Site directed mutagenesis of miRNA binding site on the 3′-UTR of choline kinase alpha gene

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By

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LIST OF ABBREVIATIONS, ACRONYMS

AMP	Ampicillin
APH (3′)-IIIa	3′, 5″-aminoglycoside phosphotransferase type IIIa
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCa	Breast cancer
bp	Base pair
CDP-choline	Cytidine 5'-diphosphocholine
ChK	Choline kinase
chka	Choline kinase alpha (mRNA or gene)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
ER	Estrogen receptor
FA	Fatty acids
g	Gram
HCC	Hepatocellular carcinoma
HCl	Hydrochloride acid
IPS	Induced pluripotent stem
Kb	Kilo base pair
LB	Luria Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate

$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
miRNAs	MicroRNAs
mRNA	Messenger ribonucleic acid
ncRNAs	Non-coding RNAs
PCho	Phosphocholine
VEGF	Vascular endothelial growth factor

LIST OF SYMBOLS

%	Percentage
~	Approximately
×	Multiply by
°C	Degree Celsius
A260	Absorbance at 260 nm
kcal/mol	Kilocalorie per mole
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
nm	Nanometer
nM	Nanomolar
nt	nucleotides
µg	Microgram
µl	Microliter
µM	Micromolar

Mutagenesis Terarah Tapak untuk Tapak Pengikat MiRNA pada 3'-UTR Gen Kolina Kinase Alfa

ABSTRAK

MicroRNA sebahagian besarnya mengawal ekspresi gen dengan melekat pada messenger RNA (mRNA) dalam sitoplasma sel. Daripada diterjemahkan segera kepada protein, mRNA yang disasarkan sama ada akan dimusnahkan dan komponennya dikitar semula, atau ia akan disimpan dan diterjemahkan kemudian. Ekspresi berlebihan Chka adalah tanda klinikal tisu berpenyakit dan sel malignan. MikroRNAs (miRNAs) adalah pengawal selia gen pascatranskrip yang berkesan. Kajian oleh pasukan kami menunjukkan bahawa ketiga-tiga miRNA ini (miR-876-5p, miR-367-3p dan miR-32-5p) mengecilkan ekspresi gen *chka*. Walau bagaimanapun, tapak pengikatan miRNA ini pada 3'-UTR gen *chka* belum disahkan. Kajian ini bertujuan untuk mengubah tapak pengikatan miRNA ini untuk pengesahan seterusnya oleh ujian luciferase. Dalam kajian ini, kami melakukan mutagenesis terarah tapak PCR bagi miR-367-3p (GAAGCAGAAAT ATAGTGCAATA) daripada tapak pengikatan nukleotida (nt) 1817-1825 dalam *chka* dan miR-876-5p (GAG TGTAGCTGTG AAATCCA) daripada tapak pengikatan nukleotida (nt) 2573-2581 tapak pengikat dan mengesahkan mutasi dengan penjujukan DNA. Kerja in vitro, selepas langkah mutagenesis, produk PCR dihantar untuk penjujukan, namun hasilnya tidak memuaskan.

Site Directed Mutagenesis of MiRNA Binding Site on the 3′-UTR of Choline Kinase Alpha Gene

ABSTRACT

MicroRNA largely controls gene expression by attaching to messenger RNA (mRNA) in the cell cytoplasm. Instead of being promptly translated into a protein, the targeted mRNA will either be destroyed and its components recycled, or it will be retained and translated later. Choline kinase alpha (*chka*) overexpression is a clinical sign of diseased tissues and malignant cells. MicroRNAs (miRNAs) are effective posttranscriptional regulators of gene. Studies by our team showed that these three miRNAs (miR-876-5p, miR-367-3p and miR-32-5p) downregulated the expression of *chka* gene. However, the binding sites of these miRNAs on the 3'-UTR of the *chka* gene have not been verified. This study aimed to mutate the binding sites of these miRNAs for subsequent verification by a luciferase assay. In this study, we performed PCR site-directed mutagenesis on the miR-367-3p (GAAGCAGAAAT ATAGTGCAATA) from nucleotides (nt) 1817-1825 binding sites in *chka* and miR-876-5p (GAG TGTAGCTGTG AAATCCA) binding site from nucleotides (nt) 2573-2581 binding sites and verified the mutation by DNA sequencing. In *vitro* work, after mutagenesis step, the PCR products were sent for sequencing, however the results were not satisfactory.

Chapter 1

1.1 Introduction

Choline kinases phosphorylate choline to generate phosphocholine, which is the first enzymatic step in phosphatidylcholine (PtdCho) production. Choline Kinase (CK) is a genuinely regular stimulus that phosphorylates extracellular choline metabolites to produce phosphorylcholine (PC), addressing the underlying increase of the Kennedy pathway, which bio-produces phosphatidylcholine (PtdCho), a factor important for the structure of eukaryotic cell membranes (ECM) (McMaster, 2018). The most common kind of phospholipid in eukaryotic cells is PtdCho. It is the main structural component of the membrane bilayer, along with other phospholipids, including phosphatidylethanolamine and neutral lipids. PtdCho has been found as a large source of second messenger molecules, in addition to contributing 40–60 percent of the phospholipid makeup of eukaryotic cell membranes (ECM) (Casares et al., 2019; Malito et al., 2006). The endoplasmic reticulum (ER), which is made up of around 60% PtdCho, is the major location of PtdCho biosynthesis (Jacquemyn et al., 2017). In addition to affecting protein trafficking to the Golgi, removing PtdCho from the ER membrane by choline depletion or inhibition of CDP-choline pathway enzymes causes an ER stress response. The outer leaflet of the plasma membrane contains 15 to 40% PtdCho, depending on the cell type. PtdCho is also used as a starting point for the production of other glycerophospholipids (Sanchez et al., 2013).

Highly multiplying cancer cells must continuously produce glycerophospholipids, specifically for membrane formation, by synthesising fatty acids from scratch (Dolce et

al., 2011), whereas cancer is one of the biggest causes of mortality in the world today. The costs in terms of social, economic, and public health are significant. In recent decades, the number of people diagnosed with cancer has risen. According to the Global Cancer Observatory yearly data, there are over 19.3 million cancer diagnoses in 2020, and around 10 million deaths from cancer globally (Sung et al., 2021). In most malignancies, there is an increased of choline kinase alpha (ChKa) expression and activation that leads to a shift in choline metabolism and a rise in phosphocholine (PCho) levels. Overexpression of ChKa is observed in a number of malignancies, including breast, lung, colorectal, and prostate cancers (Faten et al., 2021).

