The regulation of tumor suppressor PTEN by microRNAs in breast cancer

by

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Abstract

Breast cancer (BCa) is the most frequently diagnosed cancer in women worldwide. Treatment strategies often target hormone receptors on breast cancer cells. Triple-negative breast cancer (TNBC) is a BCa subtype where estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are not expressed. Although TNBC accounts for approximately 15% of all BCa cases, the prognosis of these patients is poorer when compared to patients with other BCa subtypes. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) plays an important role in cell proliferation and cell migration by regulating the PI3K–AKT signal pathway negatively. *PTEN* is one of the most commonly inactivated tumor suppressors gene in BCa, and its inactivity is associated with larger tumor size, considerable lymph node metastases, and an aggressive triple-negative phenotype. MicroRNAs (miRNAs) are small non-coding RNAs that regulate protein expression. In multiple cancers, PTEN is downregulated by miRNAs; however, very few microRNAs target PTEN directly in TNBC.

In this study, nineteen microRNAs predicted to target PTEN were screened using immunoblotting and luciferase reporter assays. Expression levels of miRNA-498 (miR-498) were measured by TaqMan microRNA assays. Clonogenic, cell cycle, and scratch wound assays were performed to examine the oncogenic role of miR-498. I demonstrate that miR-498 directly targeted the 3′ untranslated region of *PTEN* messenger RNA (mRNA) and reduced PTEN protein levels in TNBC cells. Compared with a non-tumorigenic breast epithelial cell line, TNBC cell lines overexpressed miR-498. Moreover, miR-498 promoted cell proliferation and cell cycle progression in TNBC cells in a PTEN-dependent manner. Suppressing miR-498 overexpression impaired the oncogenic effects of miR-498 on cell proliferation and cell migration. This study

identified miR-498, a novel miRNA to be overexpressed in TNBC cells and its oncogenic role in suppressing *PTEN*. These results provide new insight into the downregulation of *PTEN* and indicate a potential therapeutic target for treating TNBC.

Preface

This thesis is an original work by Chengsen Chai. A portion of this thesis has been published: Chai C, Wu H, Wang B, Eisenstat DD, Leng RP. MicroRNA-498 promotes proliferation and migration by targeting the tumor suppressor PTEN in breast cancer cells. Carcinogenesis. 2018 Sep 21;39(9):1185-96. doi: 10.1093/carcin/bgy092. The authors who contributed to this work in my publication are me (CC), postdoctoral fellows Dr. Hong Wu (HW) and Dr. Benfan Wang (BW), Chair and Professor of Department of Oncology Dr. David Eisenstat (DE), and my supervisor Dr. Roger Leng (RL). Our contributions are as follows: conception and design by CC and RL; development of methodology by CC, HW, BW and RL; data analysis and interpretation by CC, HW, BW, DE and RL; review and revision of the manuscript by CC, DE and RL. As the first author, I wrote the first draft of this publication.

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, project name “Study of the role of UBE4B in tumors”, Study ID: Pro00024519, Start Date: July 30, 2016.

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List of Abbreviations

AGO – argonaute protein

AKT – rac-alpha serine/threonine-protein kinase (PKB)

AR – androgen receptor

BAD – bcl-2 antagonist of cell death

BCa – breast cancer

BRCA1 – breast cancer type 1 susceptibility protein

CA – Canada

°C – Celsius degrees

CHEK1 – cell cycle regulator checkpoint kinase 1

Cys – cysteine

DGCR8 – DiGeorge syndrome critical region 8

ddH2O – purified water (deionized water)

DH5α – alpha Escherichia coli bacteria

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

dNTP – deoxynucleotide triphosphate

E1 – ubiquitin activating enzyme

E2 – ubiquitin transfer enzyme

E3 – ubiquitin ligase enzyme

EDTA – ethylene-diamine-tetraacetic acid

EGFR – epithelial growth factor receptor

EMT – epithelial to mesenchymal transition

ER – estrogen receptor

EVI1 – ecotropic virus integration site 1 protein

FAK – focal adhesion kinase

FBS – fetal bovine serum

FOXO – forkhead box o

g – earth's gravitational force of 9.8 m/sec2 (g)

