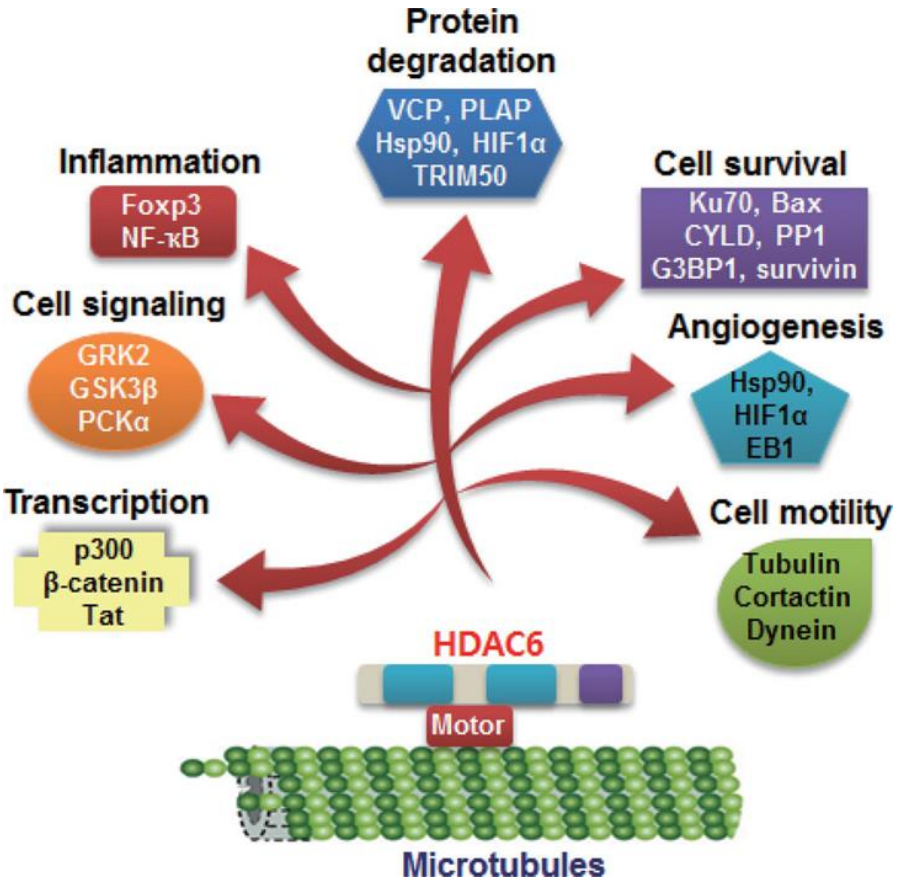


Figure 18: Cellular functions regulated by HDAC6. *Deacetylase-dependent and -independent functions of HDAC6 play a role in the recruitment of partner proteins such as Tubulin, HSP90, HIF1α, Bax, CYLC, surviving, VCP, PLAP, NF-κB, PCKα, p300, and β-catenin, and many more. Adopted from Li et. al., 2013, doi: 10.1111/febs.12079.*

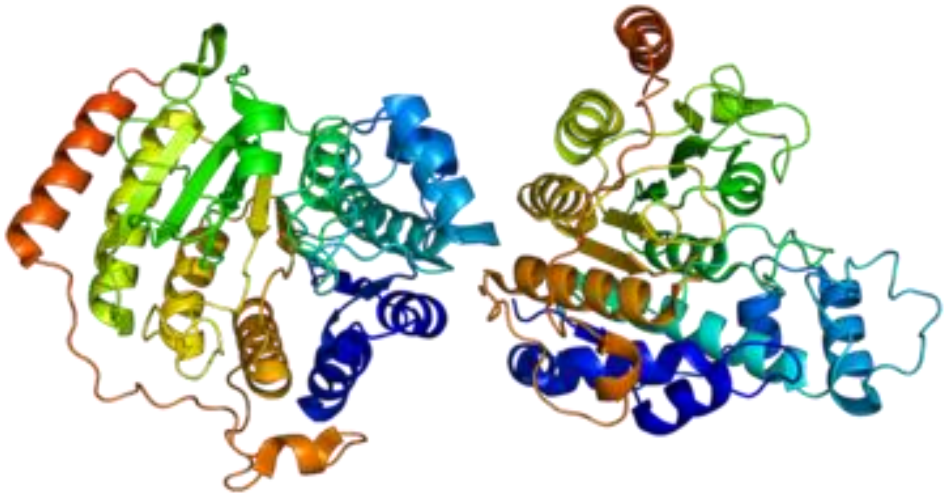


Figure 19: Crystal structure of human HDAC8. *PDB Ortholog search: https://en.wikipedia.org/wiki/HDAC8*

Functionally HDAC8 deacetylates its target substrates thereby modulating gene transcription, protein stability, and activity (Figure 20). HDAC8 preferentially deacetylates histones at H3 lysine 9/27 (H3K9/K27) (Fu et al., 2014; Ha, Reid, Meshkibaf, & Kim, 2016). This H3/H4 histone deacetylation regulates several gene repressions transcriptionally such as SOCS1/3, SIRT7, CCL4, IFNB1, MLN64, and BNIP3 (S. M. Gao et al., 2013; Ha, Han, Reid, & Kim, 2014; Meng et al., 2016; Tang et al., 2020; Yang et al., 2021). Nonetheless, several studies revealed HDAC8 as a deacetylase for multiple non-histone substrates, majorly SMC3 which is a subunit of cohesin (Dasgupta, Antony, Braithwaite, & Horsfield, 2016; Deardorff et al., 2012) and as a result, there is a proper dissociation of the cohesin complex which recycled after cell cycle completion. Tumor suppressor p53 (Lane, 1992) , upon deacetylation by HDAC8 becomes destabilized and functionally repressed resulting in tumor progression and thereby acts as a therapeutic mark in cancer of the ovary (J. Y. Kim et al., 2022), hepatocellular carcinoma (HCC) (Wu et al., 2013), and AML (Acute myeloid leukemia) (Qi et al., 2015). Moreover, HDAC8 was found as a deacetylase for many transcription factors thereby reactivating their functions such as ERRα, a nuclear receptor associated with cellular metabolisms (Wilson, Tremblay, Deblois, Sylvain-Drolet, & Giguère, 2010), and c-Jun, an oncogenic transcription factor (Emmons et al., 2019), and also represses their activity such as CREB and RUNX2 (Fu et al., 2014; J. Gao, Siddoway, Huang, & Xia, 2009). Additionally, HDAC8 also targets cytoskeletal proteins like cortactin, which bind with actin and enhance polymerization with actin and contraction of smooth muscle (J. Li et al., 2014).

Our group discovered α- tubulin as a substrate of HDAC8 (Vanaja et al., 2018), although, it is majorly deacetylated by HDAC6 (Hubbert et al., 2002). This functional redundancy of HDAC8 as tubulin deacetylase is observed only when HDAC8 is overexpressed and HDAC6 is normally expressed such as in cervical cancer cells (Vanaja et al., 2018). How

HDAC6 and HDAC8 are regulated in cancer cells and what decides which HDAC does tubulin deacetylation remains elusive.

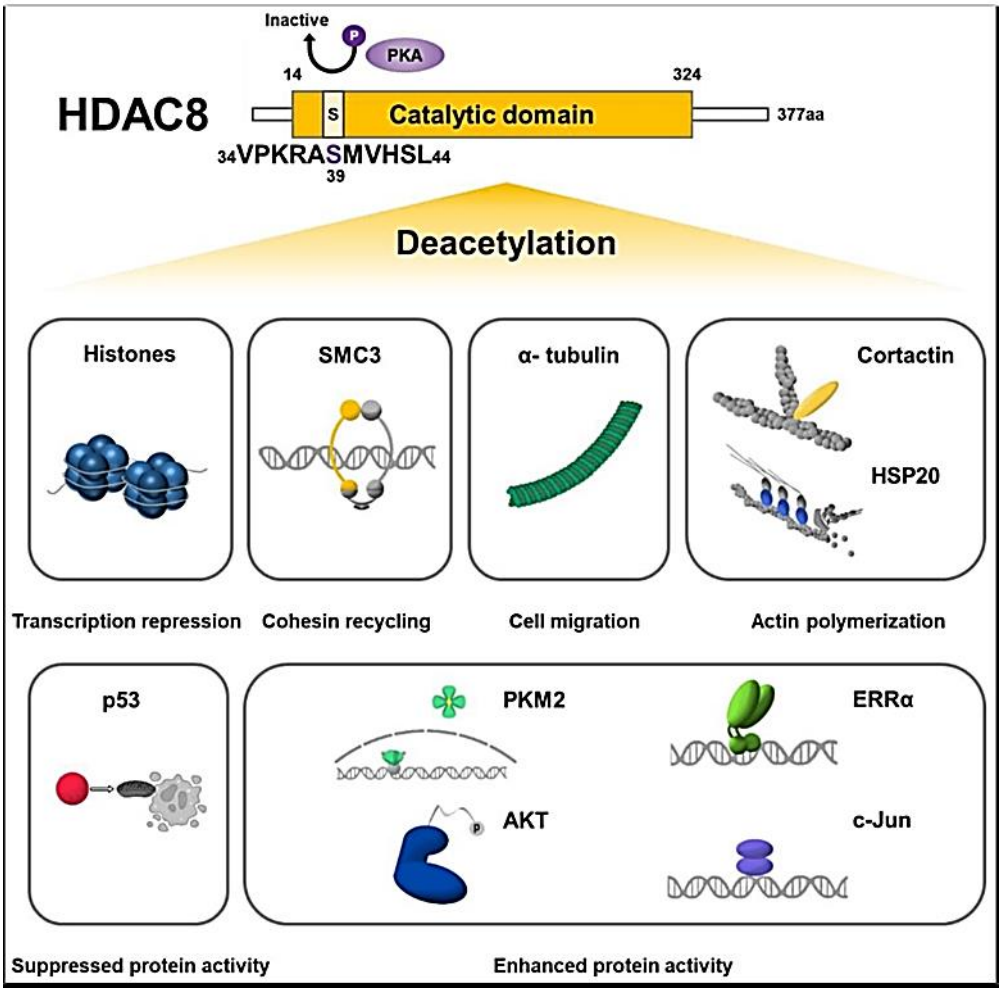


Figure 20: Schematic representation of histone and non-histone substrates of HDAC8. *HDAC8 deacetylates histones and suppresses the transcription of target genes. HDAC8 also deacetylates non-histone substrates such as SMC3, α-tubulin, cortactin, HSP20, p53, PKM2, AKT, ERRα, and c-Jun. Adopted from Kim et. al., 2022, doi: 10.3390/cells11193161.*

1.8 HDAC inhibitors (HDACi)

Aberrant expression of HDACs has been revealed to be intricate in cancer advancement and thus have been promising drug candidates. Several studies have demonstrated the in vitro and in vivo efficacy of HDAC inhibitors (HDACi) which are considered as a highly promising treatment strategy for cancer. However, due to the non-

selectivity of the identified inhibitors between 18 known HDACs, the increased toxicity has limited the use of HDACi in the clinic. Many far more effective, structurally varied HDAC inhibitors have been discovered. Pan-HDAC inhibitors, as well as class-selective or isoform-selective inhibitors, are naturally occurring or synthetically manufactured. Some HDAC inhibitors are novel salutary drugs that have been clinically proven in hematologic hostilities cancer patients, such as cutaneous T-cell lymphoma (CTCL) (Y. H. Kim et al., 2018; Wahi, Jain, Sinhari, & Jadhav, 2024).

Clinical studies utilising several HDAC inhibitors as single treatments in conjunction with traditional chemotherapies or as targeted medications are presently underway. HDAC inhibitors are highly tolerated and clinically successful against hematologic malignancies, but have limited anti-cancer effectiveness against solid tumors when taken as a monotherapy (Wahi et al., 2024).

Specific inhibitors for HDAC6 (such as Ricolinostat and Citarinostat) and HDAC8 (PCI -34051) have been well-studied for reducing cancer progression by inducing DNA damage, and advancing sensitization in transformed cells (Debnath et al., 2019; Tsai et al., 2023). However, HDACi also shows toxic side effects in patients. So, in order to reduce the toxicity, isoform-specific and class-selective HDACi need to be identified. Until then, we need to learn about the other mechanisms of HDAC regulation and one such mechanism is miRNA-mediated regulation of HDAC.

1.9 miRNAs and HDACs

Epigenetic regulation of HDACs through miRNAs shows a complex relationship that could be critically important but not yet completely understood. miRNAs play a critical role in regulating HDACs while HDACs themselves also can legalize the expression of miRNAs (Noonan et al., 2009; Rhodes et al., 2012; Scott, Mattie, Berger, Benz, & Benz, 2006). Except for few studies demonstrating miRNA-mediated HDAC1 regulation by miR-499a

(Noonan et al., 2009), HDAC4/5 regulation by miRNA-9 and miR-206 (Roccaro et al., 2010) and recent studies on HDAC8 regulation in breast malignancy by miRNA (Menbari et al., 2019; Menbari et al., 2020), HDAC6 and HDAC8 regulation mediated by miRNA in cervical Cancer is still unexplored, which forms the basis for the current study.

2.0 Rationale of the study

HDAC6 and HDAC8 are two deacetylases of α-tubulin and show differential expression in cancer cell and normal cell (Vanaja et al., 2018; Y. Zhang et al., 2003). HDAC8 behaves as a carcinogenic gene showing higher expression whereas HDAC6 shows normal expression in non-aggressive female-specific cancers like cervical, breast, and ovary cancers. However, both HDAC6 and HDAC8 show overexpression in aggressive cancers of the cervix, breast, and ovary. So the rationale of the study is to understand the discrepancy regulation of these HDACs and their involvement in cervical cancer as tubulin deacetylases. With this background, we aimed to identify miRNA-intervened HDAC6 and HDAC8 regulation in cervical cancer cells. We considered the HeLa (cervical cancer) cell line and MDAMB231 (metastatic breast cancer) cell line as cancerous cells and HEK293T (embryonic kidney) as a normal non-cancerous cell line.

The following are the objectives of the study.

1. Identification of potential miRNA (s) targeting HDAC8/6 and validation of the interaction of identified miRNA (s).
2. To study the functional significance of validated miRNA-mediated regulation of HDAC8/6 in cervical cancer cells.
3. To evaluate the functional significance of validated miRNA-mediated regulation of HDAC8 in metastatic breast cancer cells.

Materials & Methods

3.0 Materials and Method

3.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), Fetal Bovine Serum (FBS), and 100X Anti-Anti (Penicillin-Streptomycin) used for cell culture were procured from Gibco. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Himedia used in cell proliferation assay, TRizol from Invitrogen was used for RNA extraction. *E. coli* poly-A-Polymerase (NEB#MO276), cDNA synthesis kit (TaKaRa Bio#6110A), and Biorad iTaqTM Universal SYBR® Green supermix (172-5120) were used to check relative RNA expression and required primers utilized were procured from Consice Services.

Antibodies used in this study included GAPDH (SC32233), β- Actin (SC1616-R), HDAC6 (SC11420), Vimentin (SC6260), E-cadherin (SC8426), acetylated p53, acetylated HSP90 from Santa Cruz, HDAC8 (Ab187139) from Abcam, α Tubulin (T5168), and Ac-α Tubulin (T6793) from Sigma. Fluoroshield^{TM} with DAPI (F6057) was obtained from Sigma, and Alexa Fluor-488-green sourced from Invitrogen used in immunofluorescence.

For molecular cloning, enzymes were acquired from Thermo Scientific: SalI (ER0641), XbaI (ER0682), BamHI (ER0055), and T4 DNA ligase (EL0011). Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit were utilized for DNA purification. Lipofectamine 3000 from ThermoFisher (L3000001) facilitated efficient transfection, while the Dual-Luciferase assay kit (Promega, E1910) was employed for luciferase activity measurements. The use of these high-quality reagents ensured the reliability and reproducibility of experimental outcomes.

3.2 Methods

3.2.1 Integrated Prediction of miRNAs Targeting HDAC8 and HDAC6

To identify potential miRNAs targeting HDAC8 and HDAC6, a comprehensive approach was employed using three widely recognized tools: miRDB (http://mirdb.org/), Targetscan7.2 (http://www.target-scan.org/), and miRmap (https://mirmap.ezlab.org/). This integrated strategy, guided by established methodologies (Liu, Bordeaux, Hettich, & Han, 2020; Quillet et al., 2019), involved the use of TBtool software for analysis and visualization. TBtool facilitated the identification of overlapping miRNAs from the three prediction databases, addressing the challenge posed by the substantial number of predicted miRNAs across these platforms (Chen et al., 2020). A Venn diagram generated with TBtool depicted the common target genes shared by miRNAs across these databases.

The selected miRNAs were further assessed for seed region preference conservation and efficiency, prioritizing canonical sites over non-canonical sites (Figure 21) (Agarwal, Bell, Nam, & Bartel, 2015; Brennecke, Stark, Russell, & Cohen, 2005; Krek et al., 2005; Lewis, Burge, & Bartel, 2005). Gibbs free energy (ΔG) calculations were performed using mfold software to assess the stability and authenticity of miRNA-mRNA binding (Yue & Tigyi, 2010).

Subsequently, the expression levels of the identified miRNAs across various human cancers were determined using miRCancer and the dbDMEC 2.0 database. The predicted miRNAs and their roles in different signaling pathways were analyzed through miRnalyze and the DIANA-miRPath V3.0 database.

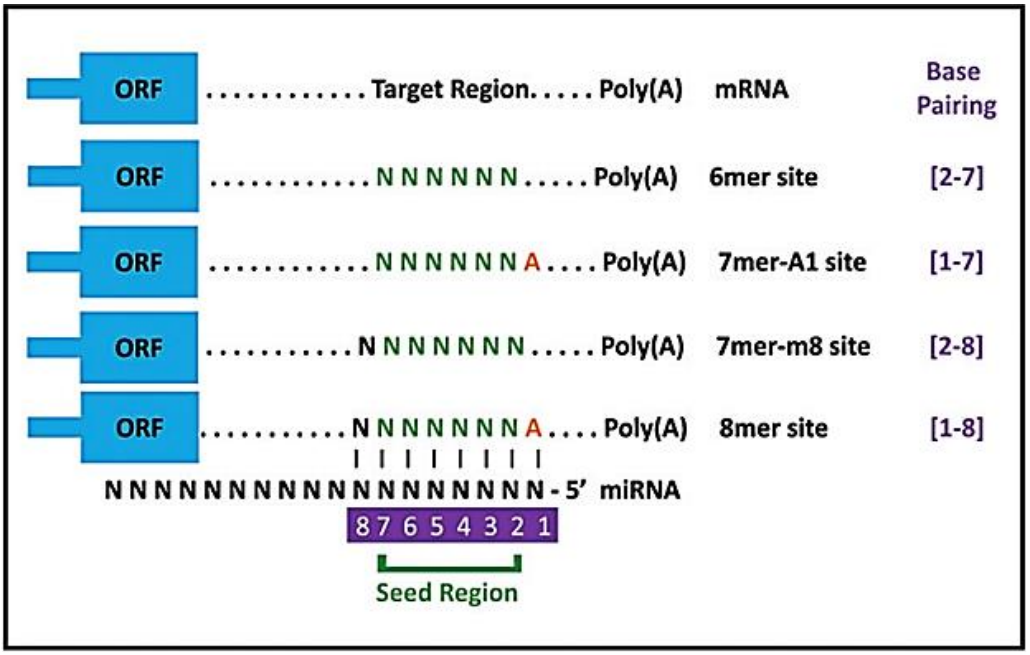


Figure 21: Different Canonical seed match regions of miRNAs to mRNA 3'UTR region (Strongest is 8mer). *miRnalyze follows a hierarchical pattern (8mer > 7mer-m8 > 7mer-A1 > 6mer) for sorting miRNAs. Abbreviation: ORF, Open Reading Frame. Subhra Das et. al., 2017, doi: 10.1093/database/bax015.*

In addition, UALCAN software (http://ualcan.path.uab.edu/) was exploited to examine the gene expression of miRNA and mRNA in tumor subgroups and the survival rate in patients with female-specific malignancy (Chandrashekar et al., 2017; Chandrashekar et al., 2022). The entire process is schematically depicted in Figure 22, illustrating the stepwise integration of various computational tools and databases for a comprehensive exploration of miRNA regulation of HDAC8 and HDAC6 in cervical cancer.