The destructive consequences of higher ChKa levels on cell growth, proliferation, cancer initiation, and progression validated the enzymes' carcinogenic character, establishing ChKa overexpression as a cancer prognostic marker and a sign of tumour response to anti-cancer treatment (Stoica et al., 2022). ChK inhibitors have been used as prospective anticancer drugs after years of research into the expressions of ChK in various cancer cells (Khalifa et al., 2020). The regulation of *chka* gene expression, particularly through epigenetic mechanisms, has received little attention (Faten et al., 2021). ChK elevation was shown to have a causative role in carcinogenesis and tumor growth (Arlauckas et al., 2016; Lin et al., 2017), while ChKa was found to be considerably higher in glioma tissues. ChKa may increase glioma formation by activating the PI3K/AKT signaling pathway, indicating that it might be used as a diagnostic and treatment method for glioma prognosis. As a result, it's been identified as a prospective cancer therapeutic target (Arlauckas et al., 2016; Rubio et al., 2021).

ChKa has been identified as a possible therapeutic target for human malignancies. This enzyme has sparked an interest in the development of pharmacological inhibitors. Interest in creating pharmacological inhibitors of this enzyme has increased after ChKa was identified as a possible therapeutic target for human malignancies. The prototype ChKa inhibitor hemicholinium-3 (HC-3) has undergone molecular changes, computer-based drug design, and natural product screening in the development of a range of ChKa inhibitors (Gokhale et al., 2021).

However, there has been very little study on the regulation of *chka* gene expression by microRNAs (miRNAs/miRs). miRNA-mediated deregulation of gene expression is a common cause of cancer (Raikundalla et al., 2021). miRNAs have long been known to suppress gene expression at the level of mRNA stability by causing mRNA degradation and at the level of translation (at initiation and after initiation) by blocking protein translation or degrading polypeptides by binding complementary to the 3′-UTR of genes (Orang et al., 2014; Sebastian et al., 2021). miRNA regulates gene expression primarily by binding to messenger RNA (mRNA) in the cytoplasm of the cell. The designated mRNA will either be destroyed and its components recycled, or it will be kept and translated later, rather than being translated quickly into a protein (Das et al., 2017). miRNA-mediated gene expression dysregulation is a prevalent cause of cancer.

miRNAs regulate gene expression by binding to the 3′-untranslated regions (UTRs) of target mRNAs and inhibiting their translation. This is the most researched function of miRNAs. On the other hand, miRNAs have recently been discovered to interact with various targets, including gene promoters, coding regions, and 5′-UTRs (Broughton et

al., 2016; Semina et al., 2021). According to an increasing body of evidence, miRNAs may move between distinct cell compartments, where they influence numerous activities like transcription, translation, alternative splicing, and DNA repair. Furthermore, miRNAs are released into the extracellular space and may be used as molecular markers for oncologic illnesses, in which they may play a major part in the development (Ratti et al., 2020). miRNAs have been proposed to operate as tumour suppressors as well as oncogenes that promote carcinogenesis (Fasoulakis et al., 2019).

1.2 Rationale of the Study

Previous studies showed that there are three miRNAs (miR-876-5p, miR-367-3p and miR-32-5p) downregulated the expression of *chka* gene (Raikundalia et al., 2021; Khan et al., 2018). However, the binding sites of these miRNAs on the 3'-UTR of the *chka* gene have not been verified *in vitro*. Hence, this study aimed to mutate the binding sites of these miRNAs which have been predicted *in silico,* for subsequent verification by the luciferase assay.

1.3 Objectives

General Objective

To mutate the binding sites of miR-367-3p and miR-876-5p on the 3′ untranslated regions (3′-UTR) of the choline kinase alpha (*chka*) gene and to verify the DNA sequence after the mutation reactions.

Specific Objectives

1. To predict the miRNAs and their binding sites on 3'-UTR of *chka* by bioinformatics tools.
2. To perform PCR site directed mutagenesis of the miR-367-3p and miR-876-5p binding sites on the 3'-UTR of *chka*.
3. To verify the mutations by DNA sequencing.

Chapter 2.0: Literature Review

2.1 Phospholipid synthesis in human and choline kinase (reaction, genes, and isoforms)

Phospholipids are widely distributed throughout the body, including the brain, nervous system, and bodily fluids like plasma. However, they are principally found in the membranes of different organelles and cells, such as mitochondria, epithelial cells, and accumulated and active platelet (Atsumi et al., 2004; Mandik & Vos, 2021). The synthesis of phospholipids is a convoluted process with several branching points. Phosphatidate (PA) is a crucial chemical in this process (Han & Carman, 2013). Diacylglycerol (DAG) or phosphatidate, which may either be an intermediary of the Kennedy route of triacylglycerol (TAG) synthesis or the result of TAG lipolysis, are the building blocks of phospholipid synthesis (Wang, 2020). To enable the production of membranes for daughter cells and organelles, phospholipid synthesis must increase in cells that proliferate quickly. Additionally, phospholipids act as stores for fatty acids (FA), which are converted into precursors for creating eicosanoids (Hanna & Hafez, 2018).

The varied spectrum of lipids produced by the intricate phospholipid synthesis impacts how membrane protein activity is carried out. Although this composition varies between tissues and subcellular organelles, the most prominent phospholipids in mammalian cells are phosphatidylcholine (PtdCho) (45–55%), followed by phosphatidylethanolamine (PE) (15–25%), phosphatidylinositol (PI) (10–15%), phosphatidylserine (PS) (5–10%), and sphingomyelin (SM) (5–10%). (456). Direct PtdCho and PE synthesis from DAG is possible, as is the conversion of PE to PtdCho (Hewgill, 2020). PS and SM are preceded

by PtdCho and PE. Cytidine-5'-diphosphate (CDP)-DAG, which must first be produced from phosphatidate through the Kennedy pathway, is the starting material for the direct synthesis of PI and PS (Blunsom & Cockcroft, 2020). Even though some of the phospholipid production enzymes are cytoplasmic, for them to be active, they need to relocate to the ER membrane (Romanauska & Köhler, 2018). A further indication that phospholipid synthesis, TAG synthesis, and -oxidation may be closely related in response to the energy and proliferative requirements of the cell is the fact that these enzymes are primarily located in the mitochondrial-associated membrane (MAM), where many enzymes involved in TAG.