GPCR – guanine nucleotide binding protein coupled receptor

GDP – guanosine diphosphate

GSK3 – glycogen synthase kinase 3

GTP – guanosine triphosphate

HAUSP – herpesvirus-associated ubiquitin-specific protease

HDAC – histone deacetylase

HEK – human embryonic kidney epithelial cells

HER2 – human epidermal growth factor receptor-2

INPP4B – inositol polyphosphate-4-phosphatase type II

kDa – kilo Dalton

L – liter

LB – lysogeny broth

Leu – leucine

Lys – lysine

M - molarity (moles/liter)

mAb – monoclonal antibody

MCL-1 – induced myeloid leukemia cell differentiation protein

MDM2 – mouse double minute 2 homolog

miR – microRNA

miRNA – microRNA

mL – milliliters

mTORC1 – mammalian targets of Rapamycin complex 1

NEDD4 – neural precursor cell expressed developmentally down-regulated protein 4; E3 ubiquitin ligase

NO – nitric oxide

OD – oligomerization domain

PAGE – polyacrylimide gel electrophoresis

PARP – poly ADP-ribose polymerase

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PH – pleckstrin homology

PI3K – phosphatidylinositol 3-kinase

PIP2 – phosphatidylinositol (4,5)-bisphosphate

PIP3 – phosphatidylinositol (3,4,5)-trisphosphate

PHLPP – ph domain leucine-rich repeat protein phosphatase

PPARγ – peroxisome proliferator-activated receptor γ

PR – progesterone receptor

PTENP1 – phosphatase and tensin homolog pseudogene 1

ROCK – rho-associated protein kinase

RT – room temperature

RTK – receptor tyrosine kinase

S – subunit

SDS – sodium dodecyl sulphate

siRNA – small interfering ribonucleic acid

SNAI1– zinc finger protein

Thr – threonine

TBS – tris-buffered saline

TBST – tris-buffered saline with 1% tween added

TE buffer – tris-EDTA buffer

TNBC – triple-negative breast cancer

TP53 – tumor protein p53

TRAIL-R – tumor necrosis factor-related apoptosis-inducing ligand receptor

Tris – tris (hydroxymethyl) aminomethane

Tris-HCl – tris (hydroxymethyl) aminomethane hydrochloride

Trp – tryptophan

TSC2 – tuberous sclerosis complex 2

Tween20 – polyoxyethylene sorbitan monolaurate

Tyr – tyrosine

UK – United Kingdom

UPS – ubiquitin proteasome system

USA – United States of America

UV – ultraviolet radiation

µL – microliters

VEGFR – vascular endothelial growth factor

XIAP – X-linked inhibitor of apoptosis protein

Chapter 1 General Introduction

1.1 Cancer and cancer treatment

1.1.1 The problem of cancer

Cancer is a global problem threatening the health of people. In 2012, there were 14.1 million people diagnosed with cancer and 8.2 million people died from cancer worldwide (Stewart BW, 2014). In Canada, cancer is the leading cause of death and makse up 30.2% of the total death cases, and more than 800 thousand Canadians live with cancer. 206,200 new cases of cancer were diagnosed in 2017 in Canada, and 103,200 cases of cancer were in women (Committee CCSA, 2017).

Generally, cancer is a collection of diseases that derive from abnormal cells with malfunctions (Weinberg RA, 2014). Some types of malfunctioning cells have uncontrolled growth and divide and form a mass of abnormal cells called as a tumor. Tumors are either benign or malignant. A benign tumor is any proliferation of abnormal cells constrained within their original site; whereas a malignant tumor can invade surrounding normal tissues or spread to distant organs of the human body through blood circulation or lymphatic system (metastasis), which impedes the function of organs and threatens life (Weinberg RA, 2014). Only the malignant (cancerous) tumors belong to cancer.