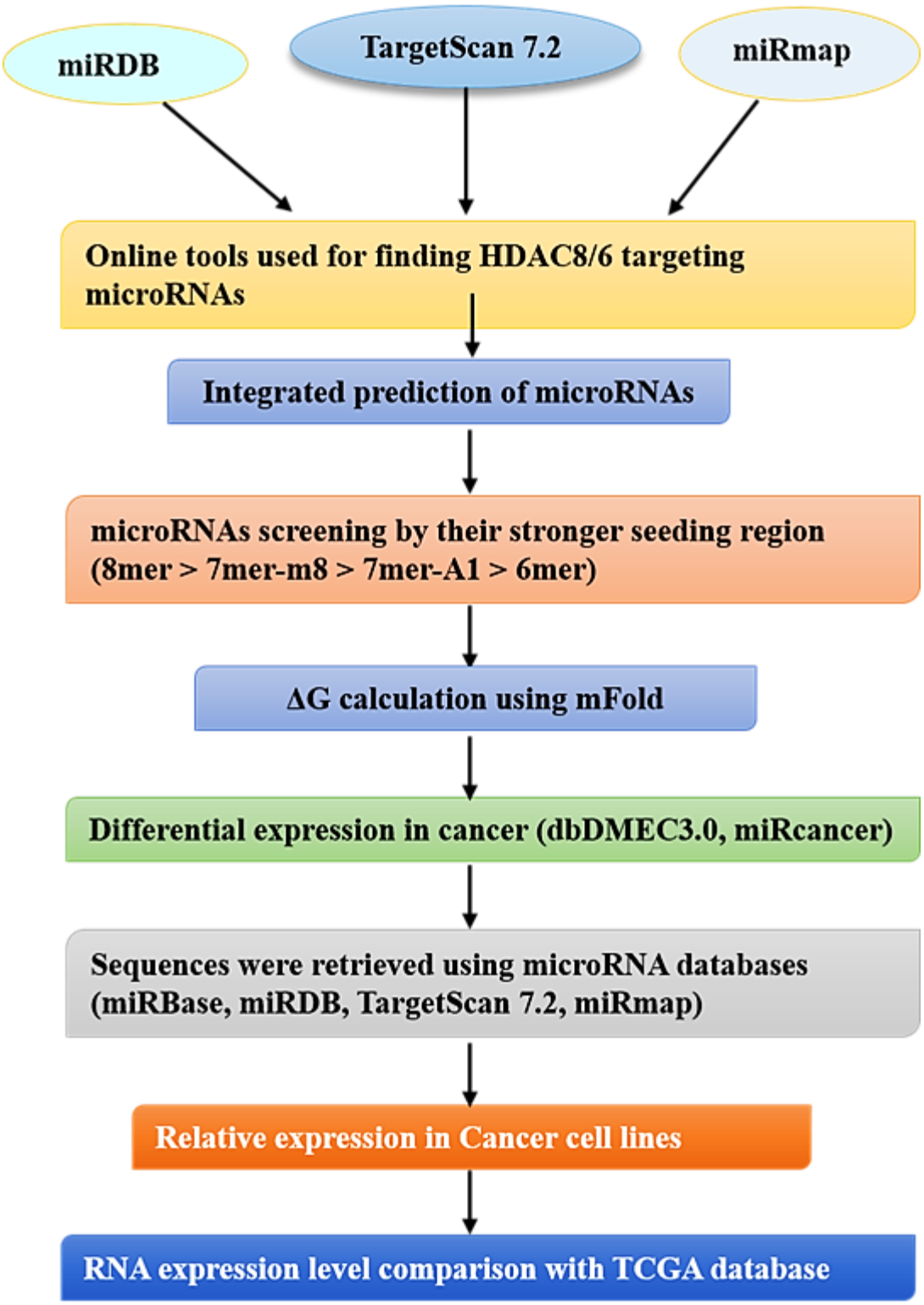


Figure 22: Schematic representation of methodology used for identifying miRNAs targeting HDAC8 and HDAC6 mRNA. *Abbreviation: TCGA, The Cancer Genome Atlas. Adopted from Naik and Kalle, 2024, doi: 10.1016/j.ncrna.2024.02.009.*

3.2.2 Cell culture

The human Breast cancer cell lines, non-metastatic luminal A MCF7, and metastatic MDAMB231, human cervical cancer cell line, HeLa, Human chronic myelogenous leukemia cell line, K562, and a normal Human embryonic kidney cell line, HEK293T, were collected from the National Centre for Cell Science (NCCS), Pune, India. Next to acquirement, HEK293T, MCF7, MDAMB231, and HeLa cells were cultivated in DMEM, whereas K562 cells were cultivated in the RPMI medium. Both cultured media were embellished with 10% FBS and 1% Anti-Anti. Cell maintenance involved incubation in a humidified atmosphere at 37 °C with 5% CO2 to ensure optimal growth conditions.

3.2.3 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Evaluation

Total RNA extraction was carried out using the TRIzol reagent. Following DNaseI treatment, 1 μg of total RNA was reverse transcribed using a cDNA synthesis kit (Takara). E. coli poly(A) polymerase (NEB) was employed to append a Poly-A tail to the miRNA, followed by cDNA synthesis using a universal adaptor primer incorporating oligo-dT (Shi, Sun, Zhang, & Chiang, 2012).

The produced cDNA was used as a template for real-time PCR (qRT-PCR) gene expression analysis with Biorad iTaqTM Universal SYBR® Green supermix and Bio-Rad CFX96 equipment, following the manufacturer's instructions. Table 1 lists all of the primers that Concise Services (Hyderabad, India) custom synthesised. To normalise gene expression findings, we used β-actin and U6 snRNA as internal standards for mRNA and miRNA detection, respectively. The $2^{-\Delta\Delta Ct}$ method was applied for comparative quantification, following established protocols (Vanaja, Ramulu, & Kalle, 2018). Each sample was used in triplicates in two independent experiments, ensuring robustness and reproducibility of the results.

Table 1: Details of primers and oligos used in Quantitative Real-time expression analysis

Name of Primer	Sequence (5'-3')
β-ACTIN FP	CTTGACAAAACCTAACTTGCGC
β-ACTIN RP	GTGAACTTTGGGGGATGCTC
HDAC8 FP	GGCTGCGGAACGGTTTTAAG
HDAC8 RP	GCTTCAATCAAAGAATGCACC
HDAC6 FP	ACCTAATCGTGGGACTGCAAG
HDAC6 RP	GAAAGGACACGCAGCGATCT
U6 FP	GCTTCGGCAGCACATATACTAAAAT
U6 RP	CGCTTCACGAATTTGCGTGTCAT
miR-150-5p FP	TCTCCCAACCCTTGTACCAGTG
miR-664b-3p FP	TTCATTTGCCTCCCAGCCTACA
miR-579-3p FP	TTCATTTGGTATAAACCGCG
miR-497-3p FP	CAAACCACACTGTGGTGTTAGA
miR-26b-3p FP	CCTGTTCTCCATTACTTGGCT
miR-324-3p FP	CACTGCCCCAGGTGCTGCTGG
miR-30b-3P FP	CTGGGAGGTGGATGTTTACTTC
miR-642a-5p FP	GTCCCTCTCCAAATGTGTCTTG
miR-181a-2-3p FP	ACCACTGACCGTTGACTGTACC
miR Universal RP	AAAGCGGCCGCTCTAGTTAGT
Tagged-OligodT	ACTAACTAGAGCGGCCGCTTTTTTTTTTTTTTT TTTTTTTTTT

3.2.4 Protein Isolation and Western Blotting

For western blot analysis, cellular lysis was conducted using 1X RIPA buffer (50 mM Tris-HCl, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.5 mM Sodium orthovanadate, and 0.5% Triton X-100), supplemented with protease (1X) and phosphatase (1X) inhibitor cocktail (Roche, Sigma). Protein lysates were isolated following a previously established protocol and quantified using the Bradford reagent (Sigma) (Vanaja et al., 2018).

Subsequently, 20-50 µg of protein was separated through 10% or 12% SDS-PAGE and transferred onto a nitrocellulose (NC) membrane. Immunoblot analysis was conducted using primary antibodies against HDAC8, HDAC6, GAPDH, β-actin, α-tubulin, acetylated-α-tubulin, p53, acetyl-Lysine, HSP90, E-Cadherin, and Vimentin. Protein bands were developed and visualized through enhanced chemiluminescence (Roche), and band intensity was quantified using ImageJ 1.34I software (NIH).

3.2.5 Construction of the miRNA Expression Plasmids

Precursor miRNA (pre-miR) cloning into a pEGFPC1 vector was carried out following its amplification along with 100 nucleotides flanking on both sides of the locus (Figure 23) including restriction sites for enzymes SalI and BamHI at the 5’ and 3’ ends respectively (Menbari et al., 2020; Shafiee, Aleyasin, Vasei, Semnani, & Mowla, 2016).

Figure 23: Schematic representation of precursor miRNA used for constructing miRNA constructing plasmid. *Precursor miRNA with 100 nucleotides flanking on either side of the locus was used to construct miRNA expression plasmid with pEGFPC1 plasmid. Adopted from Naik and Kalle, 2024, doi: 10.1016/j.ncrna.2024.02.009.*

Following PCR purification, restriction digestion was done with SalI and BamHI. Similarly, the pEGFPC1 vector was digested with SalI and BamHI restriction enzymes. Ligation of SalI and BamHI digested pre-miR and pEGFPC1 vector was carried out using T4 DNA ligase which, was followed by transformation into DH5α strain of E. coli following manufacture instruction.

Colony PCR was done to confirm the transformed colonies with pre-miR using one pre-miR-specific and one vector-specific primer. This is followed by plasmid isolation from overnight cultures of positive colonies and its single and double restriction enzyme digestion confirmation through agarose gel electrophoresis taking an empty vector as control. Finally, with Sanger sequencing, the clone of pre-miRs in the vector pEGFPC1 was confirmed (Figure 24). The primers used in cloning are mentioned in Table 2.

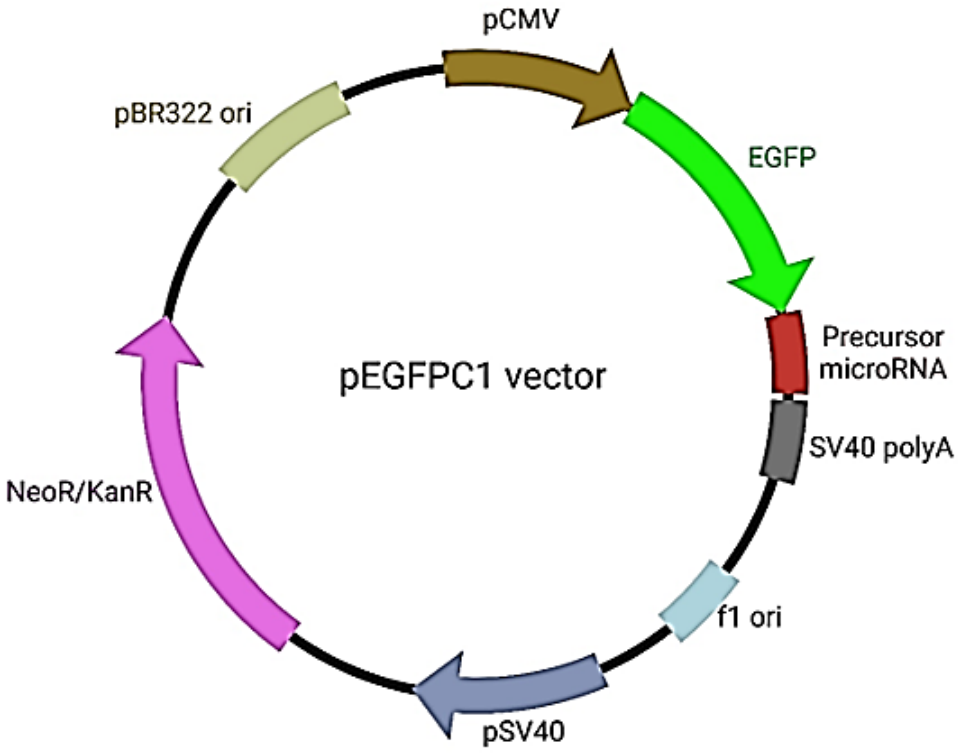


Figure 24: Schematic diagram showing miRNA expressing pEGFPC1 vector. *Abbreviations: EGFP, enhanced green fluorescent protein; NeoR, Neomycin resistance; KanR, Kanamycin resistance; SV, Simian virus. Adopted from Naik and Kalle, 2024, doi: 10.1016/j.ncrna.2024.02.009.*

3.2.6 Luciferase Reporter Construct

Wild-type (WT) HDAC8 and HDAC6 3'UTR sequences, along with mutant (MUT) sequences harboring altered miRNA binding sites and XbaI restriction enzyme recognition sites on both ends were PCR-amplified from genomic DNA (Figure 25).

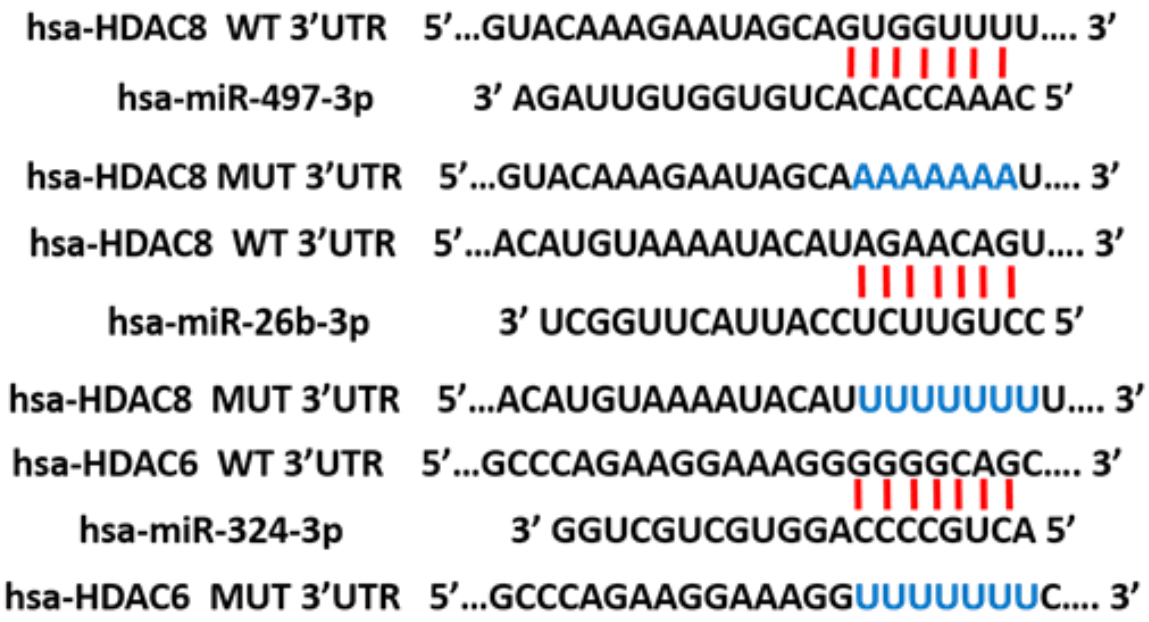


Figure 25: Schematic representation of wild type and mutation miRNA binding sequences used in the construction of luciferase constructs with pGL3-Promoter vector. *Abbreviation: WT, Wild type; MUT, Mutant, hsa, Homo sapien, UTR, Untranslated region; miR, microRNA. Adopted from Naik and Kalle, 2024, doi: 10.1016/j.ncrna.2024.02.009.*

XbaI enzyme restriction digestion for both 3'UTR and pGL3-promoter vectors was carried out. To avoid vector self-ligation, CIP treatment was done for the pGL3-promoter vector followed by ligation using T4 DNA ligase and their transformation into the DH5α strain of E. coli.

Through orientation colony PCR, the positive colonies that have 3'UTR on the sense strand of the vector using 3'UTR specific primer and vector-specific primer and their mutant having changed nucleotides at the miR seeding region was confirmed. Following

plasmid isolation, clones were further confirmed through single and double-restriction enzyme digestion where the pGL3-promoter vector lacking extra nucleotides was utilized as a negative control. Finally, Sanger sequencing confirmed clones (Figure 26).

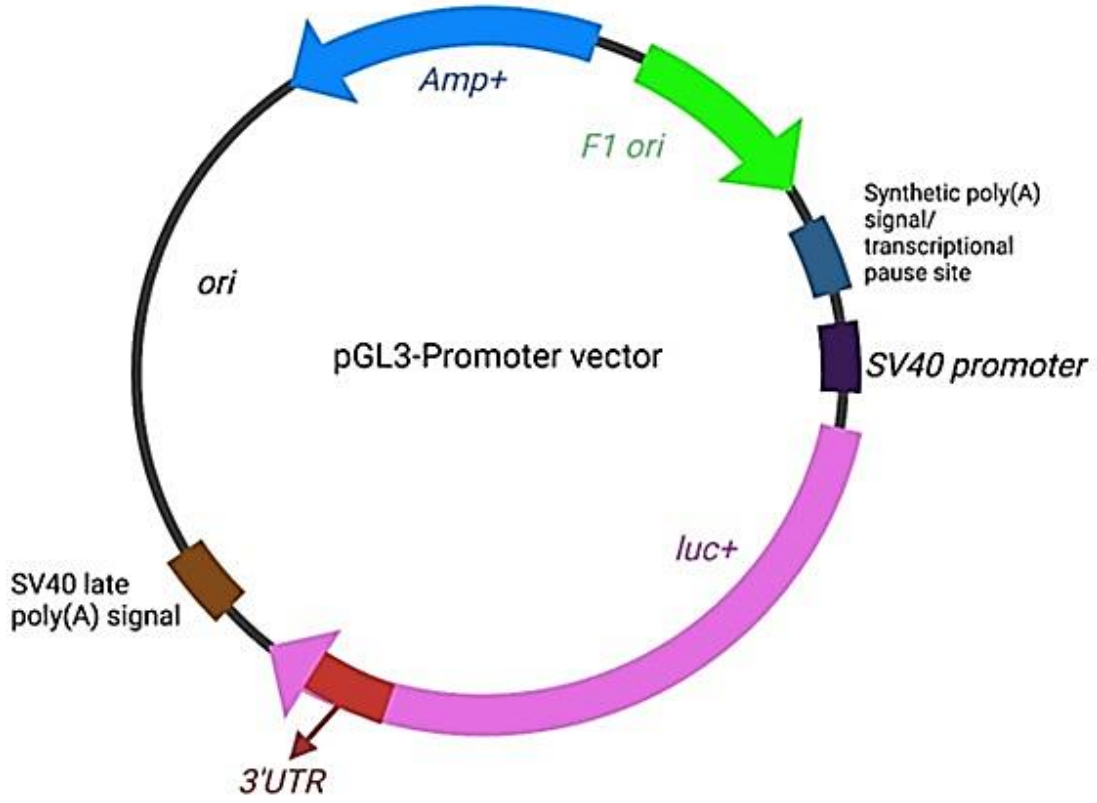


Figure 26: Schematic representation of luciferase construct. *Following cloning of HDAC6/8 3'UTR region in pGL3-promoter vector luciferase construct was made for dual luciferase assay. Abbreviation: UTR, Untranslated region; SV, Simian virus; Amp, Ampicillin; luc, Luciferase. Adopted from Naik and Kalle, 2024, doi: 10.1016/j.ncrna.2024.02.009.*

3.2.7 Dual Luciferase Assay

HEK293T cells were seeded at 0.05 x 106 in 24-well plates for investigating miRNA-mRNA interactions. Using Lipofectamine 3000, 60-80% confluent cells were transiently co-transfected with a miRNA expression vector, wild-type/mutant reporter vector, and the pRL-TK vector (Clement, Salone, & Rederstorff, 2015; Jin, Chen, Liu, & Zhou, 2013) (Promega, Madison). Luciferase activity was assessed 24 hours post-transfection with an omega FLUOstar microplate reader and the Dual-Luciferase Reporter Assay (Promega,

Madison), was normalized using Renilla luciferase activity. Transfections were conducted in triplicate and across three independent experiments.

Table 2: Details of primers used in the cloning method

Name of Primer	**Sequence (5'-3')**
PremiR497cloneFP	GTCGACGGGCCATGTTTGCCTTTTAAG
PremiR497cloneRP	GGATCCTACATACCCCACCCTCTCCTT
PremiR26BcloneFP	GTCGACAAAGTCACACAGAACCTCAAG
PremiR26BcloneRP	GGATCCACGAGGTCCCTAATTCCCAAT
PremiR324cloneFP	GCGTCGACTTAACTTCTGGTACTGCTGGC
PremiR324cloneRP	GCGGATCCAAGCTGGCACGGATGGTTATG
H6UTR3XBA1FP	GCTCTAGATAAGCCCCAGAATACGGTCC
H6UTR3XBA1RP	GCTCTAGACATATGCAACCTTGCCATGCC
XBA1H83UTR497 FP	GCTCTAGACATTTGCATAGCCTTGTCTG
XBA1H83UTR497 RP	GCTCTAGACAGTCCAGCCAAACTAAAAC
XBA1H83UTR26B FP	GCTCTAGAGAAGTTGCAAAGAAGATTGG
XBA1H83UTR26B RP	GCTCTAGACAGAATAGAGCACTGTTCTA
PEGFPC1 FP	CATGGTCCTGCTGGAGTTCGTG
PGL3PROMOTER RP	GACGATAGTCATGCCCCGCG

3.2.8 Transient Transfection

MDAMB-231, HeLa, and K562 cells were seeded in 6-well plates at a density of 1.5 x 10^5 cells per well, reaching 60-80% confluence. Lipofectamine 3000 was utilized to transfect cells with 2 µg of miRNA-expressing plasmid along with an empty vector as the negative control, following the manufacturer's instructions. Transfected media was replaced with regular culture medium six hours post-transfection, and cells were harvested 48 hours later and the RNA and protein isolated were stored at -80°C and -20°C, respectively, for future analyses.

3.2.9 Cell Viability Assay

MDAMB-231, and HeLa cells, plated in 96-well plates with 5,000 cells per well one day prior to transfection, were transfected with miRNA expression plasmids using Lipofectamine 3000. The growth medium was replaced with a transfection medium six hours post-transfection, and cell survival was measured at 24, 48, and 72 hours using the MTT assay as per standard protocol (Zhao et al., 2013).