Saccharomyces cerevisiae has been utilised as a model system to investigate phospholipid production and its control in eukaryotes, according to the paper from which (Figure 2.1) is drawn (Han & Carman 2013). Studies on molecular, genetic, and biochemical levels have shown the coordinated regulation of phospholipid production.

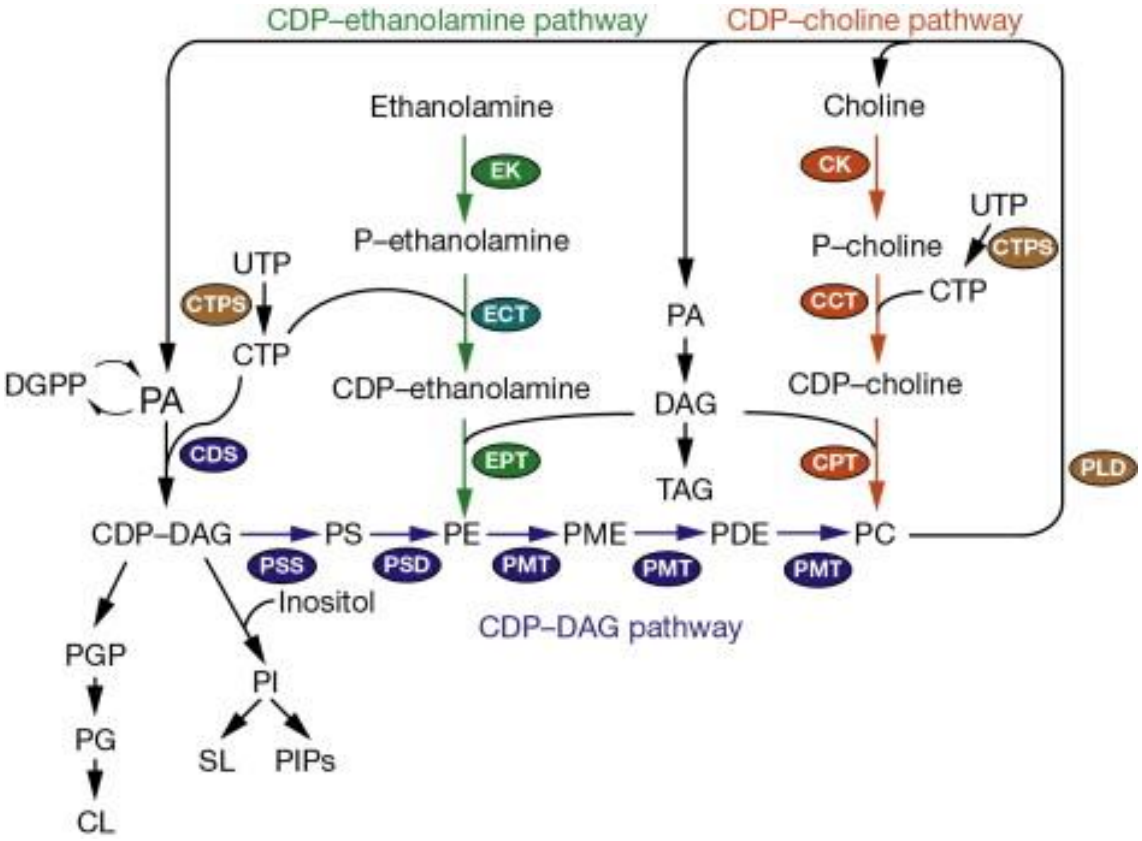

Figure 2. 1: The main *S. cerevisiae* pathways for the production of phospholipids. Relevant stages were included in the pathways for the synthesis of phospholipids. Three CDP routes are highlighted: CDP-DAG, CDP-choline, and CDP-ethanolamine. The CDP-DAG route produces PC via a series of events that are catalyzed by the enzymes CDP-DAG synthase (CDS), PE methyltransferases (PMT), and PS decarboxylase (PSD), and the PS synthase (PSS), (shown in blue). The CDP-choline route is the mechanism through which PC is formed, and it is catalyzed by the enzymes choline kinase (CK), choline phosphotransferase (CPT), and choline-P cytidylyltransferase (CCT) (shown in red). The CDP-ethanolamine route is used to produce PE, and the enzymes ethanolamine kinase (ET), ethanolamine phosphotransferase (EPT), and ethanolamine-P cytidylyltransferase (ECT) catalyze these reactions (shown in green). Brown represents the catalyzed processes by PLD and CTP synthetase (CTPS). PDE stands for phosphatidyl dimethylethanolamine. CDP-DAG stands for CDP-diacylglycerol. PS stands for phosphatidylserine. PE stands for phosphatidylethanolamine. Phosphatidylcholine (PC), diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylglycerol phosphate (PGP), phosphatidylglycerol (PG), cardiolipin (CL), sphingolipids (SL), polyphosphoinositides (PIPs), and diacylglycerol pyrophosphate (DGPP), the figure was obtained from (Han & Carman 2013).

Phosphatidylcholine makes up over 50% of the mass of the phospholipids in mammalian cells, most of which are phospholipids. The enzyme choline kinase catalyzes the first enzymatic step in the synthesis of PC by changing choline into phosphocholine (Ishidate 1997; Johnston et al., 2020). Two human genes (*chka* and *chkb*) produce three distinct choline kinase isoforms: ChKa1, ChKa2, and ChKb, and (Gruber et al., 2012). The monomeric choline kinase proteins are combined and interacted to produce homo-or hetero-dimeric active forms (Lacal et al., 2020). Although ChKa and ChKb proteins share many structural similarities and have similar enzymatic activities, they have different molecular structure domains and ways of expressing themselves in other tissues.

2.2 Choline kinase and cancer pathogenesis

Choline kinase (ChK), which is involved in the metabolism of phospholipids, has been implicated in controlling cell proliferation, oncogenic transformation, and human carcinogenesis. ChK has been identified as a human cancer prognostic factor and a therapeutic target with promise since its particular inhibitors have anticancer activity *in vitro* (de et al., 2008).