Most cancers can be categorized into four groups: carcinomas, sarcomas, leukemias and lymphomas (Cooper GM, 2000). Carcinomas, which arise from epithelial cells or tissues, are the most common type of cancer. Sarcomas, which derive from mesenchymal cells, are relatively rare and occur in muscle, bone and fibrous tissue; leukemias derive from blood-forming cells like white blood cells, while lymphomas arise from immune system cells (Weinberg RA, 2014). Among these groups, carcinomas are the dominant one and comprise of the majority of cancer

cases, which include the most commonly occurring cancers of the skin, stomach, head and neck, lung, breast, prostate, ovary, pancreas, colon, kidney, and esophagus. (Weinberg RA, 2014).

The development of cancer is a complicated process that commonly goes through five stages: hyperplasia, dysplasia, cancer in situ, malignant tumor and metastases (Johnson MD, 2004). First, a genetically altered epithelial cell grows and divides faster than a normal cell, which results in an increased number of these cells and forms cell aggregation (hyperplasia). As genetic alterations accumulate, fast-growing cells start to change their forms (transformation) and the volume of abnormal cell aggregation increases (dysplasia). Further, the population of transformed cells dramatically increases, but is still confined to its original site (cancer in situ). Eventually, some of the transformed cells (cancerous cells) break the border of epithelial tissue, invade into the adjacent normal tissue and then enter blood or lymph (malignant tumor). Through the blood circulation system or lymphatic system, cancer cells migrate to remote sites of the human body and form secondary tumors in other tissues or organs (metastases), which eventually impede the function of organs and threaten human lives (Johnson MD, 2004) (Figure 1.1). The stages of tumor development are incorporated into the grading of tissue biopsy samples under the microscope as part of the process for the diagnosis of cancer.

Genetic alterations in cells contribute to the initiation and development of cancer. Genetic alteration in cancer frequently occurs in three types of genes, including proto-oncogenes, tumor suppressor genes and genes related to DNA repair. Proto-oncogenes encode normal proteins that activate proliferative pathways and promote cell growth and division; however, genetic alteration tends to over-activate proto-oncogenes and changes them to oncogenes (Todd R, 1999; Winberg RA, 1983). For instance, proto-oncogene Myc was reported to be over-activated in a variety of cancers (Strieder V, 2002; Nesbit CE, 1999; Schwab M 2004). As a transcription factor, Myc

protein activates many genes involved in cell growth and division and thereby promotes cancer cell proliferation (Denis N, 1991; Rahl PB, 2014). Together with oncogene activation, genetic alterations of tumor suppressor genes, which encode proteins negatively regulating cell growth and division or promote programmed cell death, results in cell growth and division in an uncontrolled manner (Eliyahu D, 1989; Chen PL, 1990). For example, p53, a well-known tumor suppressor, was found to be frequently inactivated in various cancers due to the mutation or loss of its genomic DNA, which abrogated its function of negatively regulating cell division and led to uncontrolled cell proliferation (Hollstein M, 1994; Baker SJ, 1989&1990). Furthermore, DNA repair genes play an important role in tumorigenesis (Jeggo PA, 2016). Proteins encoded by DNA repairing genes, like *ATM (Ataxia Telangiectasia Mutated)* could fix DNA double-strand breaks and prevent the transformation from normal cells to cancerous cells (Bartkova J, 2005); however, the mutation of ATM protein in colon cells enhanced genomic instability and promoted the progression of tumorigenesis (Halazonetis TD, 2008; Bartkova J, 2005). Therefore, genetic alteration in cells plays a crucial role in the initiation and development of cancer.

Epigenetic aberration is also associated with the initiation and development of cancer. Increasing pieces of evidence have supported that working together with genetic alteration, the epigenetic aberration on DNA methylation, histone modification and non-coding RNA promotes the carcinogenesis and cancer progression. First, the initiation and the development of cancer is accompanied by aberrant DNA methylation such as site-specific CpG island hypermethylation and genome-wide DNA hypomethylation. For instance, the hypermethylated CpG island of tumor suppressor Rb promoter was identified in retinoblastoma, which contributed to the formation of retinoblastoma (Greger V, 1989); DNA hypomethylation on the genomic DNA of proto-oncogene RAS resulted in the activation of RAS in gastric cancer (Wilson AS, 2007).