3.2.10 Immunofluorescence

MDAMB-231 and HeLa cells were cultured on coverslips and transfected with the miRNA-expressing plasmid. Subsequently, the cells were fixed in the dark at room temperature (RT) for 15 minutes using 4% paraformaldehyde. Following two washes with 1X PBS, permeabilization was carried out for 20 minutes at RT using a 0.5% TritonX-100 solution in 1X PBS. After three additional washes with 1X PBS, cells were blocked for 1 hour in a 3% BSA solution in 1X PBST, which included 0.1% TritonX-100. After five more washes with 1X PBS, cells were incubated with α-tubulin-specific primary antibodies at 4 °C overnight. Subsequent to five 1X PBS washes the following day, cells were treated with an Alexa Fluor 488-green secondary antibody for 2 hours at 37°C in the dark, followed by a final set of five

1X PBS washes. Finally, cells were mounted using a Fluoroshield mounting medium containing DAPI, and images were captured with a trinocular immunofluorescence microscope (Leica).

3.2.11 Wound Healing Assay

MDAMB-231 and HeLa cells, cultured to 40-60% confluence in a 6-well plate, underwent miRNA-expressing plasmid transfection, along with an empty vector serving as the negative control. Wounds were generated at three different sites within each well using a 200 µl micro tip. Wound images were acquired using a bright-field microscope at 0 hours, 24 hours, and 48 hours, with wound measurements conducted using ImageJ software.

3.2.12 Statistical Analysis

Statistical analyses were performed using GraphPad Prism v6.01 software. Student t-test and one-way analysis of variance (ANOVA) were employed, and statistical significance (p-value) was set at * ≤0.05, ** ≤0.01, *** ≤0.001, **** ≤0.0001, and ns=non significance >0.05.

Results

4.0 Results

4.1 Objective 1- Identification of potential miRNA (s) targeting HDAC8/6 and validation of the interaction of identified miRNA (s)

4.1.1 Expression of HDAC8 and HDAC6

In our preliminary investigation, we assessed mRNA and protein expression levels of HDAC8 and HDAC6 across various female-specific cancer cell lines. In comparison to the non-cancerous HEK293T cell line, HDAC8 exhibited noticeable overexpression not only in the cervical cancer cell line, HeLa but also in the non-metastatic breast cancer cell line MCF7 and metastatic MDAMB231 cells (Figure 27a and 28). HDAC6 expression, in contrast moderately less in HeLa and MCF7 cells, with no significant difference in MDAMB231 cells compared to HEK293T cells (Figure 27b and 28).

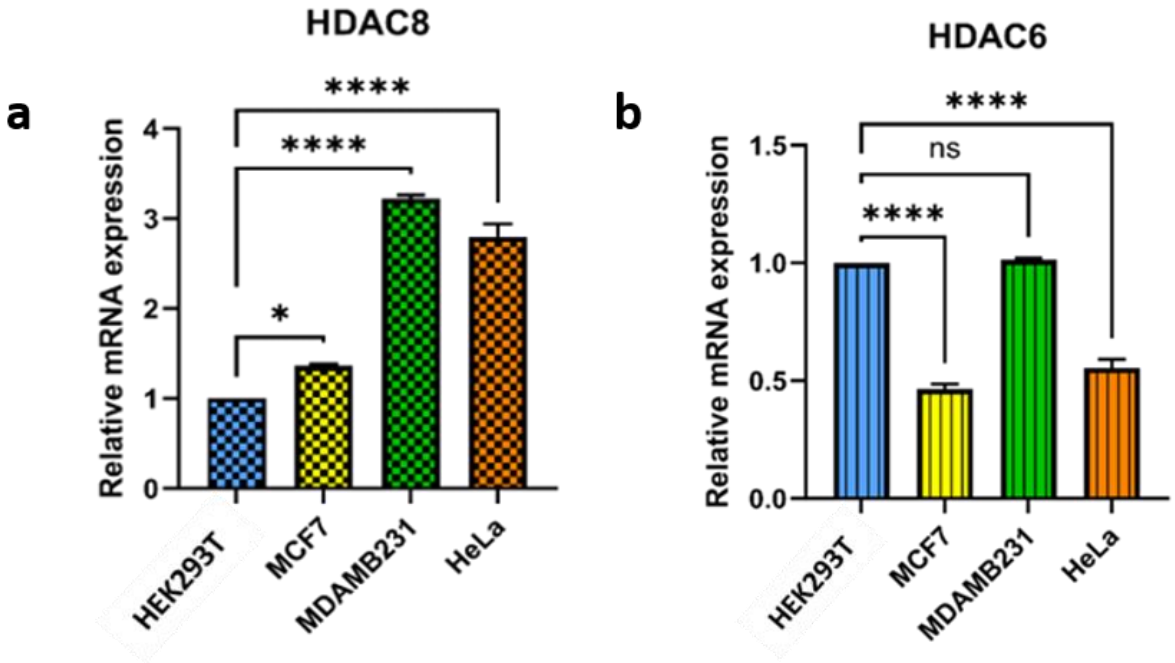


Figure 27: Relative mRNA expression of HDAC8 and HDAC6 in different female specific cancer cell lines. *(a) Relative mRNA expression of HDAC8 in HeLa, MCF7, and MDAMB-231 cell lines compared to HEK293T cell line. (b) Relative mRNA expression of HDAC6 in HeLa, MCF7, and MDAMB-231 cell lines compared to HEK293T cell line. P-value indicated as * ≤0.05, **** ≤0.0001, ns=non significance >0.05.*

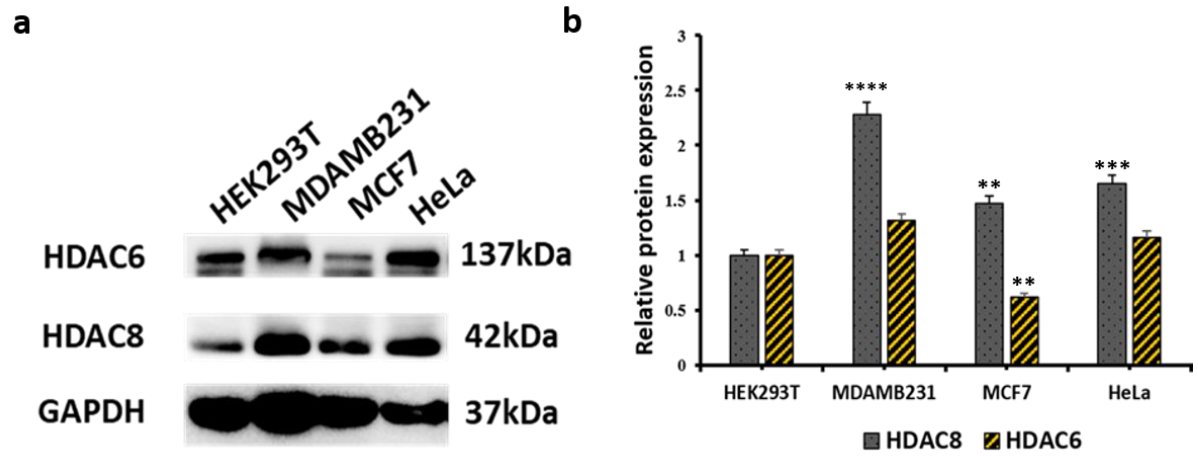


Figure 28: Relative protein expression of HDAC6 and HDAC8 in different female specific cancer cell lines. *(a) Relative protein expression of HDAC6 and HDAC8 in HeLa, MCF7, and MDAMB-231 cells compared to HEK293T cells. (b) densitometry of protein blot a. P-value indicated as ** ≤0.01, *** ≤0.001, **** ≤0.0001.*

4.1.2 MicroRNAs potentially targeting 3'UTR of HDAC8 and HDAC6 mRNA

Employing miRDB, TargetScan, and miRmap, we identified candidate miRNAs targeting the 3'UTR region of both HDAC8 and HDAC6 mRNA. TBtool software visualized the intersection genes of these target genes (Figure 29) (Chen et al., 2020). This tool facilitated cluster identification and extraction, emphasizing genes present in multiple databases. Overlapping miRNAs, selected to avoid overlooking potential information, were chosen based on their presence in two or more databases.

Furthermore, miRNAs were scrutinized for canonical binding, emphasizing stronger binding sites that significantly impact repression efficacy. Canonical site classes, including 8mer (binding site complement to 2-8 position of miRNA seed region with an A opposite to 1 position), 7mer-m8 (binding site match 2-8 position of miRNA seed region), and 7mer-A1 (binding site complement to 2-7 position of seed region with an A at opposite one position) (Brennecke, Stark, Russell, & Cohen, 2005; Krek et al., 2005; Lewis, Burge, & Bartel, 2005), were prioritized due to potent seed match characteristics (Bartel, 2009). Weaker

canonical site types, 6mer (2-7 seed regions match) and offset-6mer (position 3-8 match), were not considered.

MiRNAs with 8mer and 7mer-m8 sites, exhibiting lower ΔG values by mfold software, were further analyzed for their involvement in female-specific cancers through databases like miRcancer and dbDMEC. Consequently, miR-150-5p, miR-664b-3p, miR-579-3p, miR-497-3p, and miR-26b-3p were identified as potential mature miRNAs targeting HDAC8 mRNA (Figure 30). Similarly, miR-181a-2-3p, miR-30b-3p, miR-324-3p, and miR-642a-5p were identified as putative miRNAs targeting HDAC6 (Figure 31).

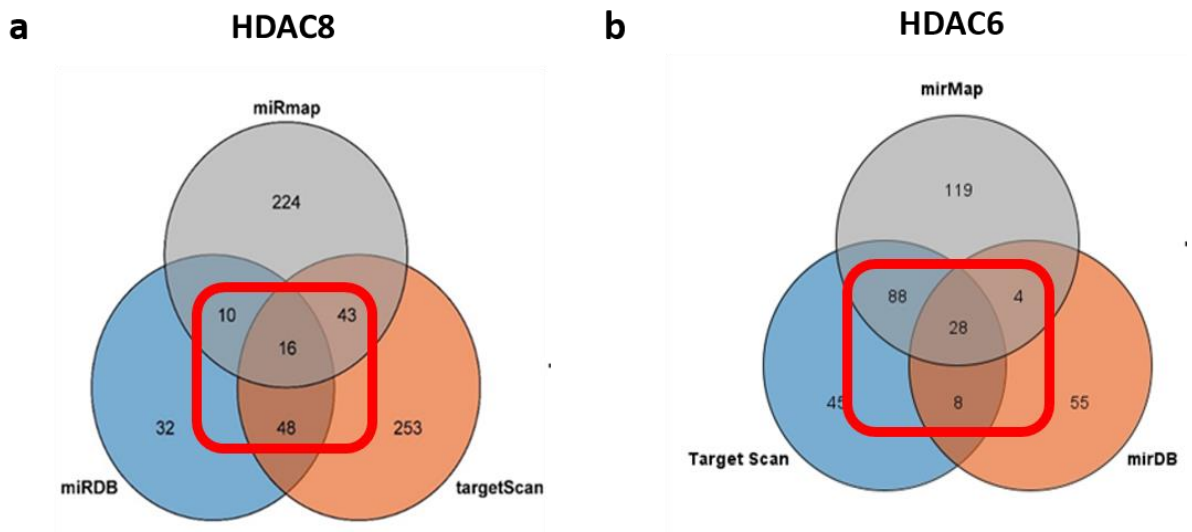


Figure 29: Schematic representation of TBtool software visualization of numeration of miRNAs targeting HDAC8 and HDAC6 from TargetScan, miRDB, and miRmap software. *(a) Figure showing numeration of miRNAs targeting HDAC8. (b) Figure showing numeration of miRNAs targeting HDAC6.*

Figure 30: Schematic representation of selected HDAC8 targeting miRNAs for validation.

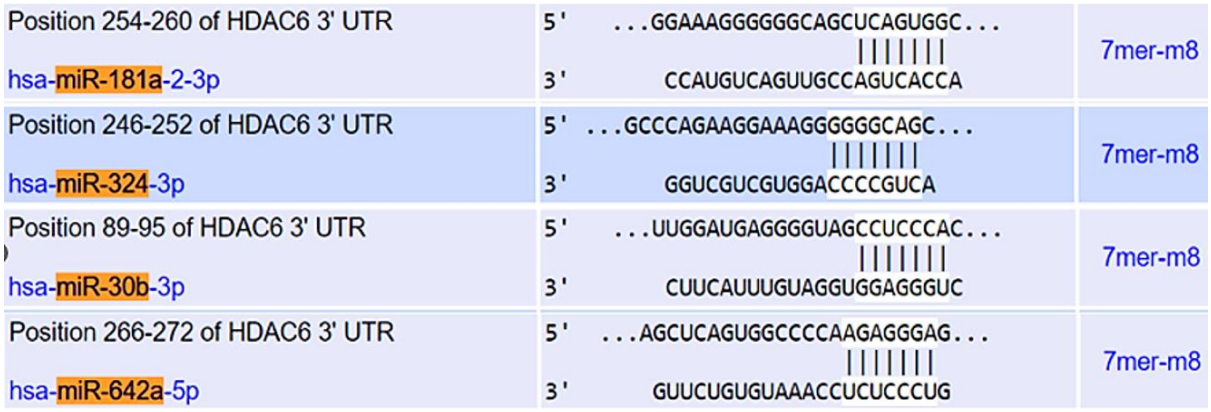


Figure 31: Schematic representation of selected HDAC6 targeting miRNAs for validation.

4.1.3 Expression of miRNAs targeting HDAC8 in cervical cancer cell line

Real-time PCR was employed to assess the expression of five selected potential HDAC8-targeting miRNAs in HeLa cells compared to HEK293T cells (Figure 32). All five HDAC8-targeting miRNAs exhibited decreased expression levels, with miR-497-3p and miR-26b-3p showing significantly ($p<0.005$) lower expression in HeLa cells, indicating a negative correlation with increased HDAC8 expression. Similar results were observed in cervical tumor samples in the TCGA database for miR-497-3p and miR-26b-3p (Figure 33). Consequently, miR-497-3p and miR-26b-3p were chosen for further studies.

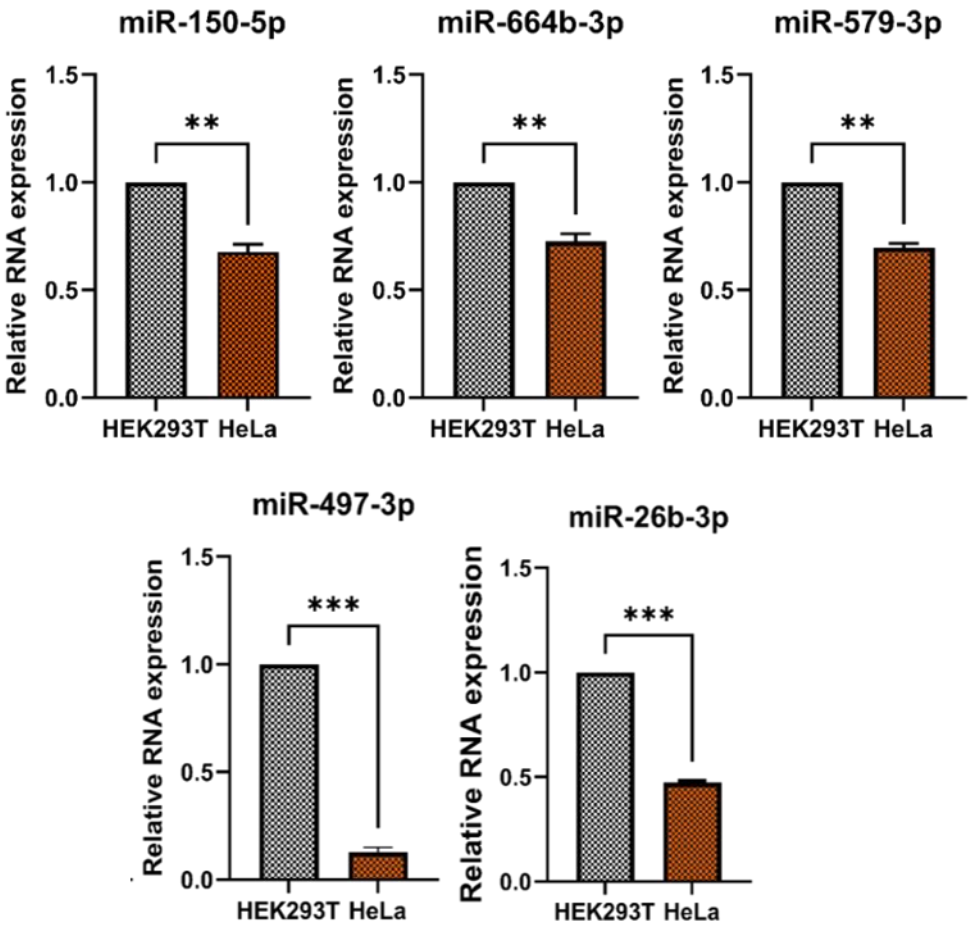


Figure 32: Real-time analysis of miRNAs expression that targets 3'UTR of HDAC8 in HeLa cells compared to HEK293T cells. *P-values indicated as ** ≤0.01, *** ≤0.001.*

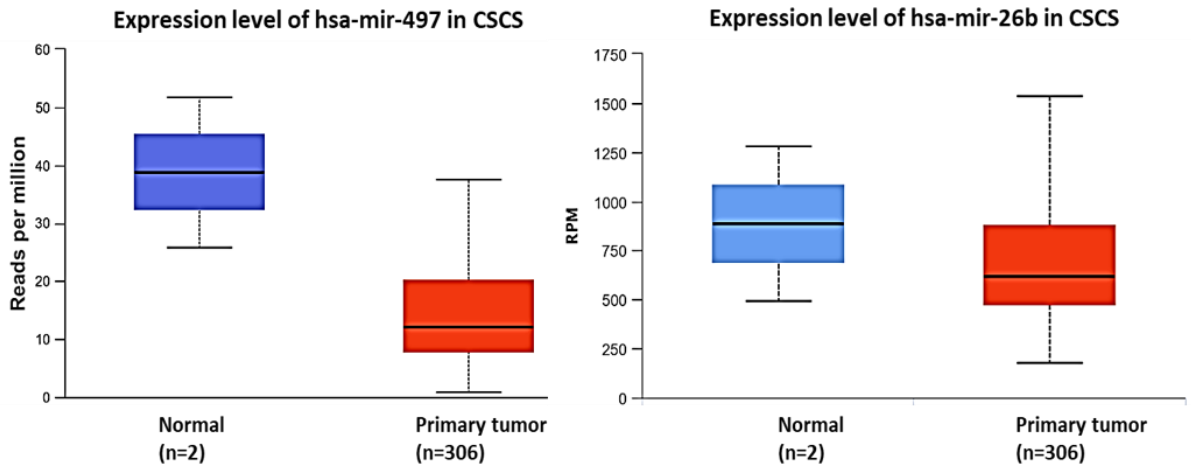


Figure 33: Expression level of miR- 497 and miR-26b in tumor TCGA sample in CSCS dataset.

4.1.4 Construction of the miRNA Expression Plasmids and Luciferase Reporter Construct

To validate the binding of the miRNA to the HDAC8 mRNA, a luciferase reporter assay was carried out for which the miRNAs were cloned into the pEGFPC1 vector. Firstly, restriction digestion of pre-miRs, HDAC8 3'UTR, pGL3- promoter, and pEGFPC1 vector was carried out (Figure 34). Following ligation and transformation, the clones were confirmed by colony PCR, restriction digestion and Sanger sequencing (as shown for miR-497 in Figure 35, 36, and 37 respectively).