Many human tumours overexpress ChK, including breast, ovary (Ramírez et al., 2002), lung, bladder, colorectal (Hernando et al., 2009), prostate (Nanni et al., 2020), endometrial, liver (Lin et al., 2016), pancreas, oesophagus and T-cell lymphoma (Xiong et al., 2015). ChKa overexpression has also been observed in cancer cells derived from osteosarcoma tumors (Li et al., 2014), Hepatitis B virus (HBV)-induced hepatocytic (Gobeil et al., 2020), breast, colon, and liver cancer cells (Kumar et al., 2015), glioblastoma and glioma-derived cell lines (Yue et al., 2020), pancreatic tumour-derived

noma lines, and T-cell acute lymphoblastic leukaemia (ALL). It has been linked to estrogen receptor (ER) status, enhanced invasiveness, and treatment resistance in breast cancer (Shah et al., 2010; Kim et al., 2015).

ChKa has been suggested as a new therapeutic target for cancer because of its well-established function in the initiation and development of human malignancies (Lacal et al., 2021). A program for designing and producing particular inhibitors was devised. The proof of concept was also supported by siRNA attenuation of Chka synthesis (Glunde et al., 2005). As a result, small compounds and particular siRNAs with potent anticancer activity have been created, both *in vitro* and in experimental animal models.

ChKa inhibition has been used in combinatorial regimes and demonstrated a potent antitumor effect that synergizes with 5-FU in breast and colon cancer cells (Mariotto et al., 2018) and reverses resistance to TRAIL in colorectal and ovarian cancer cells (Rizzo et al., 2021), supporting the significance of ChK as a target for cancer therapy. Similar synergistic effects have been observed with lung cancer cells treated with acid ceramidase inhibitors or cisplatin and pancreatic ductal adenocarcinoma-derived cancer cells treated with gemcitabine, 5-FU, or oxaliplatin (Lacal et al., 2021; Mazarico et al., 2016).

Cancer cells must synthesize PtdCho to grow and increase. As a result, ChK inhibition has been demonstrated to have therapeutic benefits in a range of tumor-derived cancer cell lines and tumor xenografts (Arlauckas et al., 2017). Human tumour tissues have enhanced ChK activity, which results in higher amounts of phosphocholine, the product of ChK (Gallego et al., 2009; Ramírez et al., 2002). Additionally, ChK overexpression makes

breast cancer cells more aggressive and resistant to treatment (Tan & Le, 2021; Shah et al., 2010). According to Rodrguez et al., 2004, ChK inhibitors are effective anticancer agents both *in vitro* and *in vivo*, and it helps increase the efficiency of chemotherapy (Rodríguez et al., 2004; Lacal et al., 2022). Apoptosis is triggered, and cancer cells are eliminated when ChK expression is specifically inhibited (Bañez et al., 2008; MacKeigan et al., 2005; Ayub et al., 2018), while normal cells are not affected.

Choline kinase was implicated in the development of human cancer not only because it was expressed at greater levels but also because cancer cells showed it to be more active (Ramírez et al., 2002). The less detectable choline kinase isoform or, the more active ChKa2 alone may also be responsible for the more excellent choline kinase activity in cancer cells (Nakagami et al., 1999). The post-translational activation, like phosphorylation or positive control by interacting partners, may also cause the increased choline kinase activity (Chang et al., 2016).

The accumulating data suggest that greater attention should be paid to the identification of ChKa isoform in light of the accumulating data that mainly links ChKa to the etiology and prognosis of many malignancies (Bañez et al., 2008; de et al., 2008; Gallego et al., 2009; Glunde et al., 2005; See Too et al., 2010; Gokhale et al., 2021). Pattern direct ChKa may be used to monitor expression at different stages of cancer development and afterward to monitor the tumor ‘response to treatment by using direct quantitative comparison in other cells and tissues. The ChKa and PCho may be utilized as tumour markers, according to these results, which firmly establish the involvement of ChKa in the etiology human cancer (Rodríguez et al., 2003; de et al., 2007; Gokhale et al., 2021).

2.3 Choline kinase inhibition as a potential anticancer strategy

A few studies have proposed ChK inhibition as a potential anticancer strategy (Cuadrado et al., 1993; Kiss 1999; Ramírez et al., 2002; See Too et al., 2010). More recent studies on the biological function of ChK isozymes have shown that ChK may play a more critical role in cancer development because only ChK was upregulated in breast cancer cell lines and because selective depletion of the ChK isoform by shRNA selectively induced apoptosis across several tumour-derived cell lines without affecting the survivability of normal primary cells (Bañez et al., 2008; Lacal et al., 2015). Furthermore, it has been proposed that the ChKa isoform may serve as a viable prognostic marker for assessing the therapeutic prognosis in non-small-cell lung cancer patients (Lacal et al., 2021).

Because of the correlation between increased lipid metabolism and mitotic activation, many inhibitors of PtdCho metabolism have been developed for related enzymes, including CT (CTP: choline phosphate CT), phospholipase D (PLD), ChK, and diacylglycerol kinase (DGK). Many inhibitors for these enzymes have been created as a result, including PLD inhibitors (suramin, xanthogenate derivatives, amino steroids, miltefosine), CT inhibitors (CT2584), ChK inhibitors, and DGK inhibitors (R59949) (Ramirez De Molina et al., 2001; Finney et al., 2000; Hernandez-Alcoceba et al., 1997, 1999; Lacal, 2001). Clinical investigations have been conducted with several of these inhibitors (Finney et al., 2000; Ramirez De Molina et al., 2001).

As innovative anticancer approaches for ChKa inhibition, a variety of small-molecule ChKa inhibitors have been created (Rubio et al., 2021). The first ChKa inhibitor developed around a bis-oxazonium pharmacophore was hemicholinium-3 (HC-3), but it had unintended effects on choline transporters, acetyltransferase, and acetylcholinesterase (Wang et al., 2021). Later, MN58B was created by structurally altering HC-3 and exhibited excellent *in vitro* and *in vivo* antiproliferative potencies and effectiveness in treating a variety of malignancies, including glioblastoma, carcinoma models and colon cancer xenografts (Campos et al., 2003; Al-Saffar et al., 2006; Kall et al., 2018). MN58B provided a framework for the development of new anticancer treatments and allowed for a greater comprehension of the mechanism of action of this novel class of antitumor medications. This inhibitor has been proven effective, against cancer cells when quinolinium replaced pyridiniums in MN58B as cationic head groups. RSM-932A is proven both *in vivo* and *in vitro* (Lacal et al., 2015, Sánchez et al., 2005, Rubio et al., 2021). However, only MN58b entered and occupied the region where an AMP molecule was used to simulate the structure of sChK with ATP, and RSM-932A appears to have an entirely novel mechanism of inhibition and is synergistic with both choline and ATP. Both MN58b and RSM-932A bound to a region that overlapped with the choline binding site (Figure 2.2) (Zimmerman et al., 2013 and 2020).