Moreover, aberrant histone modifications are also related to the initiation and the development of cancer. A global loss of acetylation on histone H4K16 was found in the tumorigenesis of lymphomas (Fraga MF, 2005); this aberrant histone modification resulted from the overexpression of histone deacetylase (HDAC) and led to the repression of tumor-suppressing genes in multiple cancers (Halkidou K, 2004; Song J, 2005). Furthermore, the aberrant expression of microRNA is associated with tumorigenesis. The tumor-suppressing microRNA let-7, which interacts with the mRNA of proto-oncoprotein RAS, was repressed in lung cancer (Johnson SM, 2005). In contrast, oncogenic microRNA miR-21 targets tumor suppressor PTEN, was upregulated in human glioblastoma (Chan JA, 2005). Therefore, the epigenetic aberration is also important for the initiation and development of cancer.

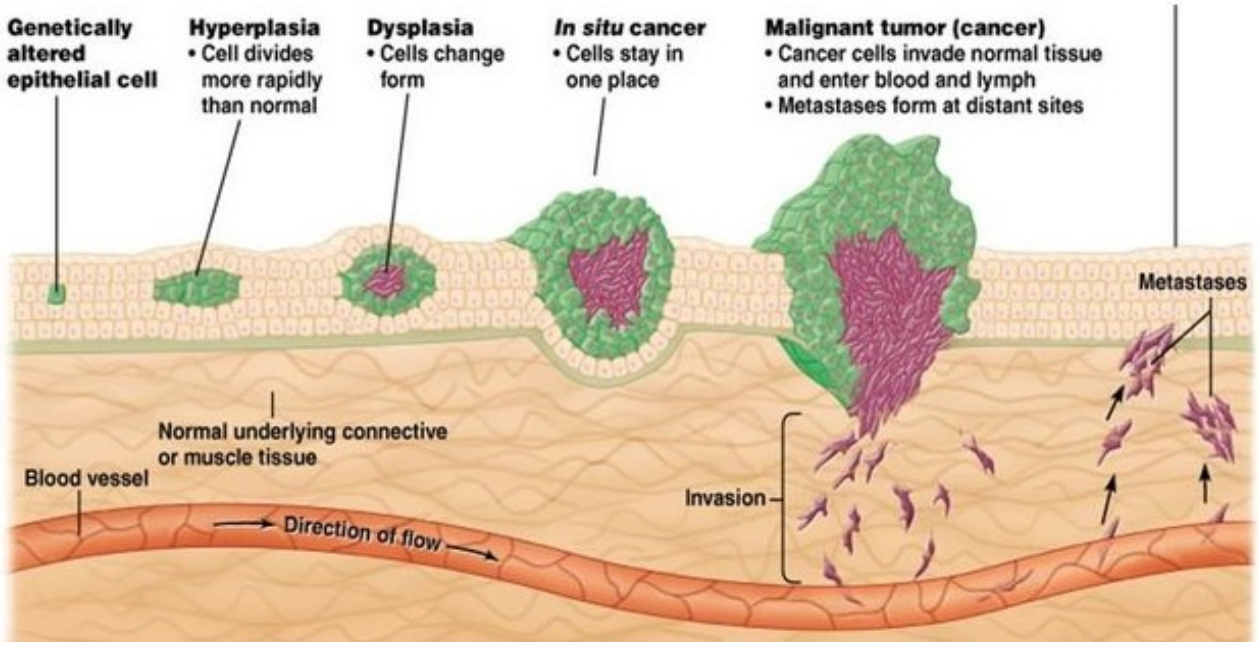


Figure 1. 1 The development of cancer.

This figure is cited from Human Biology: concepts and current issues. 8nd Edition. Chapter 18 Cancer: uncontrolled cell division and differentiation; Figure 18.2 The development of a malignant tumor. Page 422. The permission to use this figure is PE Ref # 208033.

1.1.2 Breast cancer

Breast cancer (BCa) is the most common cancer in women. According to the Committee for the Coordination of Statistical Activities (CCSA, 2017), 26,300 new cases of breast cancer were diagnosed in Canada in 2017, accounting for 26% of all new cancer cases in women.