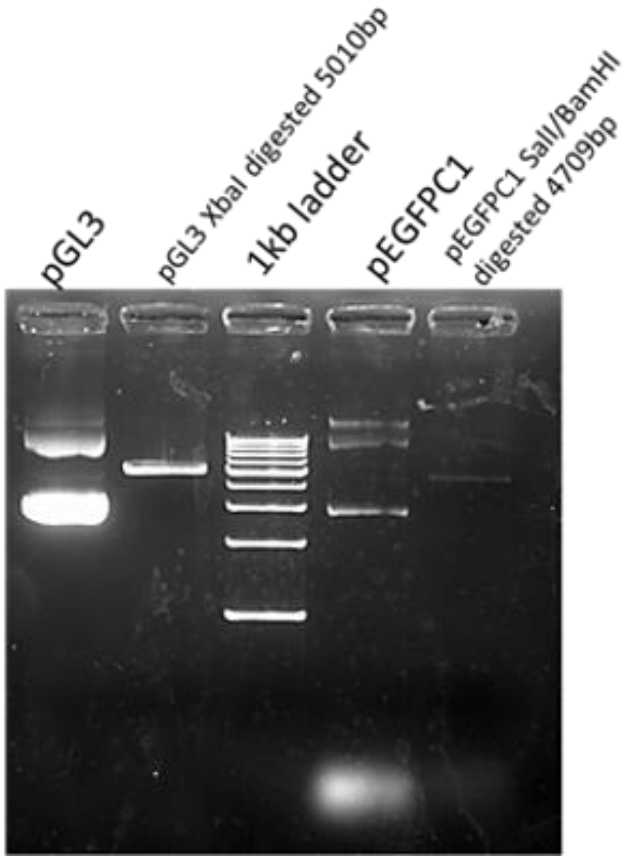


Figure 34: Restriction digestion of Vector pGL3-promoter and pEGFPC1. *Restriction digestion of pGL3-promoter vector with XbaI gives a band of size 5010bp on agarose gel electrophoresis. Restriction digestion of pEGFPC1 vector with SalI and BamHI gives a band size of 4709bp on agarose electrophoresis.*

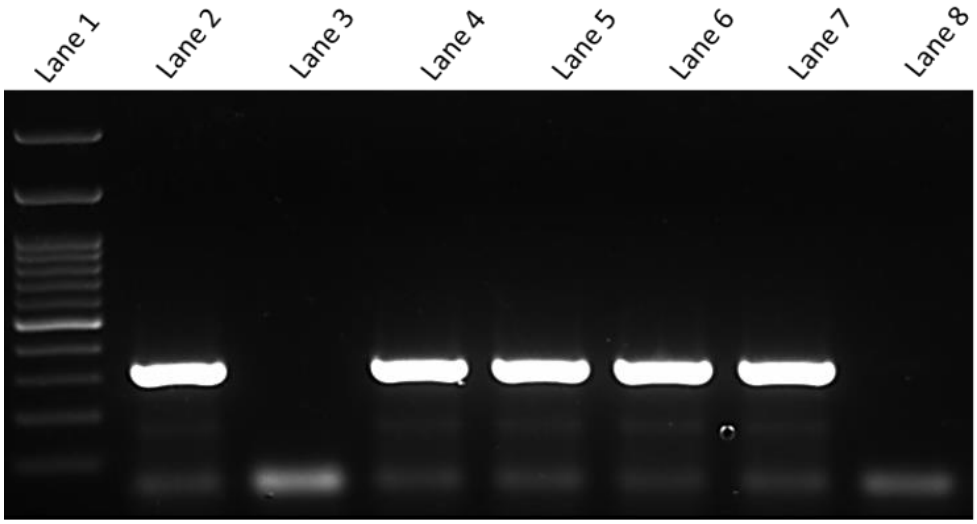


Figure 35: pEGFPC1-miR-497 clone confirmation by colony PCR with a product length 314bp. *On agarose gel electrophoresis lane 1: 100 bp DNA ladder, lane 2-6: random colonies, lane 7: positive control and lane 8: negative control.*

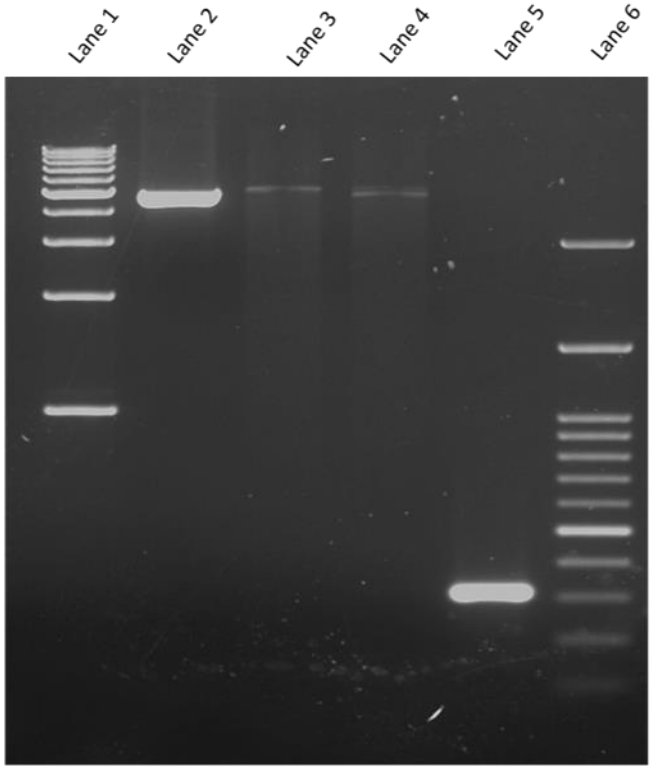


Figure 36: Verifying the accuracy of cloned miR-497 precursor within pEGFP-C1-miR-497 vector by restriction digestion and PCR analysis. *On agarose gel electrophoresis Lane 1: 1kb DNA ladder, lane 2- single digestion of pEGFP-C1 mock digested with SalI produced a 4731 bp fragment, lane 3: A single digestion of pEGFP-C1-miR-497 by SalI produced 5024 bp fragment, lane 4: Double-digest of pEGFP-C1-miR-497 with SalI and BamHI produced a 4710bp linear vector, lane 5: A 314bp PCR segment is amplified by specific primer, lane 6: 100bp DNA ladder.*

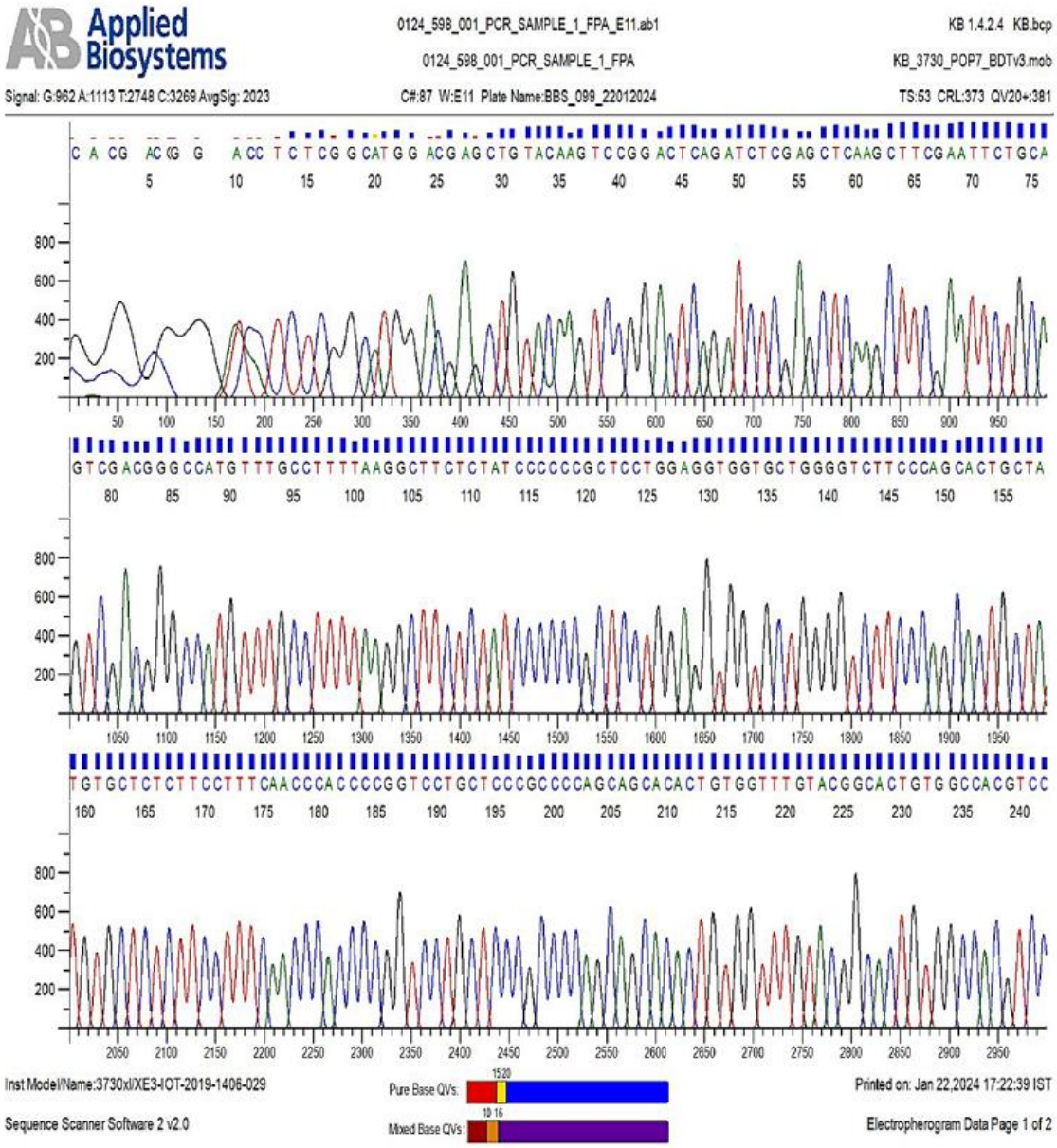


Figure 37: Sequencing result showing the presence of miR-497 in the pEGFPC1 vector.

We followed the similar methodology for constructing Luciferase constructs in pGL3-promoter vector as shown for wild type HDAC8 3'UTR having a binding site for miR-497-3p and its mutant. we confirm the clone by colony PCR as shown in Figure 38a and Figure 38b, then by restriction digestion as shown in Figure 39, and finally through sanger sequencing (Figure 40 and Figure 41).

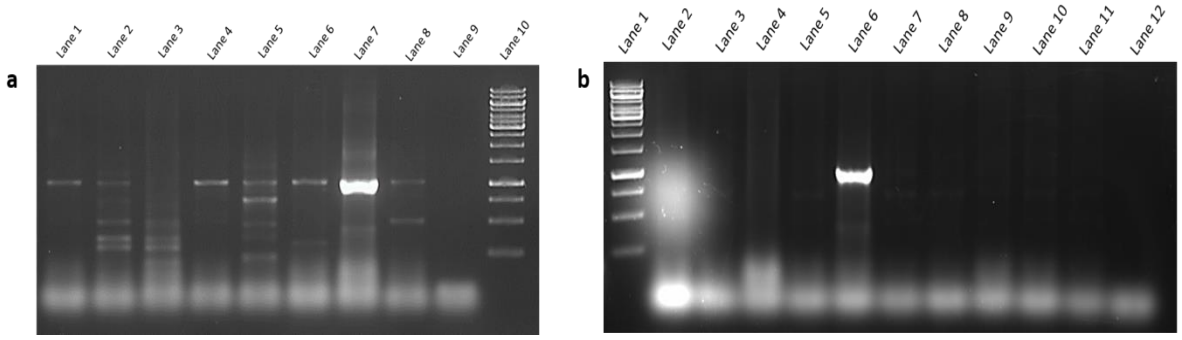


Figure 38: Orientation colony PCR confirmation of HDAC8 3'UTR having wild type and mutant seeding region for miR-497-3p. *(a) pGL3-Promoter-H83'UTR497WT (~1062bp): lane 1-8 Random colonies, lane 9- negative control, lane 10- 1kb DNA ladder. (b) Orientation colony PCR confirmation of pGL3-Promoter-H83'UTR497mutant (~1062bp): lane 1- 1kb DNA ladder, lane 2-11 Random colonies, lane 12- negative control.*

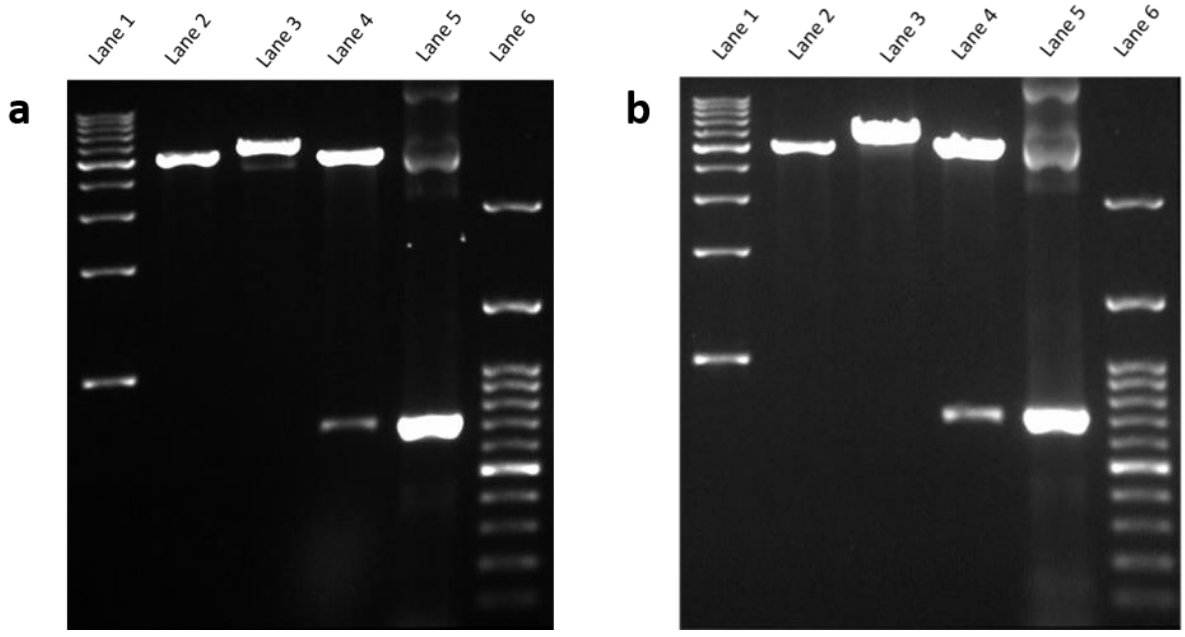


Figure 39: Verifying the accuracy of cloned HDAC8 3'UTR having seeding region for miR-497-3p within pGL3-Promoter vector by restriction digestion and PCR analysis. *(a) Wild type 3'UTR clone for miR-497-3p; b: Mutant type 3'UTR clone for miR-497-3p; Lane 1: 1kb DNA ladder, lane 2- single digestion of pGL3 promoter mock vector produced a 5010 bp fragment, lane 3: A single digestion of pGL3 promoter HDAC8 3'UTR, lane 4: Double-digest of pGL3 promoter HDAC8 3'UTR with XbaI, lane 5: A PCR segment is amplified by specific primer, lane 6: 100bp DNA ladder.*

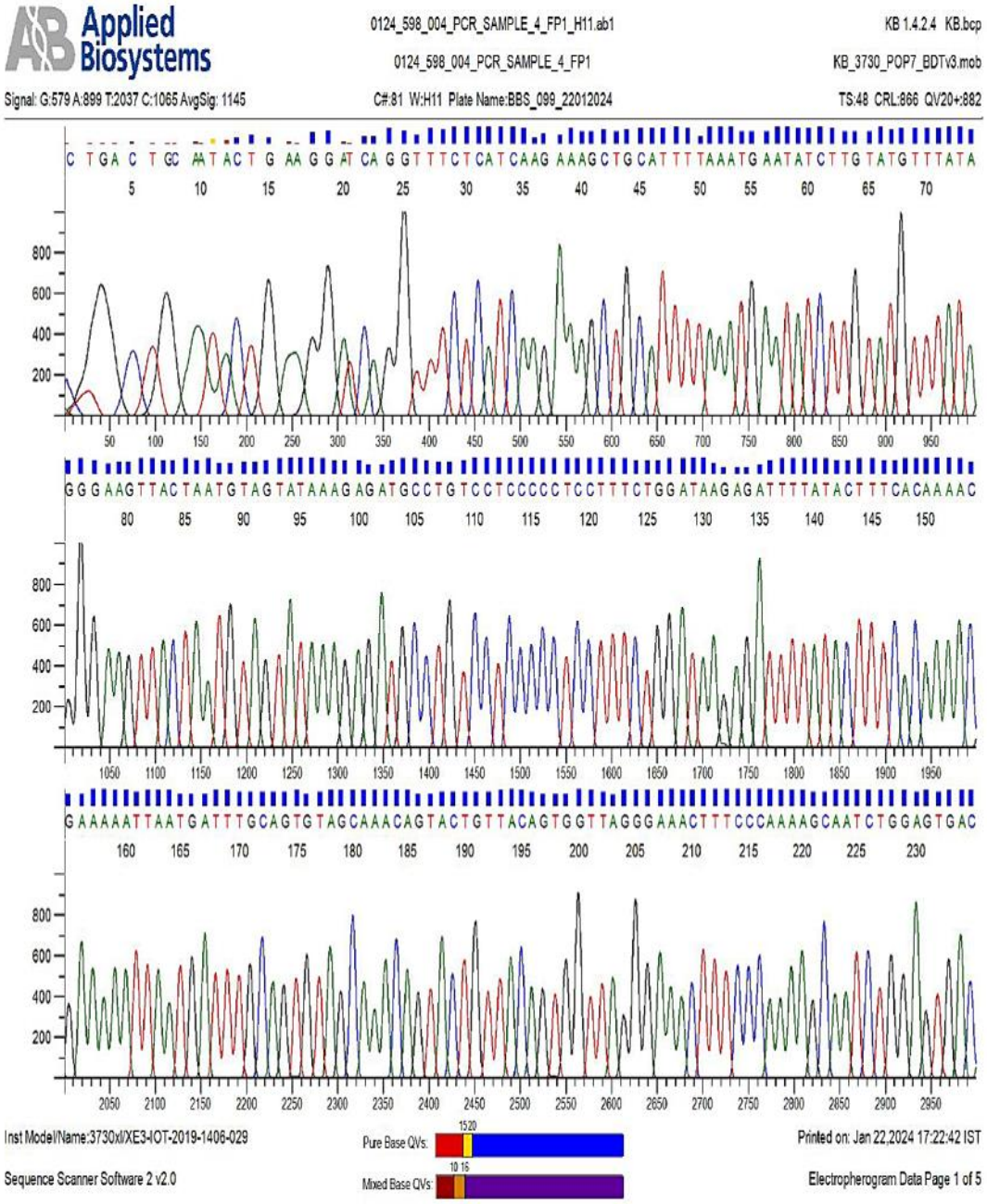


Figure 40: Sequencing result showing the presence of HDAC8 3'UTR having wild type seeding region for miR-497-3p in the pGL3-promoter vector.

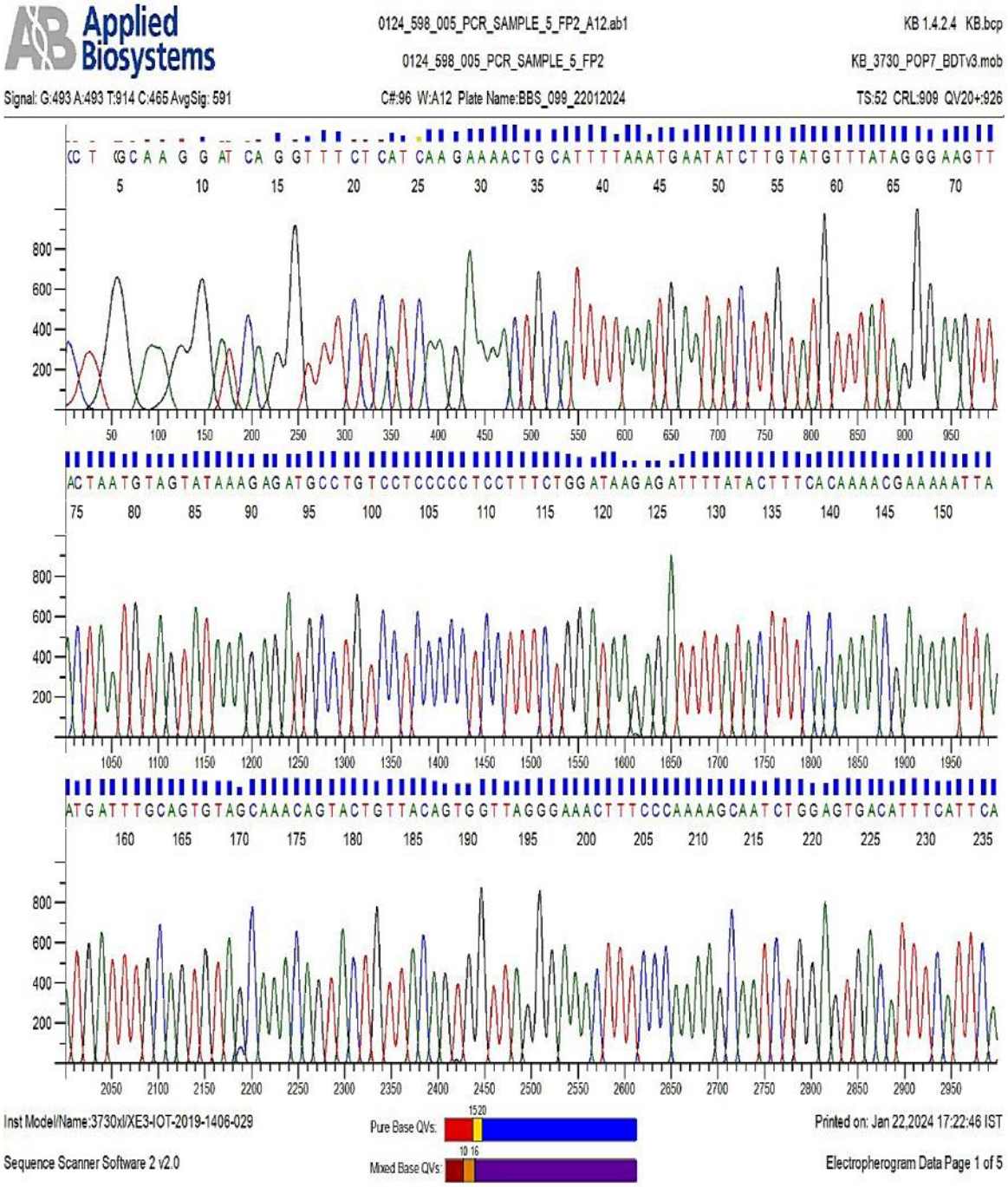


Figure 41: Sequencing result showing the presence of HDAC8 3'UTR having mutant seeding region for miR-497-3p in the pGL3-promoter vector.