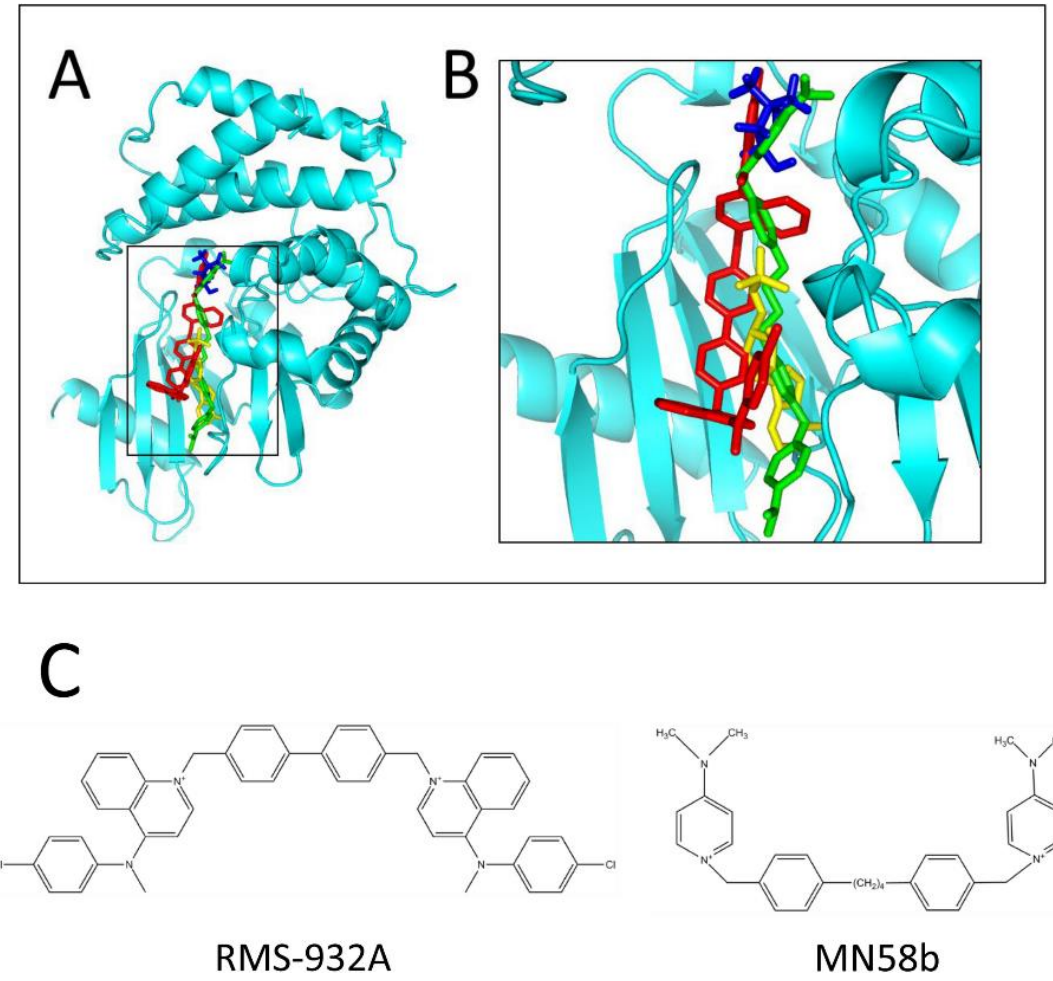

Figure 2. 2: (A) The crystal structure of apo-sChk with MN58b (green) and RSM-932A (red) docked to it, as well as the locations of AMP (yellow) and natural substrate choline (blue); (B) A close-up of the binding sites; (C) 3D structures of RSM-932A and MN58b (Zimmerman et al. 2020).

However, although ChKa inhibitor therapy is used in unresectable stage III cancer, it is associated with several side effects, including nausea, vomiting, bone marrow suppression, as well as to-, neuro-, and nephrotoxicity, which accounts for a significant decline in the quality of life (Thandra et al., 2021; Griesinger et al., 2019).

Choline kinase inhibition prevents the transmission of two different signaling pathways that are necessary for cancer cells to survive. There is growing evidence that the PI3K/AKT and MAPK pathways work together to support cell survival. The MAPK and PI3K/AKT pathways are often dysregulated in cancer. The expression of choline kinase is necessary for anchorage-independent survival *in vitro*. Multiple forms of cancer are less likely to spread and survive when the MAPK and PI3K/AKT pathways are both inhibited (McCubrey et al., 2007; Yalcin et al., 2010; Wang et al., 2021).

ChK function has been disrupted using specific molecular inhibitors and small interfering RNA (siRNA). Anticancer medicines in these reagents have been proven efficient and selective (Lacal et al., 2001; Sharifiaghdam et al., 2021). Numerous studies have examined the effects of preventing ChKa function. It could explain how *chka* silencing with particular siRNA specific inhibitors is work. The unfold protein response (UPR), ER stress, and ROS homeostasis are only a few of the impacts brought on by ChK inhibition by pharmacological or siRNA methods, as well as possible match loss and cytosolic release (Rizzo et al., 2021), the UPR, ER stress (Sanchez et al., 2013), and glutathione levels, there have been reports of comparable effects on ER stress. Similar like trial on targeted inhibition with *chka* siRNA induces apoptosis in cancer cells (Mori et al., 2007; Mariotto et al., 2018). In breast and T-cell lymphoma cancer cells, ChK is either

pharmacologically inhibited or silenced by siRNA to lower ERK and AKT phosphorylation (Clem et al., 2011; Wang et al., 2020).

Little attention is being paid to the control of *chk* transcription by cytoplasmic modulators, such as miRNAs, and the bulk of efforts have been focused on inhibiting the action of ChK by utilizing small molecule inhibitors or RNA interference (RNAi). Therefore, it would be beneficial to look for miRNAs that target *chk* to learn about their potential utility in treating certain cancers. miRNA transcription, processing by Drosha and Dicer, and other stages of the miRNA biogenesis process are all tightly regulated as mentioned in the Figure 2. 3 (Peng & Croce, 2016).