BCa includes a range of different subtypes of cancers. Histologically, breast cancer can be broadly classified as carcinoma in situ or invasive carcinoma. Furthermore, breast carcinoma in situ includes ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), while invasive breast carcinoma comprises a greater number of subtypes, including tubular, ductal/lobular, mucinous, medullary, medullary, papillary, invasive lobular, and invasive ductal carcinoma (Malhotra, 2010). However, this classification does not capture the intrinsic differences between BCa patients at the molecular level and cannot be used for determining the most appropriate and effective therapy for each individual (Malhotra, 2010). Owing to the advances in molecular biology, some molecular biomarkers have been identified and applied in breast cancer research, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2, also known as ERBB2) (Perou, 2000; Sorlie, 2001). Based on these molecular markers, breast cancer cases can be categorized into six subtypes, namely luminal A BCa, luminal B BCa, HER2-overexpressing BCa, basal-like BCa, claudin-low BCa, and normal-like BCa (Sorlie, 2001, 2003).

Luminal A BCa is a hormone-receptor positive (expressing ER or PR, or both) and HER2-negative subtype with low tumor grade, which is the most common subtype of BCa with good prognosis (Yersal, 2014). Luminal B subtype is similar to luminal A, but involves HER2

expression or high levels of antigen Ki-67. As it grows faster, it has poorer prognosis than luminal A (Perou, 2011; Yersal, 2014). On the other hand, HER2-overexpressing BCa is characterized by high expression of HER2 without expression of hormone receptors. It grows faster and has poorer prognosis than the aforementioned luminal subtypes (Malhotra, 2010). Normal-like BCa, which does not fit into any of these subtypes and originates from epithelial cells, expresses hormone receptors and has a good prognosis (Prat, 2011). Basal-like BCa, which consistently expresses genes of basal cells or myoepithelial cells including basal cytokeratins and epidermal growth factor receptor (EGFR), generally does not involve ER or PR expression and is associated with the worst prognosis among all BCa subtypes (Nielsen, 2004; Yersal, 2014). Claudin-low BCa is identified as a BCa subtype and is characterized by low expression of genes involved in tight junction and cell-to-cell adhesion, including claudin 3/4/7 and E-cadherin (Prat, 2010; Yersal, 2014). Due to the absence of ER and PR expression and low expression of HER2, claudin-low and most of basal-like BCa types can be described as triple- negative breast cancer (TNBC) (Prat, 2010, 2011; Schmadeka, 2014; Yersal, 2014).

TNBC, defined by the phenotype of negatively expressed ER, PR or HER2, is a heterogeneous group of breast cancers, which accounts for 10−20% of invasive breast cancers and comprises some distinct molecular breast cancer subtypes (Nielsen, 2004; Rakha, 2006; Schmadeka, 2014). Although the majority of TNBC cases are basal-like BCa without expression of ER, PR or HER2, other subtypes of breast cancer are included in the TNBC category, such as some cases of normal-like BCa and newly-recognized claudin-low BCa, which account for approximately 25% of all TNBC cases (Bertucci, 2008; Carey, 2011; Reis-Filho, 2008).

At the histological level, most of TNBC cases are classified as invasive ductal carcinoma; however, some cases of metaplastic breast carcinoma and medullary carcinoma can also be

categorized under TNBC (Schmadeka, 2014). Based on clinical evidence, TNBC has the following main characteristics: (1) patients are usually younger (under 50 years of age) and of black ethnicity; (2) most TNBCs are high-grade invasive carcinomas; (3) TNBC is more aggressive with higher rate of brain metastases and recurrence; (4) patients diagnosed with TNBC have shorter survival time after metastasis than patients in whom other subtypes have been identified (Carey, 2006; Nielsen, 2004; Perou, 2000; Sørlie, 2001).