4.1.5 HDAC8 is a direct target of miR-497-3p

The HDAC8-miRNA interaction was established using dual luciferase reporter assays. The human embryonic kidney cells, HEK293T, cells were co-transfected with pEGFPC1-miR-497 and pGL3-HDAC8 3'UTR (wild type or mutant) and after 24 hours, the luciferase activity was determined as mentioned in materials and methods. There was a noteworthy decrease in the activity of luciferase in cells co-transfected with miR-497-3p and HDAC8 3'UTR having wild-type miRNA binding sites but not in the cell transfected with miRNA and mutant HDAC8 3'UTR confirming the interaction (Figure 42).

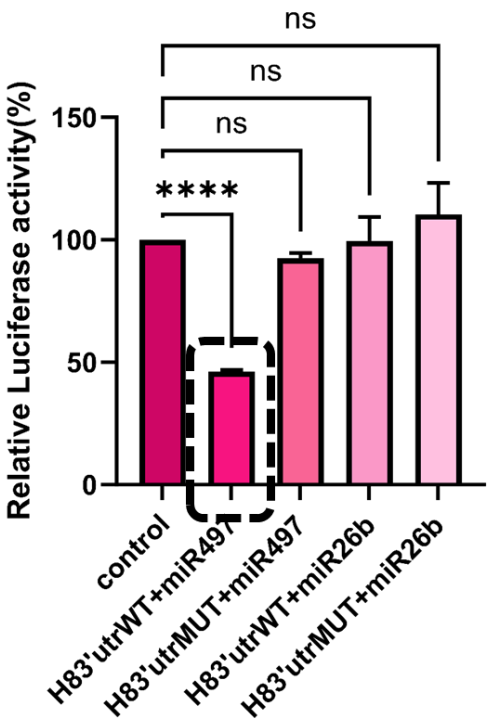


Figure 42: Dual luciferase assay showing the interaction between miR-497-3p and HDAC8 3'UTR. *Abbreviation: H8, HDAC8; utr, Untranslated region; WT, Wild type; MUT, Mutant. P-value indicated as **** ≤0.0001, ns=non significance >0.05.*

Furthermore, the negative regulation of HDAC8 by miR-497-3p was confirmed by transfecting HeLa cells (overexpressing HDAC8) with pEGFPC1-miR-497. Overexpression of miRNA in HeLa cells (Figure 43a) leads to a significant diminution in HDAC8 mRNA (Figure

43b) and protein levels (Figure 44), bringing about increased acetylation of HDAC8 targeting proteins such as p53 and alpha-tubulin (Figure 44a and Figure 44b).

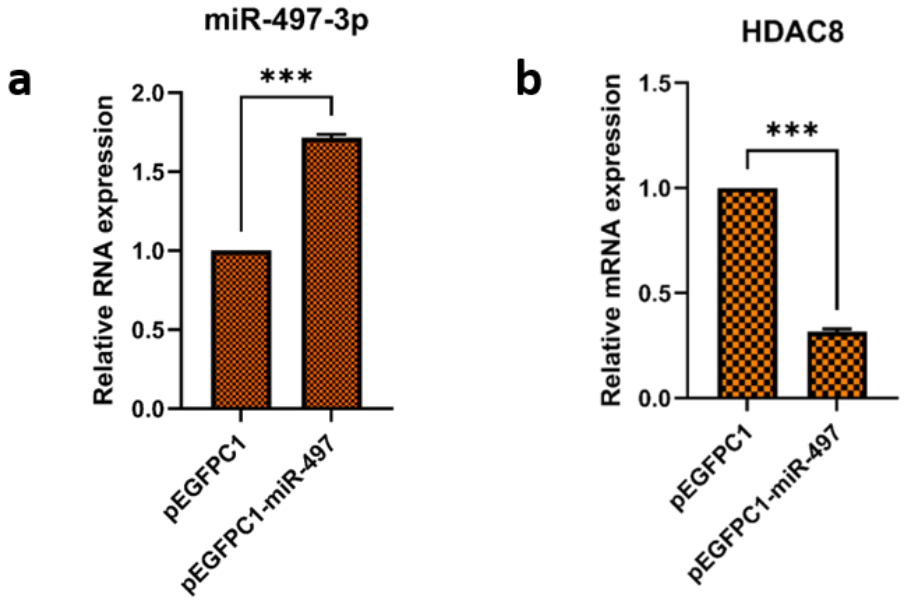


Figure 43: Real-time analysis shows the expression of miR-497-3p and HDAC8 with over expression of miR-497-3p in HeLa cells. *(a) Real-time analysis shows the expression of miR-497-3p, and (b) Real-time analysis shows the expression of HDAC8. P-value indicated as *** ≤0.001.*

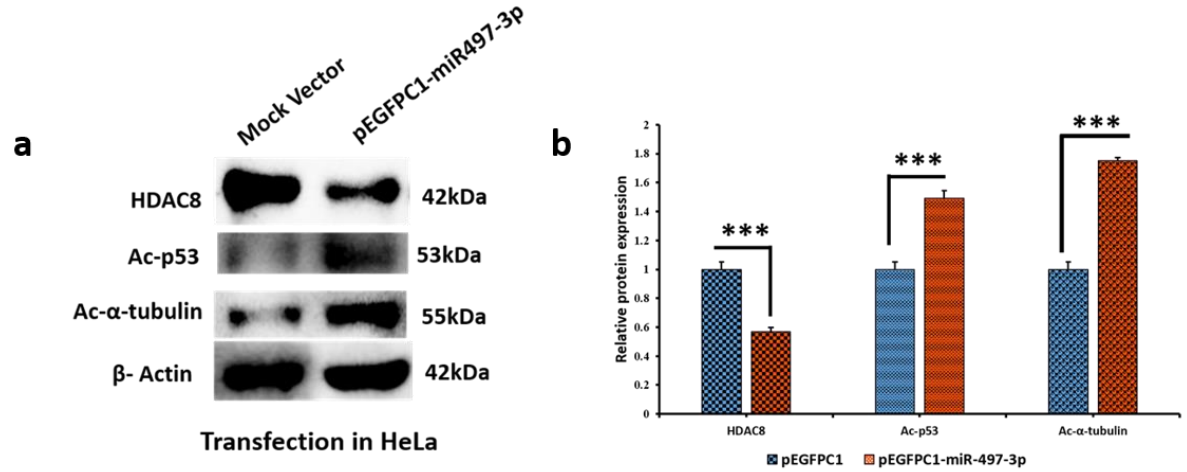


Figure 44: Protein expression of HDAC8 and its targets acetylated p53 and α-tubulin in HeLa cells with overexpression of miR-497-3p. *(a) Immunoblot shows protein expression of HDAC8, Ac-p53, and Ac-α-tubulin in reference to internal control β-Actin. (b) Densitometry analysis of immunoblot a. Abbreviation: Ac, Acetyl. P-value indicated as *** ≤0.001*

4.1.6 Expression of miRNAs targeting HDAC6 in cervical cancer cell line

We next tried to validate the *in silico* results on HDAC6-targeting miRNA. As mentioned earlier, four miRNAs (Figure 31) were identified as potentially targeting HDAC6. So, first real-time PCR was employed to evaluate the expression of the four miRNAs in HeLa cells compared to HEK293T cells (Figure 45).

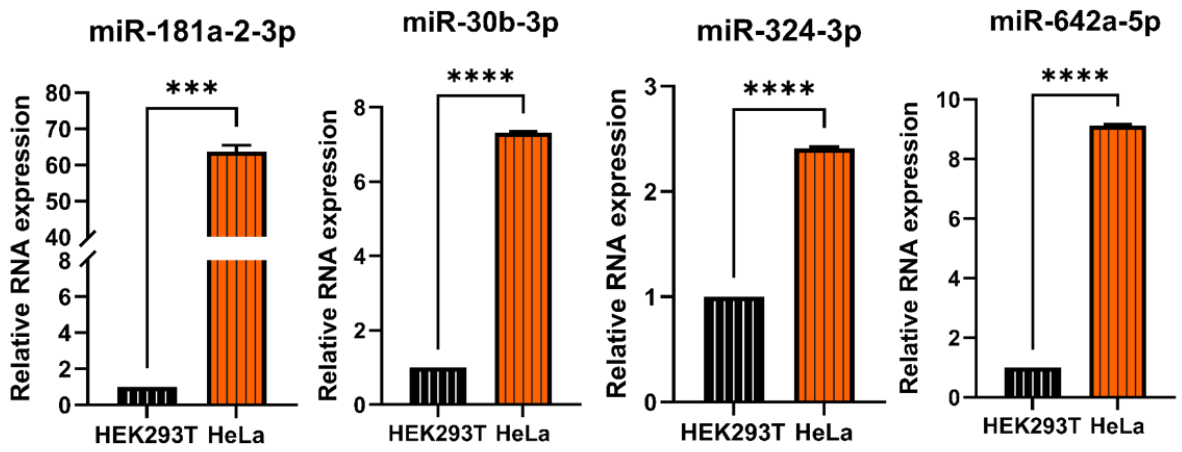


Figure 45: Real-time analysis of miRNAs expression that targets 3'UTR of HDAC6 in HeLa cells. *Expression of miR-181a-2-3p, miR-30b-3p, miR-324-3p, and miR-642a-5p in HeLa cells compared to HEK293T cells. P-value indicated as *** ≤0.001, **** ≤0.0001.*

Unlike HDAC8-targeting miRNA, all four HDAC6-targeting miRNAs exhibited increased expression levels that negatively correlated with HDAC6 expression. Similar expression was observed in the cervical tumor samples from the TCGA database for two of the four miRNAs, miR-181a-2-3p and miR-324-3p (Figure 46) and there was no data on the other two miRNAs, miR-30b-3p and miR-642a-5p. Therefore, we performed further experiments on miR-181a-2-3p and miR-324-3p.

Since HDAC6 levels were similar in both HeLa and HEK293T cells, to validate the binding of miRNA and HDAC6, we had to choose a cell line in which HDAC6 is overexpressed. According to the Human Protein Atlas database, the chronic myelogenous leukemia cells, K562, have HDAC6 overexpression and therefore, we chose K562 cells to

validate the identified miRNAs targeting HDAC6. Real-time PCR analysis was carried out to determine the expression of HDAC6, miR-324-3p, and miR-181a-2-3p expression in K562 cells and PBMCs (as normal control cells) (Figures 47a, 47b, and 47c respectively). The results clearly demonstrated a significant overexpression of HDAC6 in K562 cells compared to PBMCs (Figure 47a) and that miR-181a-2-3p as a potential negative regulator of HDAC6 as its expression was significantly lowered in K562 cells (Figure 47b). So further validation experiments were performed for miR-181a-2-3p.

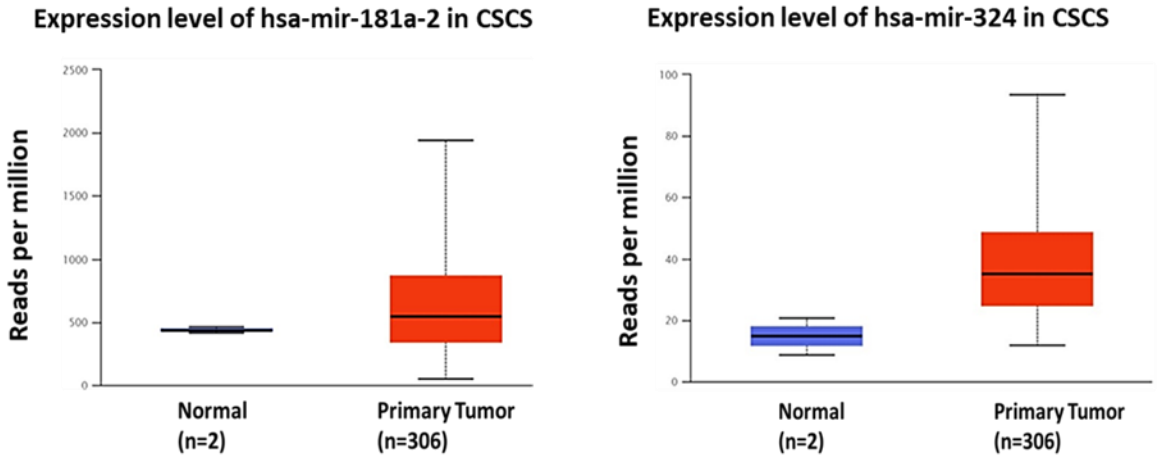


Figure 46: Expression level of miR-181a-2 and miR-324 in tumor TCGA sample in CSCS dataset.

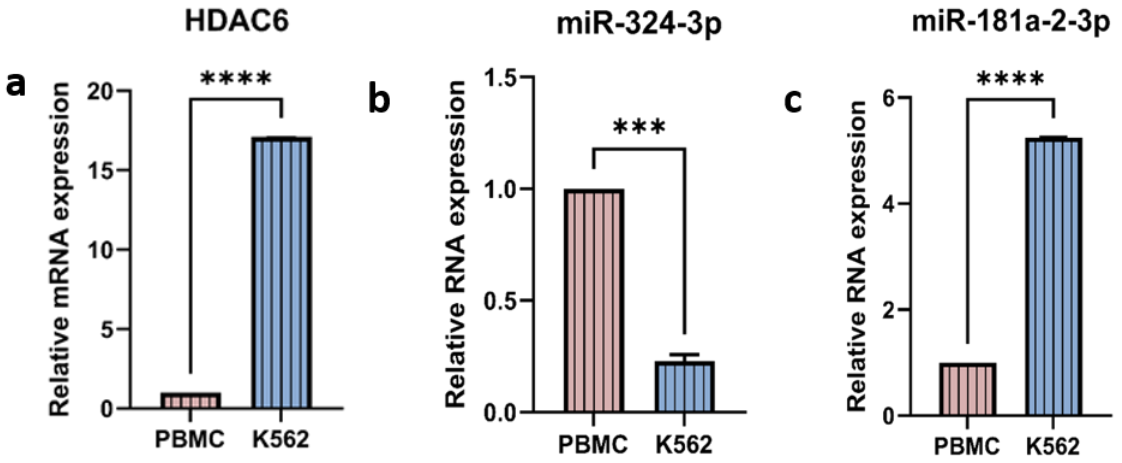


Figure 47: Real-time analysis of HDAC6 mRNA and miRNA expressions targeting HDAC6 in K562 cells. *Expression of HDAC6 mRNA, mmiR-324-3p, and miR-181a-2-3p in K562 cells compared to PBMC cells. Abbreviation: PBMC, Peripheral blood mononuclear cell. P-value indicated as *** ≤0.001, **** ≤0.0001.*

4.1.7 HDAC6 is a direct target of miR-324-3p

Dual luciferase reporter assay was performed to confirm the interaction between HDAC6 and miR-324-3p. The HEK293T cells were co-transfected with pEGFPC1- miR-324-3p and pGL3-HDAC6 3'UTR (wild type or mutant) and the luciferase activity determined after 24 hrs of transfection confirmed the direct interaction between HDAC6 and miR-324-3p (Figure 48). Overexpression of miR-324-3p in K562 cells (Figure 49a) leads to a significant decrease in HDAC6 mRNA (Figure 49b) and protein levels (Figures 50a and 50b), resulting in increased acetylation of Hsp90 and alpha-tubulin, the client proteins of HDAC6 (Figures 50a and 50b).

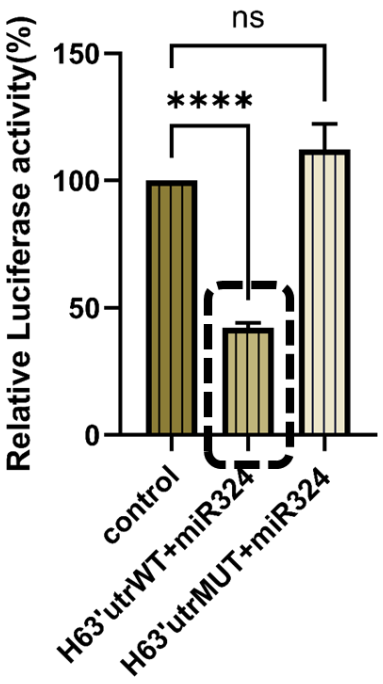


Figure 48: Dual luciferase assay showing the interaction between miR-324-3p and HDAC6 3'UTR. *Abbreviation: H6, HDAC6; utr, untranslated region; WT, wild type; MUT, Mutant. P-value indicated as **** ≤0.0001, ns=non significance >0.05.*

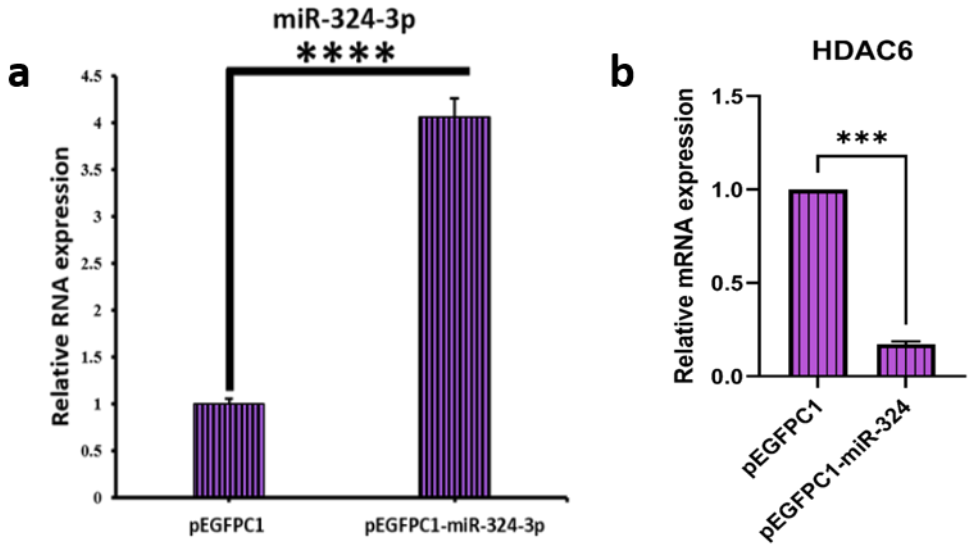


Figure 49: Real-time analysis for expression of miR-324-3p and HDAC6 mRNA with overexpression of miR-324-3p in K562 cells. *(a) Expression of miR-324-3p, and (b) expression of HDAC6 mRNA with overexpression of miR-324-3p in K562 cells. P-value indicated as *** ≤0.001, **** ≤0.0001.*

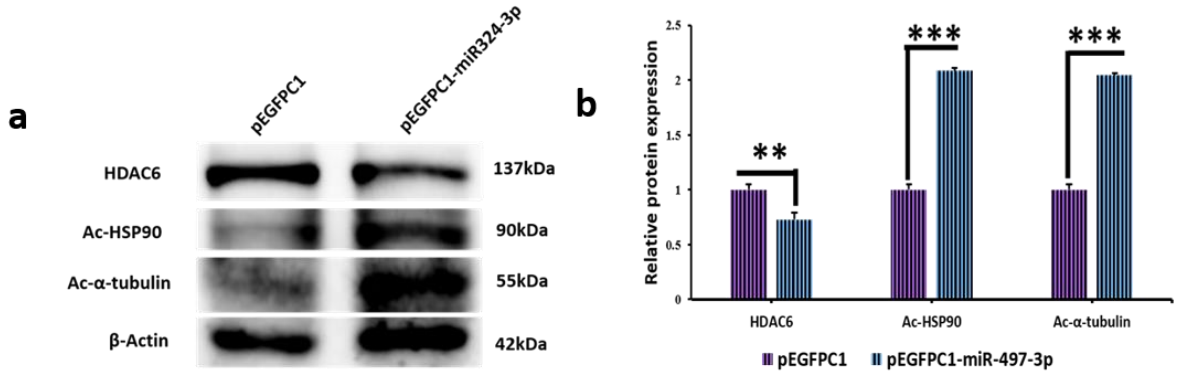


Figure 50: Immunoblot analysis for protein expression of HDAC6 and its targets acetylated HSP90 and α-tubulin in K562 cells with overexpression of miR-324-3p. *(a) Protein expression of HDAC6, Ac-HSP90, Ac-α-tubulin with reference to internal control β-Actin. (b) Densitometry of the immunoblots a. Abbreviation: Ac, Acetyl; HSP, Heat shock protein. P-value indicated as ** ≤0.01, *** ≤0.001.*

4.2 Objective 2- To study the functional significance of validated miRNA-mediated regulation of HDAC8 in cervical cancer cells

4.2.1 Functional Significance of miR-497-3p in Cervical Cancer Cell

To confirm the negative regulation of HDAC8 by miR-497-3p, we overexpressed the miR-497-3p in HeLa cells and some functional assays such as MTT cell proliferation assay, tubulin polymerization assay and revealed that miR-497-3p overexpression in HeLa cells significantly reduced cell proliferation, induced tubulin polymerization, and epithelial-mesenchymal transition (EMT) assay were performed.