2.4 MicroRNA (biology and mechanism of action)

Non-coding RNAs, known as microRNAs (miRNAs,) have significant functions in controlling the expression of genes. Most miRNAs are produced via transcription of DNA sequences into primary miRNAs with a length of 22 nucleotides on average, precursor miRNAs, and then mature miRNAs.

A multiprotein complex called polymerase II converts DNA into messenger RNA (mRNA), the majority of short nuclear RNA (snRNA), and microRNA precursor (Kornberg, 1999). Animals' canonical microRNA primary transcripts (pri-miRNAs) are broken down by the microprocessor complex, consisting of Drosha and DGCR8 (commonly known as Pasha). This cleavage marks the beginning of the maturation route for microRNAs (miRNAs) (Guo, 2012). Dicer converts miRNA precursors into useful

functional miRNAs, which are then integrated into the RNA-induced silencing complex (RISC) (Figure 2.3) (Luo et al., 2013).

However, several studies show that certain miRNA families mature through non-canonical pathways. It's significant because several studies identified miRNAs dubbed mirtrons in the brief intron sequence. The splicing apparatus initially processes the mirtrons without the aid of Drosha to produce miRNA with a lariat structure. The lariat-debranching enzyme subsequently converts the introns into pre-miRNAs, which continue the transition process along nicely route (Okamura et al., 2007; Ruby et al., 2007). Additionally, it has been noted that in one instance (miR-451) AGO2 was used in place of DICER's cleave step (Cheloufi et al., 2010; Cifuentes et al., 2010).

miRNAs cause mRNA degradation and repression by interacting with the 3′ untranslated region (3′-UTR) of target mRNAs (Figure 2.4). However, it has also been shown that miRNAs connect with other areas, including the 5′-UTR, coding sequences, and gene promoters (O'Brien et al., 2018).

Both siRNAs and miRNAs may bind to AGO proteins, which then regulate the suppression of certain target RNAs by either RNA degradation or translation inhibition (Höck & Meister, 2008).

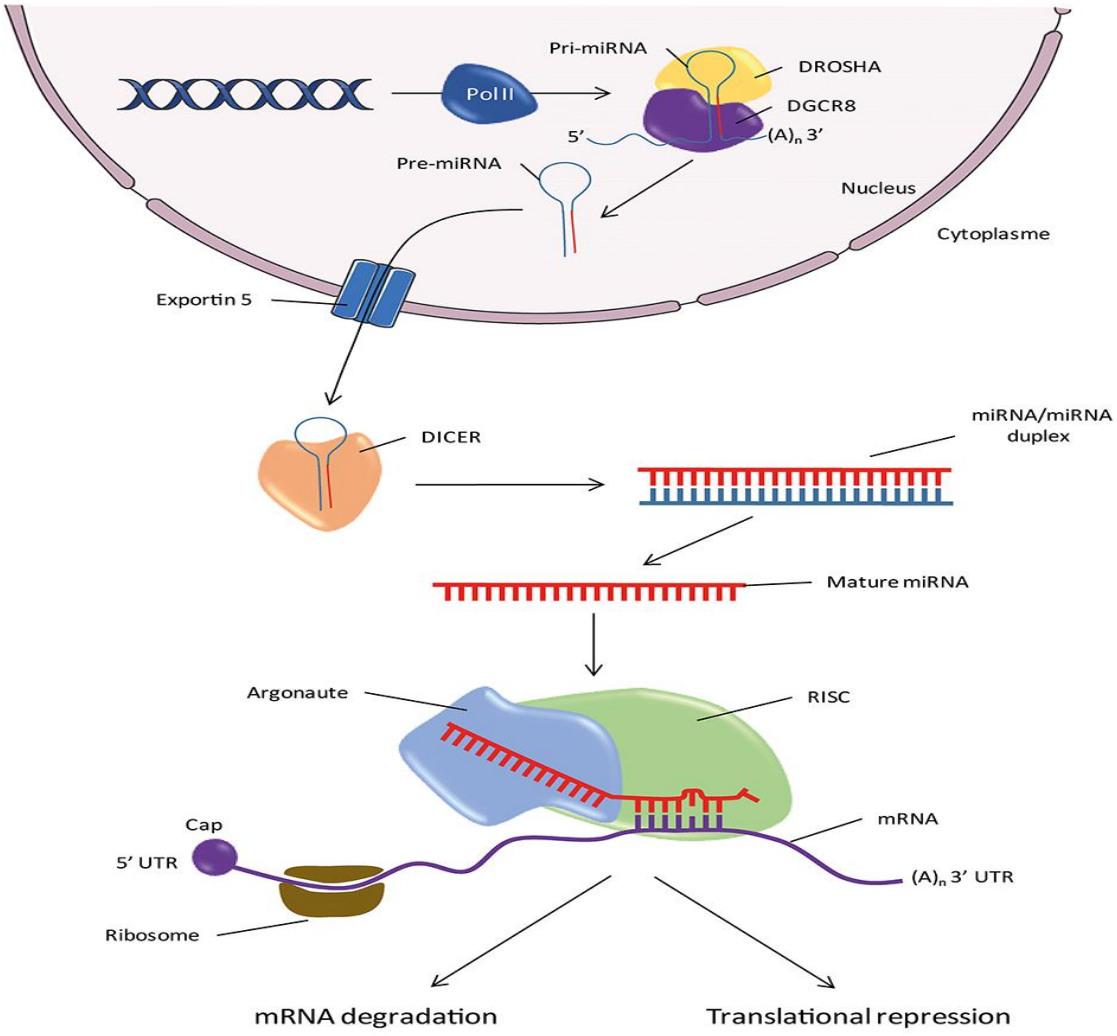

Figure 2. 3: Biogenesis and mechanism of action of microRNAs. Pri-miR is for primary microRNA, pre-miR for precursor microRNA, RISC stands for RNA-Induced Attempting to silence Complex, 5′ or 3′-UTR stands for 5′ or 3′-utr region, DGCR8 stands for DiGeorge Syndrome Critical Region 8, and (A)n stands for posttranslational modifications (Derghal et al. 2016).