The genetic characteristics of TNBC have been identified at the molecular level. According to the available evidence, the germline mutation in BRCA1 (breast cancer type 1 susceptibility protein) is highly associated with TNBC (Atchley, 2008). BRCA1 is a protein involved in DNA repair and acts as a tumor suppressor (Yoshida, 2004). Moreover, BRCA1 is implicated in breast and ovarian cancers, and mutated BRCA1 results in the failure of DNA repair for double-strand breaks and cell apoptosis, which increase the risk of breast cancer (Boulton, 2006; Wang, 2001; Warmoes, 2012). In extant studies, 60−80% of breast cancer patients with germline mutation in BRCA1 exhibited a triple-negative phenotype (Atchley, 2008; Lakhani, 2002). Moreover, the PI3K/AKT/mTOR pathway is aberrant and is particularly over-activated in a subset of TNBC patients (Kriegsmann, 2014; Mentero, 2012). In recent studies, phosphorylated mTORC1, a downstream effector of AKT related to a poor prognosis in TNBC cases, was observed to be upregulated in 40−70% of TNBC patients (Kriegsmann, 2014; Ueng, 2012; Walsh, 2012).

The treatments applied in triple-negative breast cancer are surgery, radiation therapy, chemotherapy and molecular target therapy. Similar to other invasive breast cancers, surgery to excise breast tumor mass is still the first choice for primary and locoregional TNBC tumors

(Brouckaert O, 2012; Chen FX, 2017). Breast-conserving surgery is used to remove a smaller tumor mass, while mastectomy (breast partially or completely removed) is applied to a larger tumor or multifocal tumor (Freedman G, 2009; Voduc KD, 2010; Lowery AJ, 2012). Radiation therapy is an essential supplement for the surgical treatment of TNBC, and it is demonstrated to be important for optimizing long-term outcome after surgical treatment (Brouckaert O, 2012; Chen FX, 2017). Chemotherapy is critically important for TNBC patients since TNBC is confirmed to be more sensitive to chemotherapy than other subtypes (Roché H, 2006; Carey LA, 2007). Prior to breast-conserving surgery, adjuvant chemotherapy can diminish the tumor mass to facilitate surgery (Straver ME, 2010; Carey LA, 2007; Cardoso F, 2010). Regarding metastatic TNBC, chemotherapy is the only demonstrated systemic treatment for improving the disease outcome (Rouzier R, 2005; Liedtke C, 2008). Single-agent chemotherapies used for TNBC including taxanes and platinum agents are effective in particular subsets of TNBC, while combined chemotherapy regimens such as capecitabine combining with ixabepilone, significantly improve the progression-free survival of TNBC patients (Harris LN, 2006; Rottenberg S, 2007; Perez EA, 2010). Although there is a high response rate of chemotherapy for TNBC patients, the duration of response is deficient, and the prognosis is inferior compared to other subtypes of breast cancer (Carey LA, 2007; Kennecke H, 2010; Cardoso F, 2010).

Molecular target therapy is a novel and promising treatment for TNBC patients. Since there is no expression of ER or PR in TNBC patients, hormonal therapy drug Tamoxifen, which impedes the binding of estrogen to the estrogen receptor on breast cancer cells, is useless for TNBC. Recently, potential molecular targets that drive the progression of TNBC have been identified, including androgen receptor (AR), vascular endothelial growth factor (VEGF), epithelial growth factor receptor (EGFR) and poly ADP-ribose polymerase (PARP), which

suggests the possibility of developing new therapeutic targets for TNBC (Brouckaert O,2012; Robert NJ, 2011; Schmadeka R, 2014). Some chemical agents have shown an inhibitory effect on these molecular targets of TNBC in clinical trials (Robert NJ, 2011; Milker K, 2007). For instance, a VEGF inhibitor bevacizumab, combined with first-line chemotherapy, has been reported to enhance the progression-free survival of patients with metastatic TNBC (Robert NJ, 2011; Milker K, 2007). However, the clinical use of these molecular-targets therapies is still limited due to the lack of predictive biomarkers for effectively instructing the application of these molecular-target therapies (Brouckaert O, 2012).