MTT assay demonstrated a significant decrease in the cell viability of cervical cancer HeLa cells transfected with miR-497-3p (Figure 51). Immunofluorescence revealed the formation of tubulin bundles in HeLa cells following miR-497-3p transfection (Figure 52), confirming tubulin stabilization and thus inhibition of cell proliferation. Further, Wound-healing by scratch assay demonstrated reduced cell migration (Figures 53a and 53b) in miR-497-3p overexpressed cells compared to control cells. Furthermore, Western blot analysis showed decreased expression of the mesenchymal marker Vimentin and increased expression of the epithelial marker E-cadherin, confirming the regulatory role of miR-497-3p in HDAC8-mediated processes such as cell proliferation, tubulin polymerization, and EMT (Figures 54a and 54b).

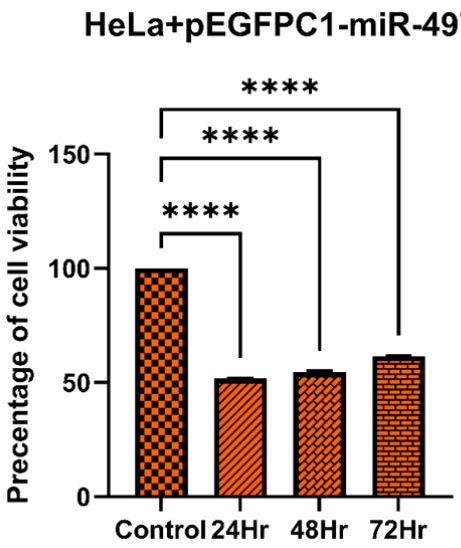


Figure 51: Cell viability checked through MTT assay for 24, 48, and 72 hours with overexpression of miR-497-3p in HeLa cells. *Abbreviation: Hr, Hour. P-value indicated as **** ≤0.0001.*

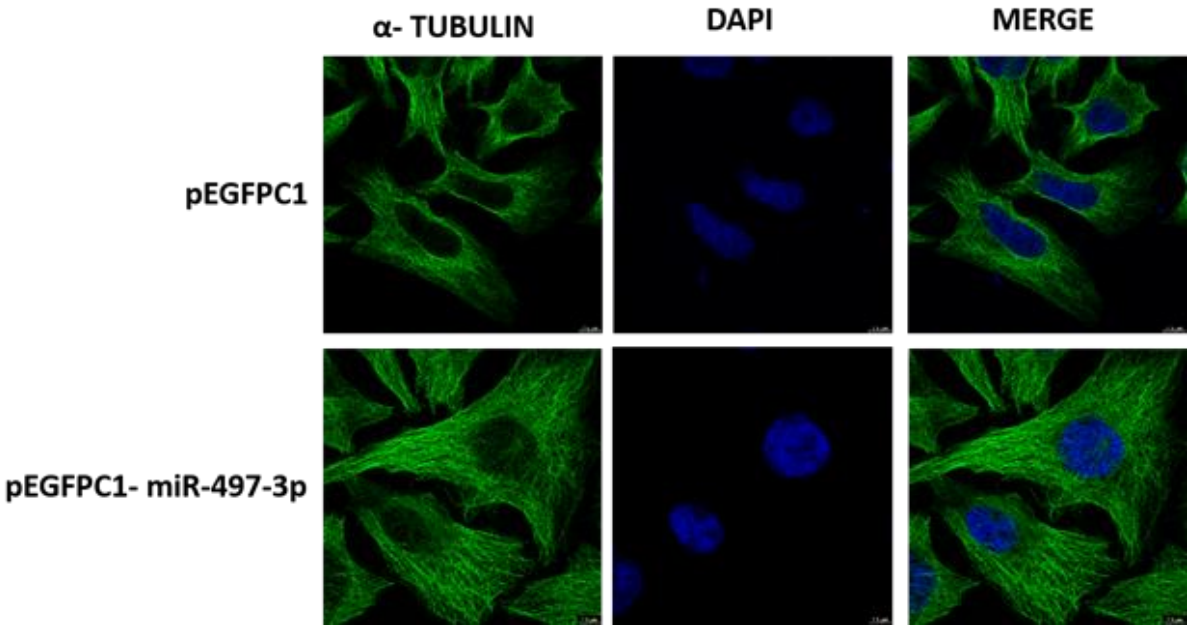


Figure 52: Immunofluorescence showing microtubule bundle formation with overexpression of miR-497-3p in HeLa cells.

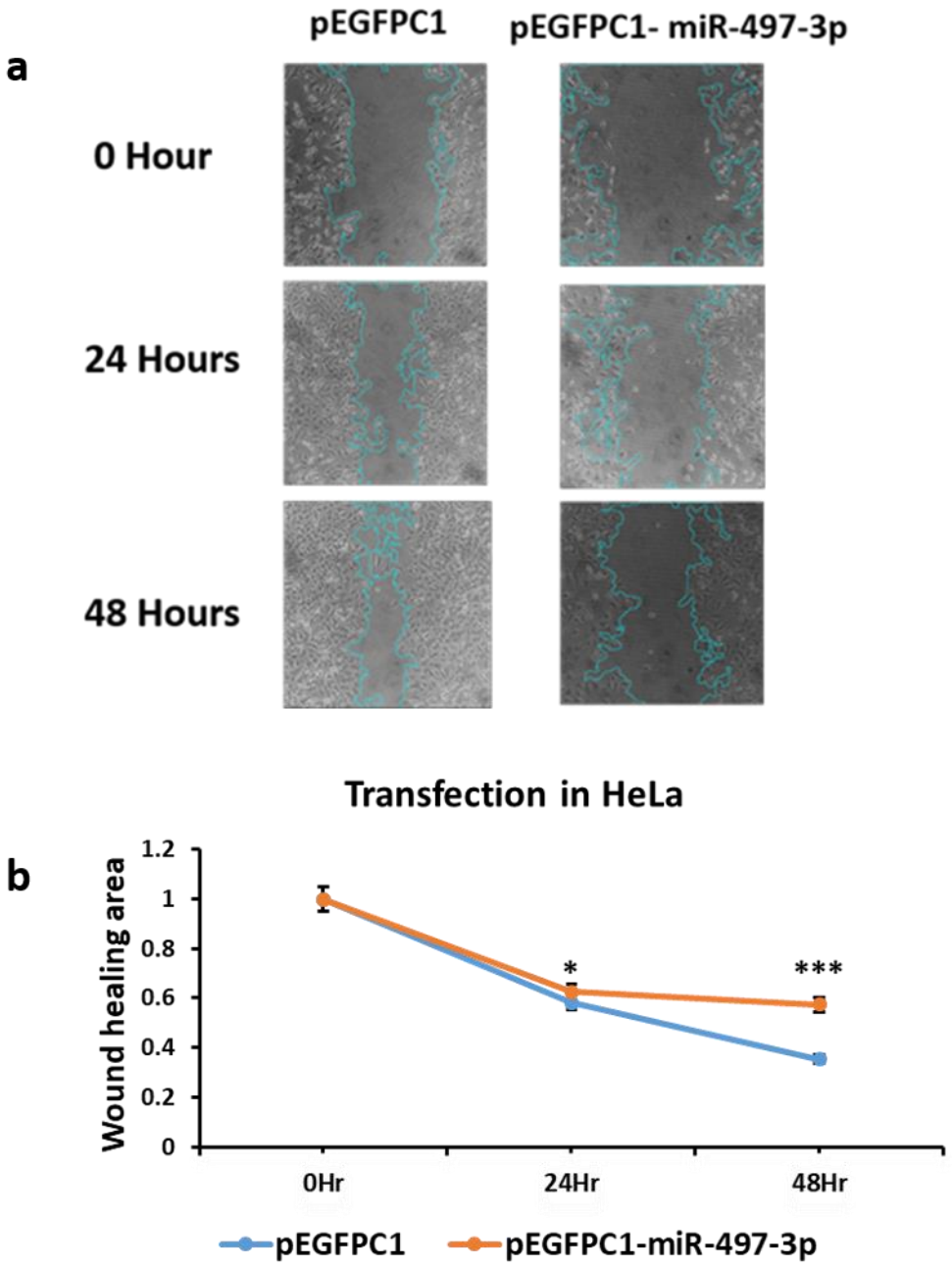


Figure 53: Image shows wound healing assay at 0, 24, and 48 hours-time points with overexpression of miR-497-3p in HeLa cells. *(a) Microscopic image showing wound healing at different time points. (b) Graphical representation of wound healing assay. Abbreviation: Hr, Hour. P-value indicated as * ≤0.05, *** ≤0.001.*

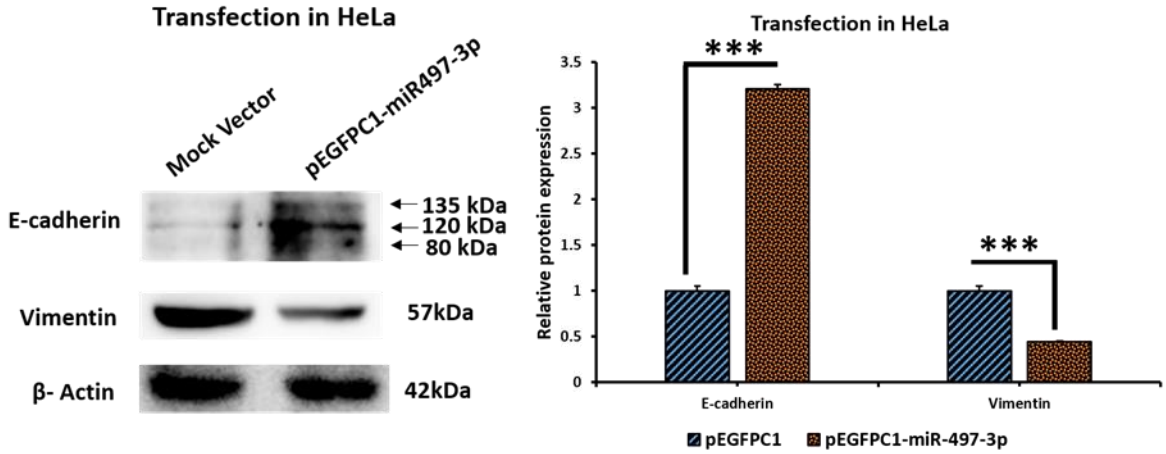


Figure 54: Expression of EMT marker proteins E-cadherin and Vimentin with overexpression of miR-497-3p in HeLa cells. *(a) Immunoblot showing protein expression of E-cadherin and Vimentin in reference to internal control β-Actin. (b) Densitometry of the immunoblot a. P-value indicated as *** ≤0.001.*

4.3 Objective 3- To study the functional significance of validated miRNA-mediated regulation of HDAC8 in metastatic breast cancer cells.

Our study results from Objective 1 & 2 demonstrated that miR-497-3p inhibited HDAC8 in cervical cancer cells, HeLa, and thus inhibited cancer progression. Based on our preliminary data, we observed that HDAC8 is overexpressed in breast cancer cells MCF7 and MDAMB231 (Figure 27a). We therefore tried to check the regulation of HDAC8 by miR-497-3p in metastatic breast cancer MDAMB231 cells.

4.3.1 expression of miR-497-3p in metastatic breast cancer cell

We first evaluated the expression of the miR-497-3p in MDAMB231 cells by qRT-PCR and as expected, the miR-497-3p was significantly lowered in both the cell lines (Figure 55) which is in strong negative correlation with HDAC8 expression in the cell lines.

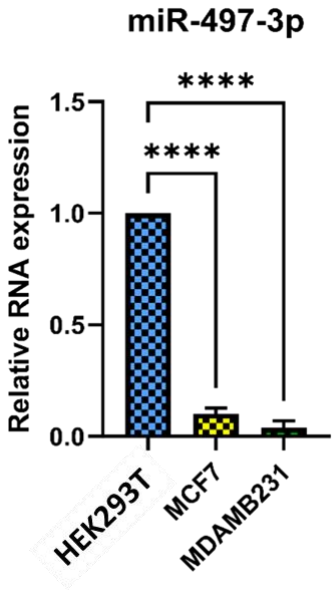


Figure 55: Real-time analysis of miR-497-3p expression in Breast cancer cell lines. *Expression of miR-497-3p in MCF7 and MDAMB231 cells compared to HEK293T cells. P-value indicated as **** ≤0.0001.*

Additionally, we could find the similar result in breast tumor samples from the TCGA database (Figure 56) and high expression of miR-497-3p shows a better survival rate in breast cancer patients (Figure 57).

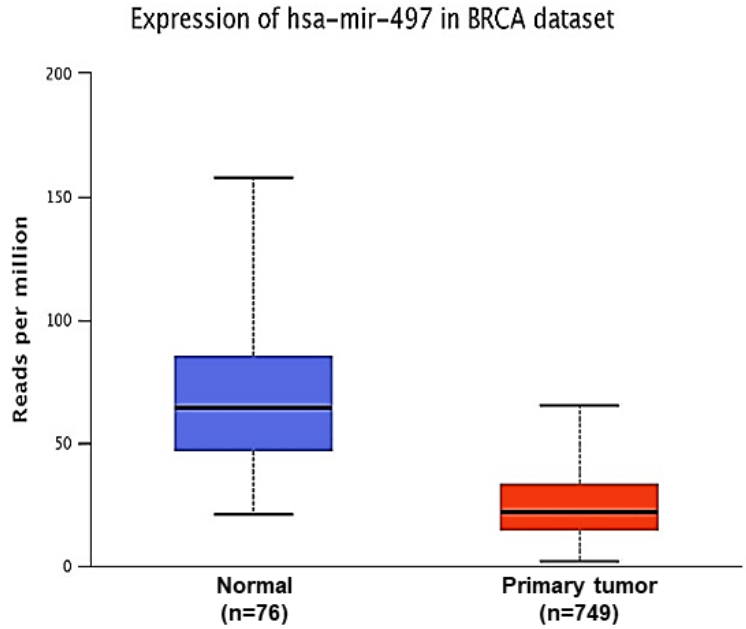


Figure 56: Expression level of miR- 497 in tumor TCGA sample in BRCA dataset.

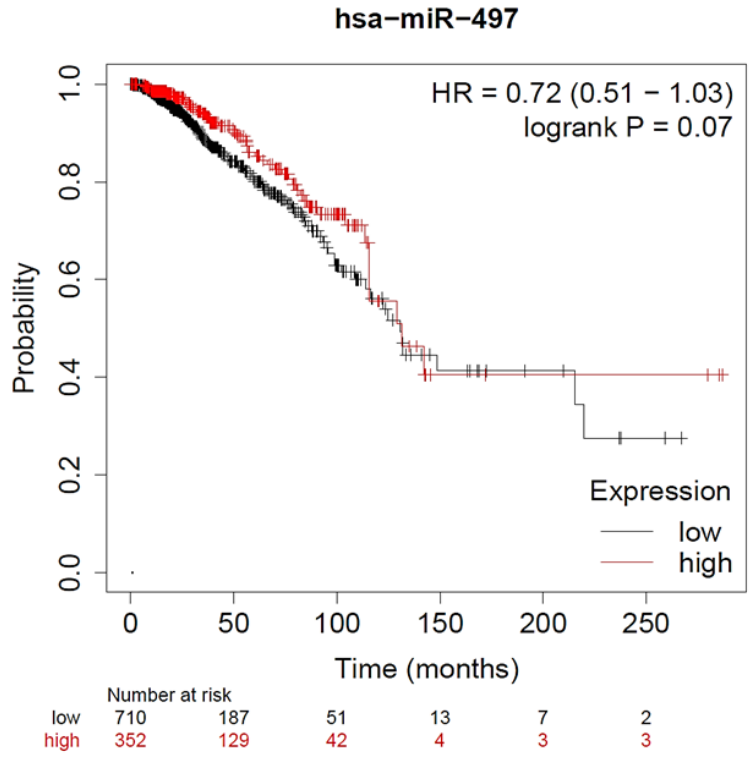


Figure 57: Overall Survival (OS) of miR-497 was analyzed based on breast cancer clinical samples taking TCGA dataset through Kaplan Meier plotter and the result shows high expression significantly correlated with a high survival rate in patients.

4.3.2 Functional Significance of miR-497-3p in Metastatic Breast Cancer Cell

We tried to confirm the negative regulation of HDAC8 by miR-497-3p in breast cancer cells by some functional assays similar to that in HeLa cells. Transient high expression of miR-497-3p in MDAMB231 cells (Figure 58a) significantly reduced HDAC8 at the mRNA level (Figure 58b) as well as at protein level and significantly increased the acetylated levels of its target proteins (Figure 59).

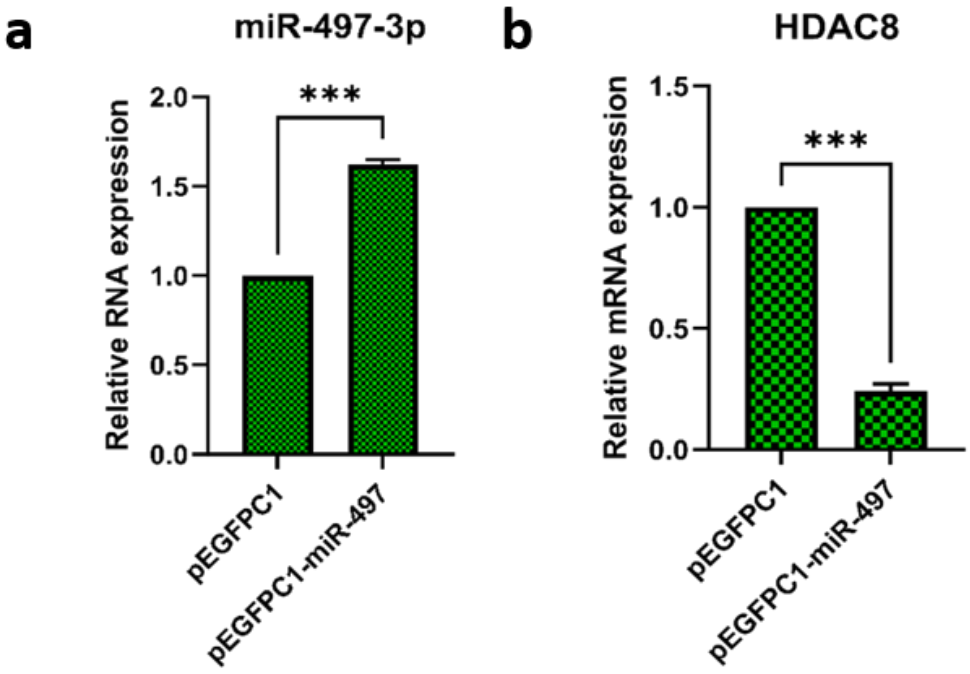


Figure 58: Real-time expression of miR-497-3p and HDAC8 mRNA with overexpression of miR-497-3p in MDAMB231 cells. *(a) Expression of miR-497-3p, and (b) HDAC8 mRNA with overexpression of miR-497-3p. P-value indicated as *** ≤0.001.*

MTT assay demonstrated reduced cell proliferation (Figure 60) of MDAMB231 cells transfected with miR-497-3p compared to untransfected cells. Immunofluorescence revealed the formation of tubulin bundles in MDAMB231 cells following miR-497-3p transfection (Figure 61), confirming tubulin stabilization.

Wound-healing Scratch assay demonstrated inhibition of cell migration (Figure 62), and Immunoblot analysis showed declined protein expression of Vimentin, the mesenchymal marker, and increased protein expression of E-cadherin, the epithelial marker (Figure 63).