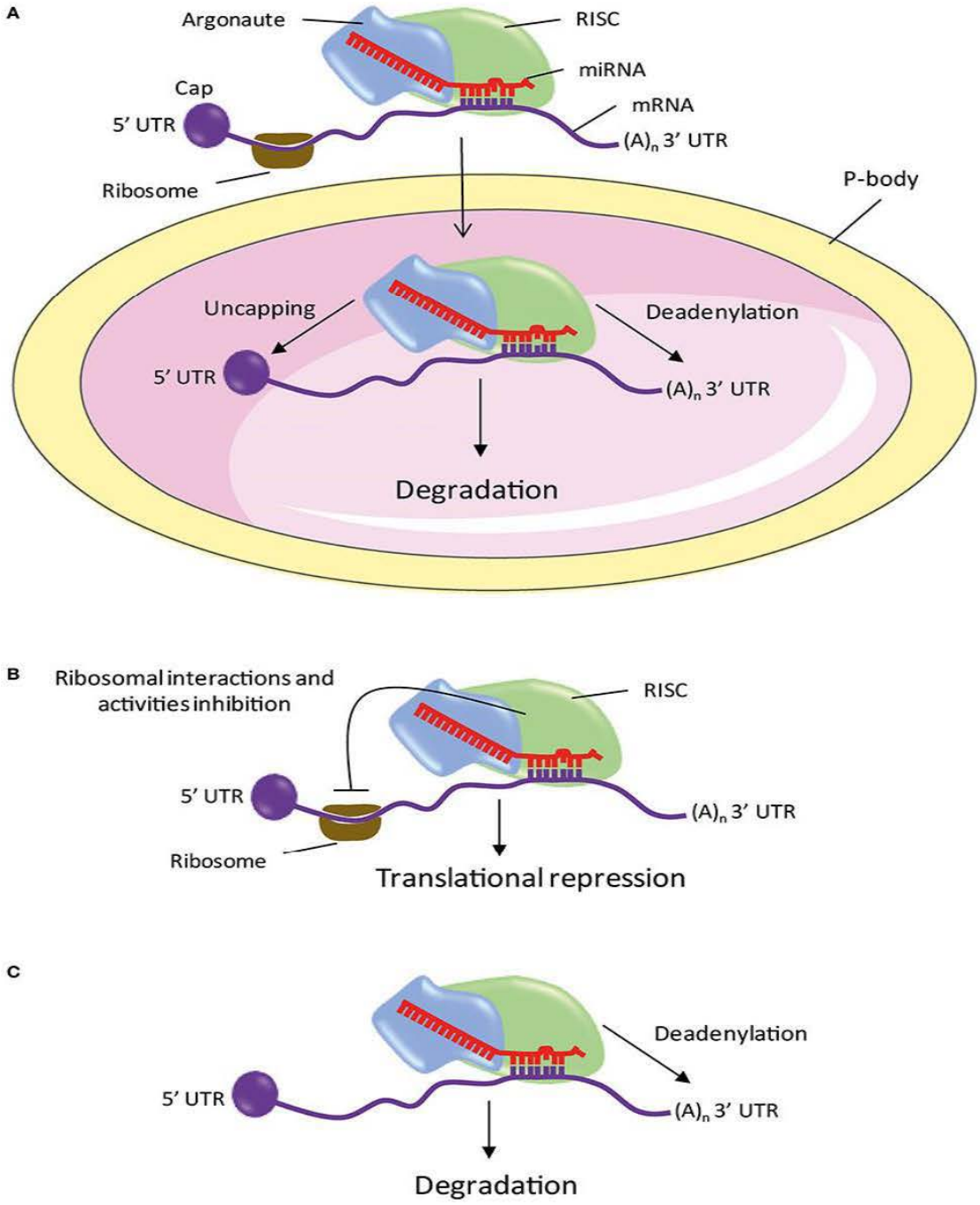

Figure 2. 4: The various mechanisms by which miRNA repress or degrade mRNA. Shows the several methods by which miRNAs inhibit or destroy mRNA. (A) The bodies that process data. (B) Intervention in the post-initiation stages of transcription initiation and repression. Deadenylation, letter C. P-bodies are processor bodies; RISC stands for RNA-Induced Silencing Complex; 5′ or 3′-UTR stands for 5′ or 3′-UTR region; and (A)n stands for polyadenylation (Derghal et al. 2016).

The field of molecular genetics has undergone a revolution since the Ambros & Ruvkun teams discovered the first miRNA, lin-4, in *Caenorhabditis* worms in 1993 (Lee et al., 1993; Wightman et al., 1993). Years ago, the Horvitz group identified lin-4 as one of the genes that control the temporal development of *C. elegans* larvae (Horvitz et al., 1980; Chalfie et al., 1981). Later in 1987, the same group discovered that a lin-4 suppressor mutation in a null-lin-4 line was wildtype while having the opposite phenotype to a lin-14 suppression mutant in a different gene (Ferguson et al., 1987). Lin-4 and lin-14 involvement in gene regulation was then established (Lee, 2004). Since that time, miRNAs have been discovered in many types of animals and plants (Li et al., 2010). More miRNAs are continually being identified (De et al., 2017) and their functions in regulating gene activity are widely known.

2.5 miRNAs and cancers

Chromosome abnormalities, alterations in transcriptional regulation, epigenetic modifications, and deficiencies in the miRNA biogenesis machinery are some of the underlying causes of cancer. One of them is miRNA gene amplification or deletion (Peng & Croce, 2016). Alterations in genomic miRNA copy numbers and gene locations are often implicated in abnormal miRNA production in malignant cells compared to normal ones (amplification, omission, or translocation). The first evidence of a miRNA gene placement alteration was the deletion of the miR-15a/16-1cluster gene at chromosome 13q14, which is typically seen in people with B-cell chronic lymphocytic leukemia (Calin et al., 2002). The miR-143 and miR-145-containing 5q33 region is often deleted in lung cancer, which lowers the expression of both miRNAs (Calin & Croce, 2006). On the other hand, upregulation of these miRNAs has been seen in B-cell lymphomas (Tagawa & Seto,

2005), lung cancers (Hayashita et al., 2005), and T-cell acute lymphoblastic leukemia (Mavrakis et al., 2010) due to amplification of the miR-17-92 cluster gene and translocation of this gene, respectively. High-resolution array-based comparative genomic hybridization on 227 specimens from human ovarian cancer, breast cancer, and melanoma demonstrated the high frequency of genomic changes in miRNA loci (Zhan et al., 2006). Additional genome-wide analyses showed that many miRNA genes are situated in genomic areas linked to cancer. A minimum zone of loss of heterozygosity, which may include tumour suppressor genes, a minimal region of amplification, which may contain oncogenes, fragile sites, or common breakpoint regions are some examples of these regions (Peng & Croce, 2016).