TNBC has a poorer prognosis than luminal breast cancer subtypes (Perou CM, Nature, 2000; Sørlie T, PNAS, 2001; Rakha EA, 2006). The recurrence risk of TNBC is high within the first few years after surgery, with the peak risk of recurrence in the third year; in contrast, over 50% of the luminal breast cancer patients relapse between 5 to 10 years after surgery (Kennecke H, 2010; Jatoi I, 2011). Following locoregional recurrence, the risk of developing distant metastasis, especially brain metastasis in TNBC patients, is also higher than the other types of BCa patients (Dent R, 2007; Smid M, 2007). Moreover, the survival time after metastatic recurrence is shorter for TNBC compared to other BCa subtypes (Kennecke H, 2010). Once the distant metastasis is observed, the progression from distant metastasis to death is fast; the median survival time of patients is nine months, and most deaths occur within five years (Dent R, 2007; Montagna E, 2012). These data indicate that TNBC is an aggressive subgroup of breast cancer with poor prognosis. Therefore, it is highly urgent and necessary to investigate the mechanism of TNBC progression and metastasis to be able to develop more effective therapies.

1.2 PI3K-AKT pathway and PTEN

1.2.1 PI3K-AKT pathway

1.2.1.1 Activation of PI3K-AKT

The PI3K-AKT pathway is one of the most important signal pathways that are hyper-activated in multiple cancers. It has been reported that in breast cancer the activation of PI3K-AKT pathway is frequently aberrant (Baselga J, 2011; Yuan TL, 2008). The PI3K-AKT pathway is activated by extracellular signals such as IGF1 (insulin-like growth factor 1) and then various sets of substrates are activated through the phosphatase activity of AKT, which control cell metabolism, growth, survival, proliferation and apoptosis (Ward PS, 2012; Jason SL, 2016; Xue G, 2013).

AKT (also PKB, protein kinase B) is a serine/threonine protein kinase, and was first found in the AKT8 transforming retrovirus (Staal SP, 1977). So far, three isoforms of AKT have been identified in mammalian cells (AKT1, AKT2 and AKT3) and they exhibit a high homology between these isoforms (Manning BD, 2007; Gonzalez E, 2009). Due to the tissue-specific expression of AKT isoforms, AKT1 is ubiquitously expressed in most human tissues; AKT2 is highly expressed in skeletal muscle and liver; AKT3 was highly expressed in brain (Yang Z, 2003; Fayard E, 2005; Dummler B, 2006; Gonzalez E, 2009). AKT consists of four domains: an N-terminal PH domain, an HR domain, a kinase domain and a C-terminal regulatory domain (Barnett SF, 2005; Zhou GL, 2006). The PH domain can interact with the kinase domain and inhibit kinase activity, which is known as the auto-inhibitory effect of AKT; while the C-terminal regulatory domain is also involved in the regulation of AKT activity through post-

translational modification (Hanada M, 2004). Initially, the activation of AKT requires the dissociation of the PH domain from the kinase domain, which is induced by intracellular second messenger PIP3 and alters the inactivated conformation of AKT through interaction between PIP3 and PH domain; then the kinase domain and regulatory domain of AKT are phosphorylated, and the activated kinase domain is capable of catalyzing the phosphorylation of AKT substrates and regulating their functional activity by phosphorylation (Fayard E, 2005; Hanada M, 2004). Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) is an important second messenger and is the phosphorylated production of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) catalyzed by phosphoinositide 3-kinases (PI3K) (Maehama T, 1998; Stephens L, 1998). PIP3 is mainly located at the plasma membrane and recruited and interacted with the PH domain of the inactivated AKT, which results in the dissociation of PH domain with AKT kinase domain, thereby promotes the activation of AKT through the modification of phosphorylation by other protein kinases (Fayard E, 2005; Stephens L, 1998). Specifically, AKT1 is activated by phosphorylation at Ser473 and Thr308 which is catalyzed by mTORC2 (mammalian target of Rapamycin complex 2) and PDK1, respectively (Fayard E, 2005; Hanada M, 2004). The phosphorylation at Thr308 is recognized as the initial step of AKT1 activation while phosphorylation at Ser473 enables the maximal activation of AKT1 (Fayard E, 2005; Hanada M, 2004). The activated AKT functions as a survival factor by catalyzing the phosphorylation of numerous down-stream substrates including GSK3, FOXOs and TSC2, which promotes cell proliferation and metabolism, enhances cell migration and inhibites apoptosis (Manning BD, 2017).