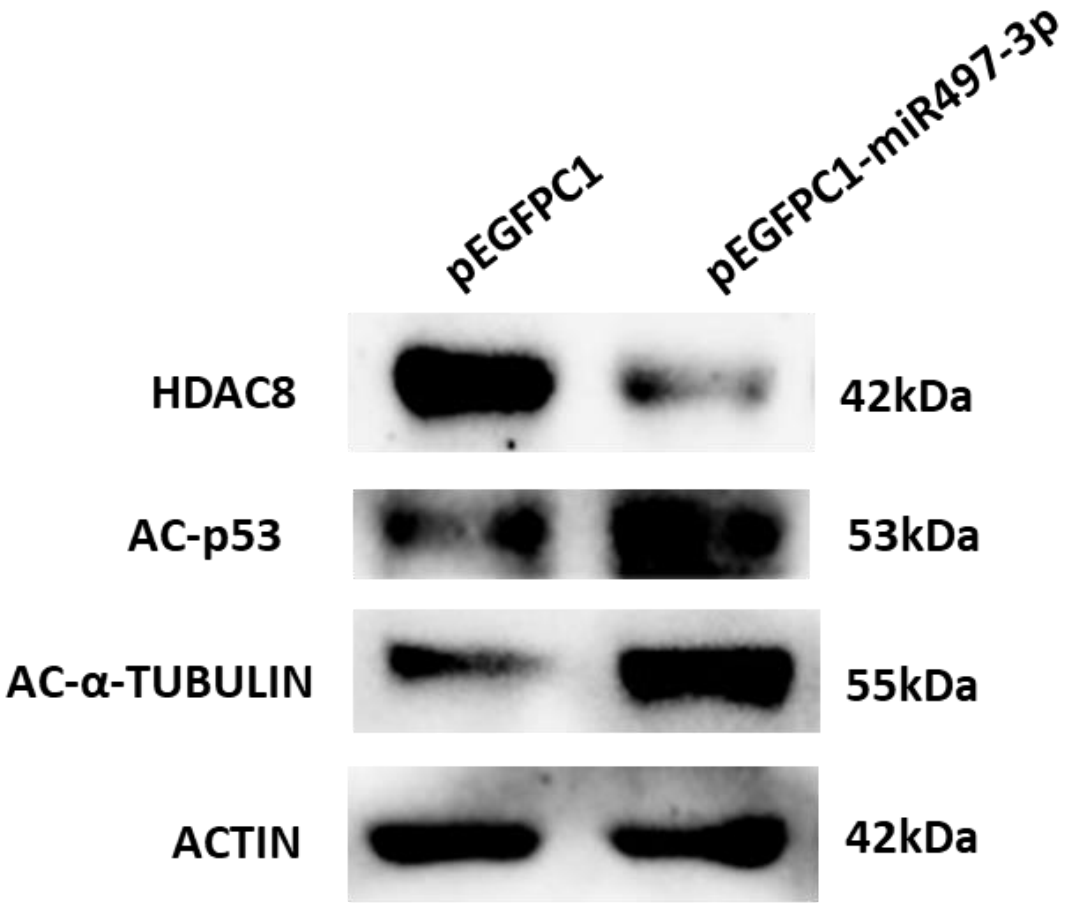


Figure 59: Immunoblot shows a protein expression of HDAC8 and its targets acetylated p53 and α-tubulin in MDAMB231 cells with overexpression of miR-497-3p. *Abbreviation: Ac, Acetyl.*

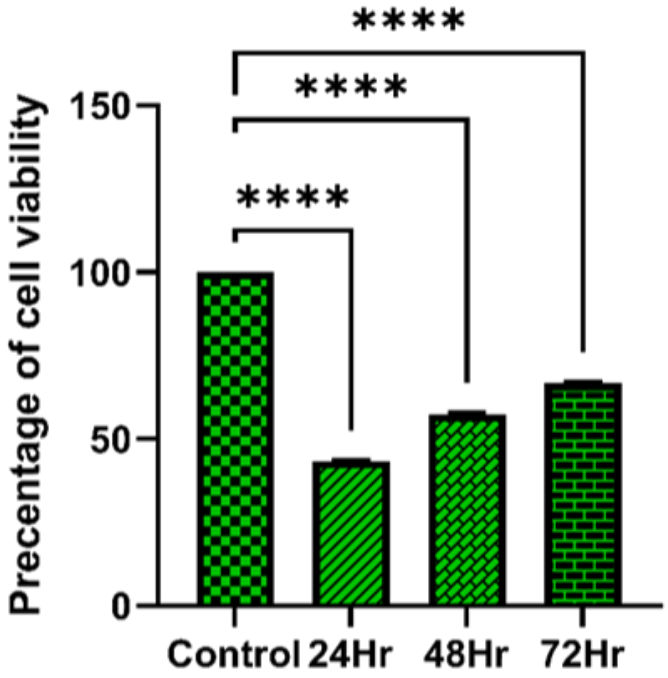


Figure 60: Cell viability analyzed through MTT assay for 24, 48, and 72 hours with overexpression of miR-497-3p in MDAMB231 cells. *Abbreviation: Hr, Hour. P-value indicates as **** ≤0.0001.*

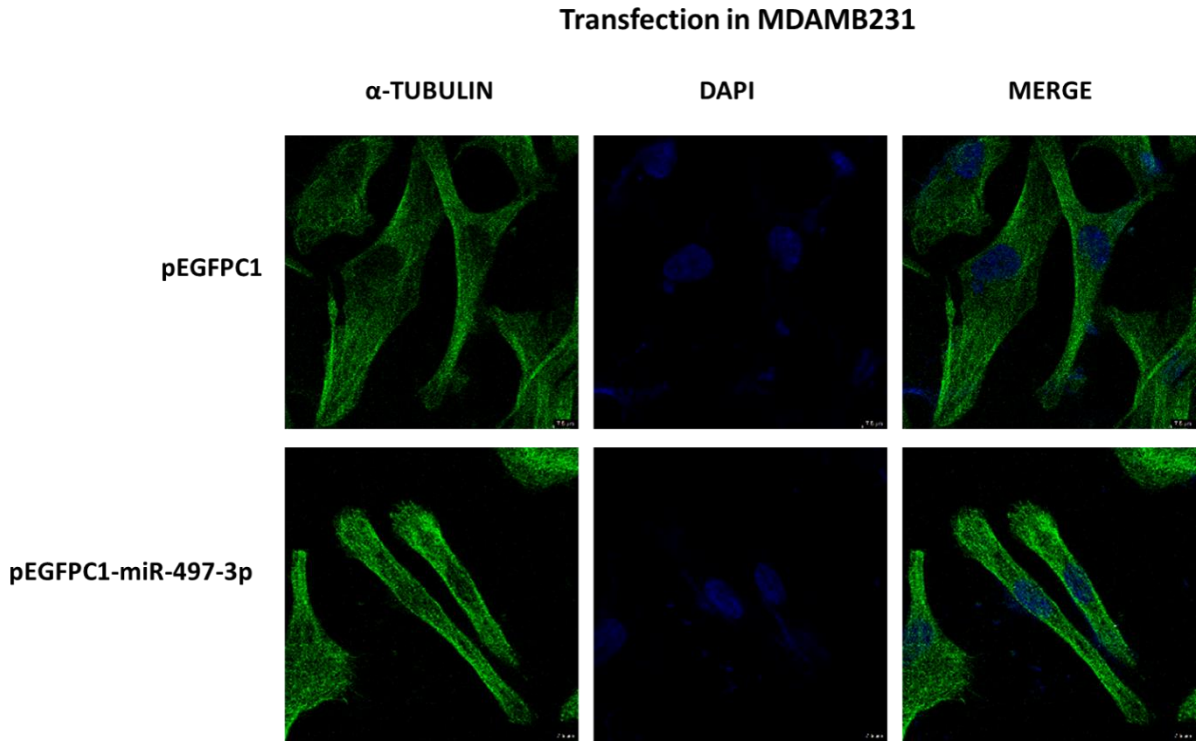


Figure 61: Immunofluorescence showing microtubule bundle formation with overexpression of miR-497-3p in MDAMB231 cells.

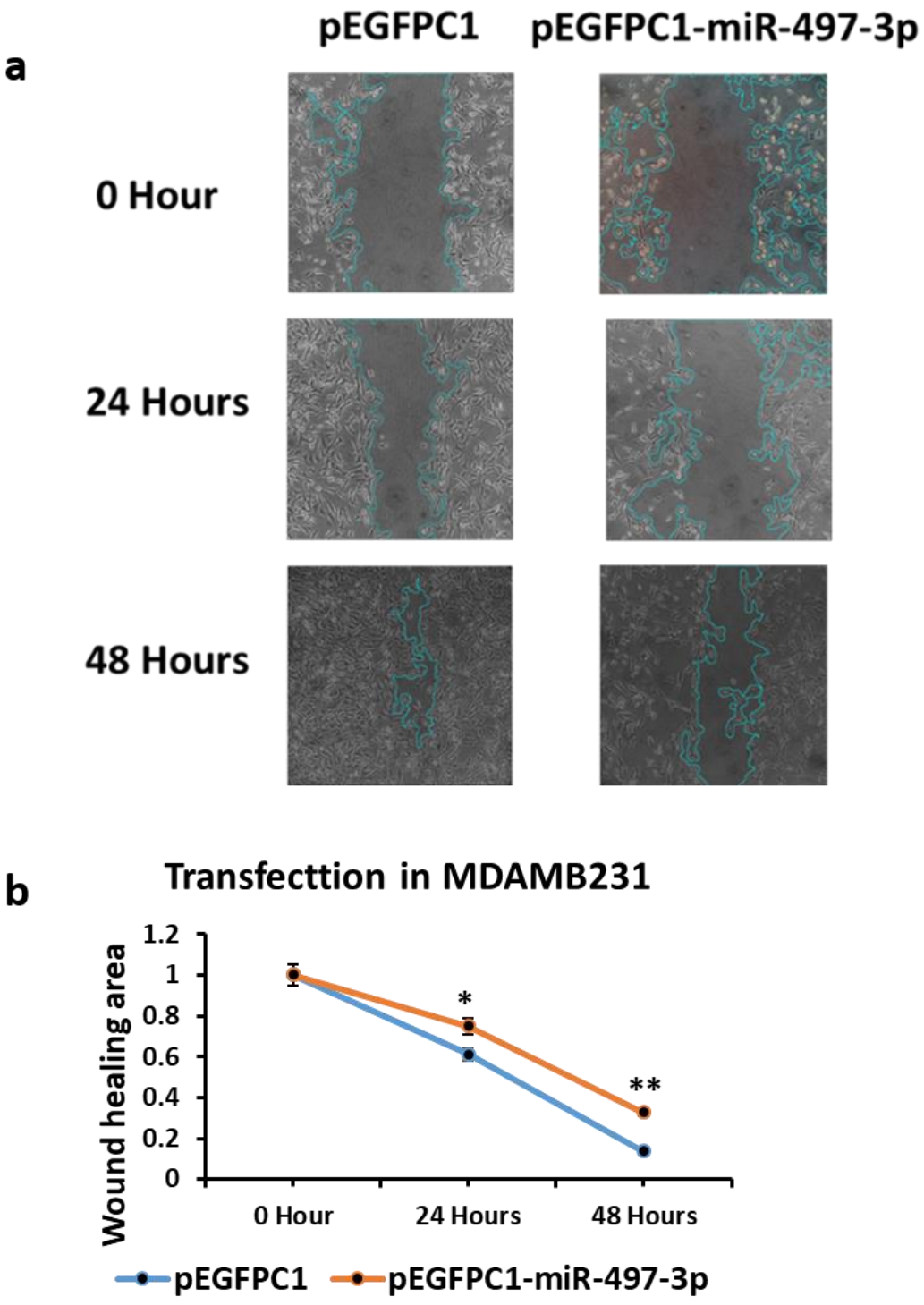


Figure 62: Wound healing assay at 0, 24, and 48 hours-time points with overexpression of miR-497-3p in MDAMB231 cells. *(a) Microscopic image showing wound healing assay, and (b) Graphical representation of wound healing assay a. P-value indicated as * ≤0.05, ** ≤0.01.*

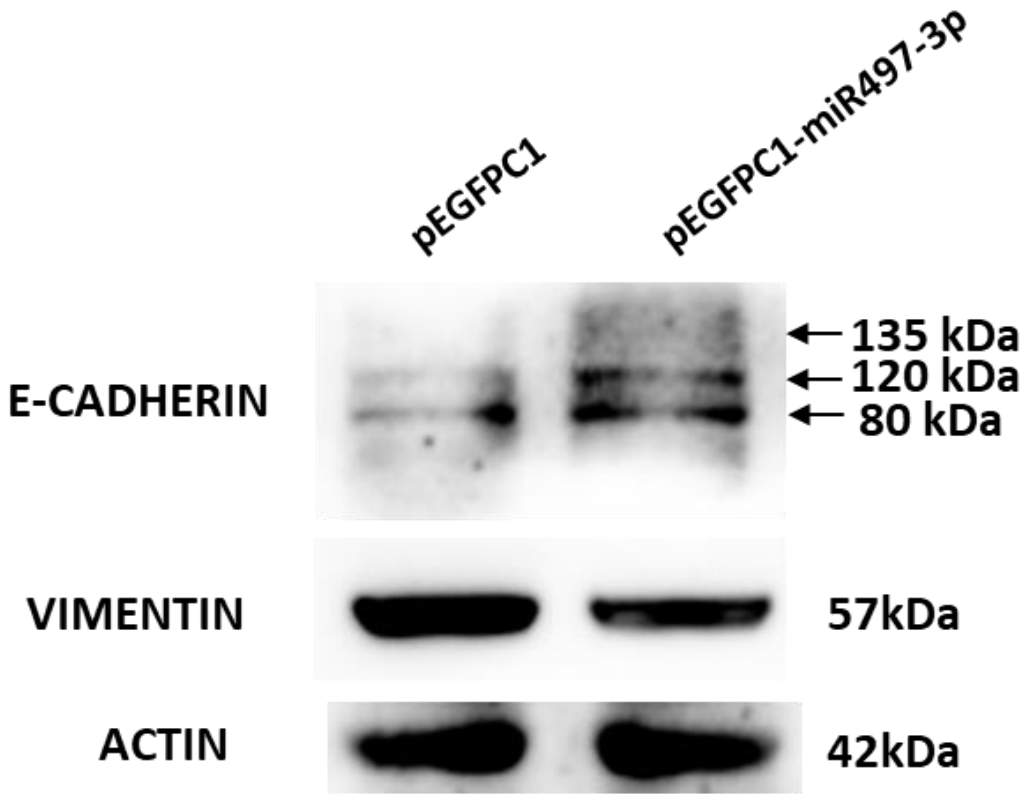
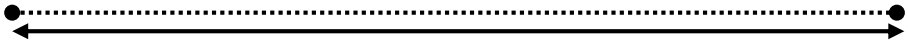


Figure 63: Immunoblot showing protein expression of EMT markers E-cadherin and Vimentin with overexpression of miR-497-3p in MDAMB231 cells.

Discussion



5.0 Discussion

Breast and Cervical cancers are the most frequent malignancies in females, producing substantial mortality worldwide. Cervical cancer is commonly triggered by chronic infection with high-risk type human papillomavirus (HPV), a sexually transmitted infection, while there are various risk factors for breast cancer development. The treatment generally involves surgery, chemotherapy, radiation therapy, immunotherapy, or a combination of these. The untargeted chemotherapy although not so expensive, has several side effects, and the targeted chemotherapy, though effective, is very expensive. Therefore, there is an immediate urgency to identify a targeted and economical treatment strategy to treat these cancers.

To identify a druggable target, understanding the fundamental molecular pathways that contribute most to the genesis and progression of these malignancies is critical. It is very well established that several changes at the genomic and molecular level such as chromosomal alterations, galvanization of oncogenes, tumor suppressor genes inactivation, and aberrant changes in the signaling pathways result in the formation of tumors. Furthermore, EMT changes and angiogenesis lead to the metastasis of tumors to the distant organs forming secondary tumors (Vogelstein & Kinzler, 2004). Apart from these mechanisms, epigenetic alterations have now been the focus of research due to their impact on cancer progression.

Epigenetic changes refer to the alterations in gene expression without any changes in the DNA sequence itself. Instead, these changes affect how genes are turned on or off, influencing cellular processes. There are several epigenetic modifications such as chromatin remodeling, DNA methylation, Histone modifications (for example, Histone acetylation and deacetylation), RNA interference, post-translational

modifications of proteins and so on. Among several histones as well as non-histone protein post-translational modifications, acetylation by HATs and deacetylation by HDACs have been well studied from the perspective of cancer development and advancement. In fact, HDACs are established as a druggable target for the treatment of cancer. Another epigenetic modulator, miRNAs have gained importance in recent years due to their involvement in regulating the other epigenetic modifiers including HDACs. Therefore, for the present study we focused on understanding and identifying miRNAs that specifically regulate HDACs in cervical and breast cancer.

HDACs are enzymes that remove acetyl chemical structures from histone proteins, causing chromatin condensation and transcriptional inhibition. Dysregulation of HDAC activity can result in aberrant gene expression patterns, contributing to cancer initiation, progression, and metastasis. HDACs also modulate non-histone protein function by deacetylation resulting in another level of regulation. For example, the deacetylation of Hsp90 protein by HDAC6 is crucial for its chaperonic activity. On the other hand, lysine deacetylation of tumor suppressor protein p53 results in its inactivation. However, aberrant expression of HDACs is observed in several cancers.

Overexpression of HDACs has been experiential in various cancers, including breast and cervical cancer. HDACs can stop tumor suppressor genes that are part of cell cycle regulation, apoptosis, and DNA repair, promoting oncogenesis. Inhibition of HDAC activity has materialized as a favorable therapeutic strategy for cancer treatment, as it can re-activate silenced tumor suppressor genes and encourage arrest of cell cycle, differentiation, and cell death in cancer cells. Several HDAC inhibitors, such as vorinostat, romidepsin, and panobinostat, have been approved for the treatment of definite types of cancer, including cutaneous T-cell lymphoma and multiple

myeloma. HDAC inhibitors are also being investigated in clinical trials for the treatment of other cancers, both as monotherapy and in combination with other drugs.

MiRNAs are short non-coding RNAs that post-transcriptionally control gene expression by interacting with the 3'UTR of destined mRNAs, resulting in mRNA destruction or translational inhibition. Dysregulation of miRNAs can impact various cellular progressions, including proliferation, cell death, differentiation, and metastasis. Aberrant expression of miRNAs is a common feature of cancer, with many miRNAs acting as oncogenes (oncomiRs) or tumor suppressors. Oncogenic microRNAs can promote cancer growth by affecting tumor suppressor genes or genes associated with apoptosis and cell cycle control. Tumor suppressor miRNAs, when downregulated, can lead to increased expression of oncogenes, facilitating tumor growth and metastasis. Altered miRNA expression profiles have been identified in various cancer types, and specific miRNAs have been associated with prognosis, response to therapy, and metastatic potential. miRNAs hold promise as therapeutic targets and biosignatures for cancer judgment, prediction, and medication response prediction. Therapeutic strategies targeting miRNAs include miRNA mimics (to restore tumor suppressor function) and miRNA inhibitors (to block oncogenic miRNA activity). Ongoing research attempts to understand the functions of various miRNAs in cancer biology and create miRNA-based therapeutics for a variety of cancers.

HDACs can regulate the miRNAs expression by modulating chromatin structure and transcriptional activity. Conversely, miRNAs can target and regulate the expression of HDACs, forming regulatory feedback loops that influence gene expression patterns. HDAC inhibitors (HDACis) can restrain the expression of definite miRNAs, leading to variations in gene expression outlines and cellular phenotypes in cancer cells.

Combination therapies targeting both HDACs and miRNAs hold promise for enhancing anti-cancer effects and overcoming drug resistance in cancer treatment. Understanding the intricate interplay between miRNAs and HDAC regulation is essential for unraveling the complex molecular mechanisms underlying cancer development and identifying novel therapeutic targets and strategies for cancer treatment.

HDAC8, a well-established class I histone deacetylase (HDAC) and X-linked gene, has been extensively implicated in the progression of various cancers, including breast, cervix, lung, liver, and colon (Ba et al., 2020; Dasgupta, Antony, Braithwaite, & Horsfield, 2016; Kim et al., 2022; Menbari et al., 2019; Thakur et al., 2005; Vanaja, Ramulu, & Kalle, 2018; Wu et al., 2013). Notably, HDAC8 has also been associated with the enhancement of cancer metastasis (Chen et al., 2022; Pantelaiou-Prokaki et al., 2022; Tang et al., 2020). And a recent study from our lab found alpha-tubulin as a direct non-histone substrate of HDAC8, which is one of the major components involved in metastasis. HDAC8 inhibition through selective inhibitors like PCI- 34051 was well-studied in cervical cancer, however, miRNA-mediated regulation has not yet been discovered.

HDAC6, a class IIb HDAC, is primarily known for regulating alpha tubulin thereby maintaining normal physiology like cell structure and cell cycle in non-cancer cells (Hubbert et al., 2002) like HEK293T.

HDAC6 and HDAC8 are two deacetylases of α-tubulin and show differential expression in cervical cancer cells in judgment to normal cells. HDAC8 functions as an oncogene showing higher expression whereas HDAC6 shows normal expression in non-aggressive female-specific cancers like cervical, breast, and ovary cancers. However,

both HDAC6 and HDAC8 show overexpression in aggressive cancers of the cervix, breast, and ovary.

Our research showed the involvement of overexpressed HDAC8 as α-tubulin deacetylase rather than HDAC6, a known tubulin deacetylase, specifically in cervical cancer (Vanaja et al., 2018). In an attempt to identify the mechanism for such differential expression of HDAC8 and HDAC6 in cervical cancer, we hypothesized miRNA-based regulation of HDACs and aimed to find novel miRNAs targeting and regulating HDAC8 and HDAC6 functional activities in cervical cancer.

The results of the study showed that indeed, miRNAs differentially regulate the two alpha tubulin deacetylases, HDAC8 and HDAC6, in human cervical cancer. We showed miR-497-3p was significantly downregulated in HeLa cells, while the HDAC8 gene expression was increased compared to normal HEK293T cells. However, most studies have been done on miR-497-5p in cervical cancer and it was found it deeds as a tumor suppressor showing poorer expression (Luo, Shen, Zhou, Chen, & Wang, 2013; Y. Zhang et al., 2015), and miR-497 was also discovered as a probable prognostic marker and a biosignature as cisplatin chemosensitivity regulator (Yang et al., 2016). Also, miR-497-3p was revealed to show a different part in cancer such as it inhibits breast cancer metastasis targeting EMT (Dong et al., 2023), hinders ovarian cancer regulating CLDN4 axis (Jie et al., 2020), plays a role in gefitinib resistance in NSCL cancer (Chandrashekar et al., 2017), and in thyroid cancer as a tumor suppressor (Fan et al., 2023).

To the best of our acquaintance, this work is the first to authorize the anticancer action of miR-497-3p on the carcinogenic effect of HDAC8. Following miRNA-mRNA binding through Dual-luciferase assay, we showed that elevated expression of miR-497-3p inhibits the expression of HDAC8 both at mRNA as well as

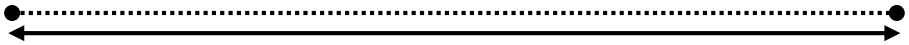
protein levels in HeLa cells. Additionally, with HDAC8 lower expression, we found there is a significant increase in the acetylation levels of its downstream target substrates like p53 and alpha-tubulin. Moreover, we showed here a decrease in cell viability and mesenchymal protein vimentin as well as an increase in epithelial protein E-cadherin with miR-497-3p over-expression in HeLa cells. Studies showed instability of alpha-tubulin increased cancer cell metastasis and with its stability there is a decrease in cancer metastasis, here showed with miR-497-3p there is an increase in the formation of alpha-tubulin bundles which shows a stable nature of alpha-tubulin in HeLa cells. Moreover, we found somewhat similar results in MDAMB231 cells with overexpression of miR-497-3p that target HDAC8.