Cell miRNAs have crucial roles in the immune system, cell death, differentiation, and cancer (Hirschberger et al., 2018). Numerous cancers form often exhibit dysregulated miRNA expression, and miRNA profiling of clinical samples may be used for cancer diagnosis and prognostic applications (Lan et al., 2015). MiR-195-5p suppresses the invasion, migration, and proliferation of hepatoma cells in humans by acting as an anti-oncogene that targets PHF-19 (Xu et al., 2015). miR-6 and miR-7 have been shown to inhibit the spread of gastric cancer by targeting the insulin-like growth factor-1 receptor (Zhao et al., 2015). The combined effects of many miRNAs (miR-200b, miR-203, miR-429, and miR-205) have been shown in certain circumstances, such as the repression of the Myc oncogenic pathway (Bueno et al., 2011; Conacci et al., 2014; Gabay et al., 2014). It has also been suggested to use several miRNAs (miR-34a, let-7b and miR-1-3p) to treat lung cancer (Kasinski et al., 2015). The development of new anti-miR chemistries (chemical changes with increased therapeutic qualities) and the associations between

miRNAs and various disorders have raised the possibility that control of miRNAs may usher in the next advancement in pharmaceutical research (van et al., 2012). Up until now there were around 20 clinical studies using treatments based on miRNA and small interfering RNA (siRNA) (Chakraborty et al., 2017). The artificial introduction of miRNAs or the use of antisense oligonucleotides to block miRNAs are strategies for miRNA therapies that have been suggested (Broderick & Zamore, 2011). Previously our laboratory discovered that miR-367-3p was able to control the expression of the *chka* gene in breast cancer MCF-7 cells. *chk* expression was drastically downregulated, apoptosis was triggered, and cell migration was reduced when miR-367-3p was transfected into MCF-7 cells (Raikundalia et al., 2021).

miRNAs have been shown to have antiproliferative effects on human cells. For instance, miR-195-5p suppresses the invasion, migration, and proliferation of hepatocellular carcinoma cells by acting as an anticancer agent targeting PHF-19 (Xu et al., 2015). And in the research conducted by our team, it was shown that miR-367-3p targeted the 3'-UTR of the *chka* transcript and that this miRNA controlled the production of ChKa, when ChKa was decreased, MCF7 cells exhibited more significant levels of apoptosis and less migration. Even though miR-367-3p expression was not determined to mimic or inhibit it after transfection, downregulation of target gene expression (*chka*) by miR-367-3p was constant throughout all experiments in the current investigation. According to the present study's findings, miR-367-3p may be a good miRNA candidate for future research into the functions of miRNAs in cancer formation, including deregulation of *chka* gene expression. To completely comprehend the process of *chka* attenuation by this miRNA in cancer, more research is required to clarify the impact of *chka* downregulation by miR-

367-3p on cell proliferation, invasion, and anticancer treatment resistance in more cancer cell lines (Raikundalia et al., 2021).

Numerous cancers have been linked to ChK overexpression. Suppression of this enzyme activity and downregulation of its expression is one of the promising anticancer methods (Wu & Vance, 2010) However, it is still unclear what causes ChK to express itself stronger in cancer cells. The current research looked at the possibility of miRNAs controlling the expression of the *chka* gene. As the experimental validation of miRNAs is time-consuming and expensive, target prediction analysis is essential (Oliveira et al., 2017). Based on the low values for mirSVR and minimum free energy (MFEs), it was expected that miR-367-3p has a significant affinity for the 3'-UTR of the *chka* mRNA transcript. When rating the result is necessary, mirSVR performs better than other prediction methods (Oliveira et al., 2017). The miR-302/367 cluster, which is the expression of the hairpin loop sequence at the 3' end of miR-367, is the source of miR-367-3p. This miRNA cluster may convert somatic cells into pluripotent stem cells and is mainly expressed in embryonic stem cells (Rosa et al., 2013). By strengthening the route involving the androgen receptor, miR-367-3p has been shown to improve the effectiveness of sorafenib treatment in decreasing hepatocellular carcinoma metastasis (Xu et al., 2016). However, it has been demonstrated that miR-367-3p is expressed ectopically in non-small cell lung cancer, inhibiting apoptosis while promoting cell proliferation and cell cycle progression (Xiao et al., 2017). The same miRNA may play diverse functions in various cancer cell types by functioning as an oncogene or tumor suppressor (Yan et al., 2020). There have been conflicting reports on miR-367 impact on the development of cancer cells and how well they respond to treatment (Chen et al., 2014; Zhu et al., 2015). It has been shown that

overexpressing of miR-367-3p in paclitaxel-sensitive ovarian cancer cells increases their sensitivity to this medication (Chen et al., 2014). Additionally, has been shown that miR-367-3p prevents the migration and invasion of gastric cancer (Bin et al., 2015). In contrast, it has been shown that a greater expression of miR-367-3p is linked to a poor prognosis for high-grade gliomas (Guan et al., 2015), resected non-small cell lung cancer (Campayo et al., 2013), and pancreatic ductal adenocarcinomas (Zhu et al., 2015). As a result, the impact of miR-367-3p on cancer cell survival can vary depending on the kind of cell. MiR-135 and miR-203 directly target the bone-related transcription factor Runx2 to operate as *in vivo* suppressors of breast cancer metastasis and osteolytic bone disease. Expression investigations showed that miR-135 and miR-203 were downregulated in bone metastases with high Runx2 levels, indicating that the pathologic rise in Runx2 protein is caused by the absence of these particular miRNAs in breast cancer metastases. Restoring miR-135 and miR-203 to metastatic cells did indeed reduce their oncogenic potential in a lab setting, but more crucially, it reduced tumour development at the orthotopic location in a living organism (Taipaleenmäki et al., 2015).

2.6 miRNA therapeutics

The systematic identification of miRNA candidates by analysis of patient samples is the first step in the development of miRNA treatments. Next, the biology and relevance of the miRNA candidates to illness are clarified through tissue culture and *in vivo* model-based validation. At present, genomic and proteomic information from diverse healthy and sick tissues is available in a number of public databases. Combining this data with biological validation may make it easier to choose possible candidates for miRNAs. The creation of chemical alterations and delivery mechanisms for miRNA mimics and anti-miRs for *in*