On the other hand, we also showed that compared to HEK293T cells, HDAC6 has lower expression and miR-324-3p was highly expressed in HeLa cells, and similar results we found in the TCGA sample. However, one study shows miR-324-3p higher expression in HeLa cells related to End/E6E7 cells (Shi, Huo, Gao, Cai, & Zhu, 2020). Additionally, in other female-specific such as breast and ovarian cancer miR-324-3p was found as a tumor suppressor (Ba et al., 2020; Fang et al., 2020; Geng, Wang, & Tian, 2022; Hou, Cai, Yu, & Lin, 2021; Li et al., 2022; Liu et al., 2020; N. Zhang et al., 2019).

In our study, we tried to find the miRNA-mediated regulation of HDAC6 in HeLa cells, and the finding results were validated using HDAC6 overexpressed K652 cells. First, we authenticated the miR-324-3p and HDAC6 3'UTR binding through dual-luciferase assay and studied their molecular regulation in K562 cells by over-expressing miR-324-3p. In a result, we found with the greater expression of miR-324-3p in cells of K562 there is inhibition of HDAC6 both at mRNA and protein levels and enhancement in its downstream target proteins like acetylated HSP90 and acetylated alpha-tubulin.

In summary, our study shows miR-497-3p, a tumor suppressor miRNA, mediated HDAC8 regulation that inhibits HeLa cell metastasis by enhancing acetylated p53 expression, increasing the stability of ac-alpha-tubulin, and suppressing the EMT. The study also identified miR-324-3p-mediated HDAC6 regulation in HeLa cells. Overall, our study helps in understanding the miRNA-mediated regulation of HDAC8 and HDAC6 and paves a path toward identifying the miRNA-based alternate therapeutic method for treating cervical cancer.

Conclusions



6.0 Conclusions

In conclusion, our investigation has unraveled the pivotal roles played by miRNAs in orchestrating the intricate dance of histone deacetylases, specifically HDAC8 and HDAC6, within the realm of cervical cancer. At the forefront, miR-497-3p emerges as a potent tumor suppressor, steering HDAC8 regulation to inhibit metastasis in HeLa cells. This regulatory paradigm manifests through the elevation of acetylated p53 expression, fortification of acetylated alpha-tubulin stability, and the concurrent suppression of EMT. Crucially, our study extends its reach to illuminate the nuanced regulatory landscape surrounding HDAC6 in HeLa cells, mediated by miR-324-3p. By delineating the molecular intricacies through dual-luciferase assays and probing the downstream effects in K562 cells, we discerned a landscape of inhibited HDAC6 expression at both mRNA and protein levels. Furthermore, this regulatory cascade reverberates across the cellular milieu, amplifying the acetylation levels of HSP90 and alpha-tubulin. Collectively, our findings provide a comprehensive understanding of miRNA-mediated regulatory mechanisms governing HDAC8 and HDAC6 in cervical cancer. This not only deepens our insight into the molecular intricacies of cervical carcinogenesis but also charts a promising course toward the development of miRNA-based therapeutic strategies. Additionally, we found miR-497-3p showing similar regulation to HDAC8 in breast cancer MDAMB231 cells. By identifying miR-497-3p and miR-324-3p as key players in modulating HDAC8 and HDAC6, respectively, our study lays the foundation for future endeavors aiming at precision interventions in the treatment landscape of cervical and breast cancer.

Summary

7.0 Summary

Cancer is a disease distinguished by its ability to alter cell phenotypes, resulting in uncontrolled growth, invasion, and metastasis, while also disrupting normal body functions thereby hampering human health and causing death. Cervical cancer is the fourth utmost fluent cancer of adult women in emergent countries causing 87% of deaths. Early diagnosis and medication of cervical cancer in the premalignant stage can decrease incidence and mortality rates. Pap smear test is a productive diagnostic method for cervical cancer. In more than 85% of cervical cancer cases, it is known that human papillomavirus (HPV) is the cause and can be prevented with HPV vaccination. However, lack of awareness is one of the major reasons for increased mortality rates in cervical cancer.

The second most frequent female-specific cancer showing the highest mortality and morbidity globally is breast cancer. Although mammogram is used as a diagnostic method, early diagnosis of breast cancer is challenging and is associated with high rates of metastasis.

Paclitaxel is used as a front-line therapy in both cervical cancer and breast cancer. Paclitaxel is a tubulin polymerization inhibitor. Tubulin polymers make up human microtubules, the most widespread intracellular cytoskeletal structures. These structures serve a critical role in regulating a variety of cellular functions, including cell growth, division, motility, intracellular transportation, organelle placement, and signal transduction. Notably, in the context of cancer, the dynamic instability of microtubules is linked to processes such as cell proliferation and invasion. Paclitaxel is known to inhibit this polymerization thereby stabilizing the microtubules thus leading to cell death. However, paclitaxel not only inhibits tubulin in cancer cells, but also inhibits tubulin in all normal dividing cells causing adverse effects such as anaemia, hair loss, gastric ulcers etc.

Tubulin is epigenetically regulated by acetylation and deacetylation. Histone deacetylases (HDACs) are one of the epigenetic regulators that reverse lysine acetylation thereby regulating the protein function. Of the 18 known human HDACs, HDAC6, a class IIb HDAC, is known as tubulin deacetylase that deacetylates a conserved lysine 40 residue (K40) of alpha-tubulin and leading to dynamic microtubule polymerization in addition to controlling various other cellular and disease activities. However, our group discovered that HDAC8 of class I HDACs also deacetylates alpha-tubulin. This functional redundancy of HDAC8 as tubulin deacetylase is observed only when HDAC8 is overexpressed and HDAC6 is normally expressed such as in cervical cancer cells but not in normal cells.

Both HDAC6 and HDAC8 are X-chromosome-specific genes and are close to the centromere (Xp11.23 and Xq13.1 respectively). Despite being in the X-inactivation center (XIC, Xq13) HDAC8 escapes inactivation and gets overexpressed in cervical cancer cells where as HDAC6 remains normally expressed. We hunted to understand this differential expression of the two HDACs, HDAC6 and HDAC8, in cervical cancer.

RNAi (RNA interference), another epigenetic modifier, was shown to have an important role in gene regulation. Majorly, it includes non-coding RNAs (ncRNAs), of which microRNA (miRNA) is a major player allowing cells to fine-tune gene expression, and respond to numerous physiological and environmental motions. MiRNAs are small ncRNAs guiding the RISC (RNA-induced silencing complex) to the destined mRNA 3' untranslated region (UTR). Interestingly, they have been shown to directly and indirectly control HDACs, and their participation in this regulation is part of the cell's complex epigenetic and gene expression governing linkage. Furthermore, while few research studies have indicated miRNA-mediated HDAC6 and HDAC8 regulation in breast cancer, no studies have revealed miRNA-mediated regulation of HDAC8 in cervical cancer.

With this background, we aimed to identify miRNA-mediated regulation of HDAC6 and HDAC8 in cervical cancer cells with a rationale of empathetic the differential regulation of these HDACs and their involvement in advancement of cervical cancer as tubulin deacetylases. To fulfil our study, we framed the following objectives,

1. Identification of potential miRNA (s) targeting HDAC8/6 and validation of the interaction of identified miRNA (s).

2. To study the functional significance of validated miRNA-mediated regulation of HDAC8/6 in cervical cancer cells.

3. To evaluate the functional significance of validated miRNA-mediated regulation of HDAC8 in metastatic breast cancer cells.

Moreover, we utilize the following materials and methods to validate our objectives,

1. miRNAs targeting HDAC8 and HDAC6 were predicted using online in silico tools.

2. The predicted miRNAs were validated using in vitro studies on HeLa (human cervical cancer), HEK293T (human embryonic kidney) and MDAMB231 (human metastatic breast cancer) cancer cell lines.

3. First, using co-expression analysis by RT-PCR, the predicted miRNAs were validated. Out of 5 miRNAs predicted for HDAC8, 2 were finalized for further studies and out of 4 predicted miRNAs, for HDAC6, 1 was selected for further analysis.

4. The direct targeting of miRNAs to the 3’ UTR of target mRNA was confirmed by dual luciferase assay. miR-497-3p and miR-324-3p showed direct binding to the 3’ UTR of HDAC8 and HDAC6 mRNA respectively.

5. The miRNA-mediated regulation of HDAC8 and HDAC6 was confirmed by transfection assays followed by expression studies using RT-PCR and Western blot analysis.

6. The functional significance of the identified miRNA-mediated regulation of HDAC8 and HDAC6 was further analyzed by immunoblot analysis, immunofluorescence analysis, cell migration by wound healing assays.

7. GraphPad Prism v6.01 software was used for the statistical analysis, which included a student t-test and one-way analysis of variance (ANOVA), and statistical significance (p-value) was set at * ≤0.05, ** ≤0.01, *** ≤0.001, **** ≤0.0001, and ns=non significance >0.05.

In our initial investigation, we evaluated HDAC8 and HDAC6 expression in cervical and breast cancer cell lines compared to the non-cancerous HEK293T cells. HDAC8 was notably overexpressed in HeLa, MCF7, and MDAMB231 cells, while HDAC6 showed lower expression in HeLa and MCF7 and comparable levels in MDAMB231 cells. We also introduced the K562 cell line, known for high HDAC6 levels, for further exploration, and our findings aligned with Protein Atlas data, indicating elevated HDAC6 mRNA in K562 cells versus PBMCs.

Using miRDB, TargetScan, and miRmap, multiple miRNAs targeting the 3'UTR region of HDAC8 and HDAC6 mRNA were identified. Each database predicted a set of target genes, and TBtool helped visualize the shared genes among these databases, facilitating easy extraction of cluster genes. Given that several genes were annotated across multiple databases, the presence of a specific miRNA in different databases increased its likelihood of effectively targeting HDAC6 and HDAC8. Hence, we selected overlapping miRNAs to ensure comprehensive coverage, choosing those present in two or more databases to avoid missing potential information.

The screening process prioritized miRNAs with robust canonical binding sites, known for their stronger repression efficacy due to potent seed matches. Canonical site classes were ranked based on their effectiveness, with the 8mer site, 7mer-m8 site, and 7mer-A1 site listed as the most influential, followed by weaker site types like 6mer and offset-6mer, which have less potent canonical characteristics within the miRNA-pairing motifs in the 3' UTRs of target mRNA.

Further, miRNAs having sites of 8mer and 7mer-m8 were prioritized emphasizing those with lower ΔG values from mfold software calculations. Considering their association with female-specific cancer from databases like miRcancer and dbDMEC, miR-150-5p, miR-664b-3p, miR-579-3p, miR-497-3p, and miR-26b-3p were identified as potential mature miRNAs targeting HDAC8 mRNA, while miR-181a-2-3p, miR-30b-3p, miR-324-3p, and miR-642a-5p were selected as potential miRNAs targeting HDAC6.

Through real-time PCR, we examined the expression of five miRNAs predicted to bind HDAC8 in HeLa cells compared to HEK293T cells. The findings revealed reduced levels for all five HDAC8-miRNAs. Notably, miR-497-3p and miR-26b-3p displayed substantially lesser expression in HeLa cells, representing a negative association with higher HDAC8 expression. Comparable results were seen in cervical cancer TCGA tumor samples for miR-497-3p and miR-26b-3p. Consequently, we focused on confirming these two miRNAs.

For HDAC6, all four miRNAs showed increased expression inversely correlated with HDAC6. In cervical TCGA data, similar results were observed for miR-181a-2-3p and miR-324-3p. Further analysis in HDAC6-overexpressing K562 cells highlighted higher expression of miR-181a-2-3p and lower expression of miR-324-3p compared to PBMC cells, leading us to concentrate on validating miR-324-3p as a potential negatively regulating miRNA of HDAC6.

The study employed a dual luciferase reporter assay to validate the direct interface between predicted miRNAs and HDAC8/HDAC6. Co-transfection of miR-497-3p with wild-type HDAC8 3'UTR into HEK293T cells notably reduced luciferase activity compared to the mutant HDAC8 3'UTR, indicating a direct interaction. Conversely, miR-26b-3p didn't demonstrate significant changes in luciferase activity, suggesting no direct interaction with HDAC8 under these experimental conditions.

Furthermore, miR-497-3p overexpression in HeLa cells resulted in a significant mRNA and protein decrease of HDAC8. We also observed that HDAC8 hindrance by miR-497-3p resulted in the improved acetylation of proteins targeted by HDAC8, such as p53, and alpha-tubulin.

The dual luciferase reporter assay validated the straight binding between miR-324-3p and HDAC6. Overexpressing miR-324-3p in K562 cells, characterized by high HDAC6 and low miR-324-3p levels, solidified the interaction. This led to a substantial reduction in mRNA and protein of HDAC6, elevating acetylation levels of Hsp90 and α-tubulin, both HDAC6's client proteins.

Additionally, we checked for miR-497-3p mediated regulation of HDAC8 in metastatic breast cancer, MDAMB231 cells. With qRT-PCR we found miRNA has lesser expression compared to expression of HDAC8 mRNA. Also, with miRNA overexpression, we could find a similar result to that in HeLa cells.

In HeLa and MDAMB231 cells, overexpressing miR-497-3p reduced cell growth, prompted tubulin bundle formation, and introverted cell migration, demonstrating the role of miR-497-3p in regulating HDAC8. The observed tubulin stabilization following miR-497-3p transfection corroborates HDAC8 suppression. Furthermore, miR-497-3p impacted Epithelial-Mesenchymal Transition (EMT), decreasing the Vimentin protein expression, a mesenchymal

marker, and increasing E-cadherin, an epithelial marker, highlighting its involvement in modulating cell phenotypes.

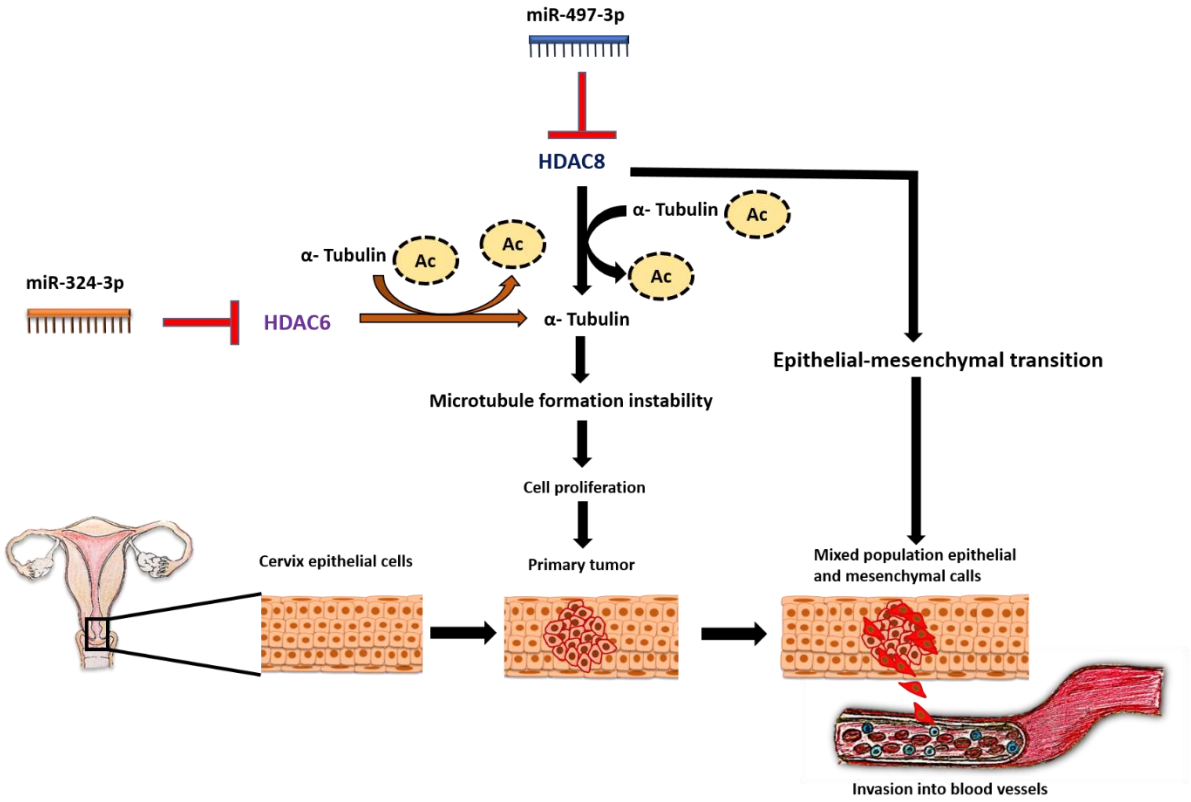


Figure 64: Schematic representation of overall summary of the study.

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Original Research Article

MicroRNA-mediated epigenetic regulation of HDAC8 and HDAC6: Functional significance in cervical cancer

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ABSTRACT

Cervical cancer, a leading global cause of female mortality, exhibits diverse molecular aberrations influencing gene expression and signaling pathways. Epigenetic factors, including histone deacetylases (HDACs) such as HDAC8 and HDAC6, along with microRNAs (miRNAs), play pivotal roles in cervical cancer progression. Recent investigations have unveiled miRNAs as potential regulators of HDACs, offering a promising therapeutic avenue. This study employed in-silico miRNA prediction, qRT-PCR co-expression studies, and Dual-Luciferase reporter assays to identify miRNAs governing HDAC8 and HDAC6 in HeLa, cervical cancer cells. Results pinpointed miR-497–3p and miR-324–3p as novel negative regulators of HDAC8 and HDAC6, respectively. Functional assays demonstrated that miR-497–3p overexpression in HeLa cells suppressed HDAC8, leading to increased acetylation of downstream targets p53 and α-tubulin. Similarly, miR-324–3p overexpression inhibited HDAC6 mRNA and protein expression, enhancing acetylation of Hsp90 and α-tubulin. Notably, inhibiting HDAC8 via miRNA overexpression correlated with reduced cell viability, diminished epithelial-to-mesenchymal transition (EMT), and increased microtubule bundle formation in HeLa cells. In conclusion, miR-497–3p and miR-324–3p emerge as novel negative regulators of HDAC8 and HDAC6, respectively, with potential therapeutic implications. Elevated expression of these miRNAs in cervical cancer cells holds promise for inhibiting metastasis, offering a targeted approach for intervention in cervical malignancy.

1. Introduction

Cervical cancer poses a significant health burden, ranking as the fourth most prevalent malignancy in developing nations, particularly in countries like India. Its incidence and mortality rates among women are escalating, and contributed to 7.5% of all cancer cases in 2020, as reported by the International Agency for Research on Cancer (IARC). Despite being preventable and treatable through effective screening and vaccination, the disease remains a formidable challenge in economically disadvantaged regions, necessitating urgent preventive interventions [1]. The complexity of cervical cancer treatment is compounded by the disease's stage-dependent therapeutic strategies, with late-stage presentations posing formidable health risks and challenges due to widespread metastasis, often culminating in fatal outcomes.

Microtubules, composed of tubulin polymers, represent ubiquitous cytoskeletal structures crucial for regulating diverse cellular functions, including growth, division, motility, intracellular transportation, organelle placement, and signal transduction [2–5]. In the context of cancer, the dynamic instability of microtubules is intricately linked to processes such as cell proliferation and invasion, positioning them as potential targets for cancer therapeutics [6,7]. Notably, the anticancer agent Paclitaxel functions as a cytoskeletal inhibitor by disrupting tubulin polymerization.

Epigenetic regulation of tubulin through acetylation and deacetylation is a pivotal cellular mechanism influencing microtubule stability and, consequently, cancer dissemination. Histone deacetylases (HDACs), key epigenetic histone modifiers, control alpha-tubulin by reversing lysine acetylation, inducing dynamic microtubule instability. While HDAC6, a class IIb HDAC, has been recognized as a significant regulator of alpha-tubulin [8–12], our research has identified HDAC8 of class I as an additional contributor to alpha-tubulin deacetylation in cervical cancer [13].

RNA interference (RNAi), another epigenetic regulator, plays a crucial role in gene regulation, including the suppression or silencing of specific genes or their messenger RNA (mRNA) products [14,15]. MicroRNAs (miRNAs), a subset of non-coding RNAs, emerge as key players in fine-tuning gene expression and responding to physiological and environmental signals. MiRNAs, by targeting the 3′ untranslated

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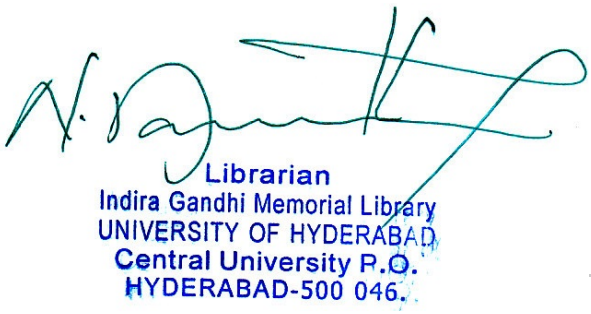

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