1.2.2.2 The functions of AKT and its substrates

In PI3K-AKT signal pathway, AKT acts protein kinase to phosphorylate a great variety of substrates, and these substrates include transcription factors, kinases, ubiquitin E3 ligase, metabolic enzymes, cell cycle regulator, which involved in many vital cellular events(Hanada M, 2004; Manning BD, 2007). Most AKT substrates shared a consensus recognition motif of R-X-R-X-X-S/T-ϕ (X represents any amino acid and ϕ denotes a preferred hydrophobic residue) and AKT phosphorylated substrates on Ser or Thr residue in this motif (Fayard E, 2005; Manning BD, 2017). However, the existence of the recognition motif is not a determinant factor for the phosphorylation by AKT, and there are other factors affecting the recognition of potential substrates, like secondary interactions by other molecules, accessibility of conformation by AKT and subcellular localization (Manning BD, 2017). It is noticeable that the well-established AKT substrates are also involved in other signal pathways and not exclusively regulated by AKT, which is a good example of the complexity and redundancy of signal networks (Fayard E, 2005; Manning BD, 2017). As a protein kinase, the physiological function of AKT mainly depends on these substrates. Due to the numerous downstream substrates of AKT, PI3K-AKT signal pathway involved in cell metabolism, cell proliferation, cell migration and cell survival (Ward PS, 2012; Jason SL, 2016; Xue G, 2013; Kim D, 2001). Here, I will introduce some well-established substrates of AKT (FOXOs, GSK3, TSC2), which transmit upstream signals and play a vital role in regulating cellular function (Figure 1.2).

Forkhead Box O (FOXO) is a subfamily of transcription factors and consists of four isoforms (FOXO1, FOXO3, FOXO4 and FOXO6). As transcription factors, FOXOs control the

transcription of a great set of downstream genes, which are involved in the cell proliferation, cell cycle arrest and apoptosis (Brunet A, 1999; Accili D, 2004; Zhang X, 2011). Firstly, FOXOs negatively regulates cell proliferation and cell cycle progression through cell cycle kinase inhibitor (CKI) p27kip1 (Kops GJ, 2002; Medema RH, 2000). FOXOs promoted the transcription of p27kip1 which was a negative regulator of cell cycle. The accumulation of p27kip1 resulted in the inhibition of cell cycle progression which was dependent on cyclin/CDK activation. The phosphorylation of FOXOs by AKT resulted in the nuclear export of FOXOs and the abrogation of transactivation of p27kip1 by FOXOs, thereby promoted the cell cycle progression (Medema RH, 2000). Moreover, FOXOs also triggers cell apoptosis in both intrinsic and extrinsic pathways (Fu Z, 2008; Zhang X, 2011). In the extrinsic pathway, FOXOs promoted cell apoptosis through positively regulated the pro-apoptotic genes such as TRAIL and TNFR (Modur V, 2002; Ding B, 2009). FOXOs increased the transcription of TRAIL and TNFR and helped the activation of cell apoptosis from extracellular signals. In the intrinsic pathway, FOXOs promoted the expression of pro-apoptotic BH3-only protein BMF and induced mitochondrial-dependent apoptosis (Westphal D; 2011; Hornsveld M, 2016). Detailly, FOXOs increased the expression of BMF and BMF could bind to the BAX/BAK on the outer mitochondrial membrane, which activated the BAX/BAK and released the apoptogenic factors Cytochrome C from mitochondria (Du H, 2011; Westphal D; 2011). The released cytochrome C associated with Apaf-1 and caspase 9 and formed an apoptosome complex to activated caspase cascade, which induced cell apoptosis (Du H, 2011). However, AKT-mediated phosphorylation of FOXOs blocked the activation of these pro-apoptotic genes by FOXOs and thereby inhibited cell apoptosis and promoted cell cycle progression, which was reported in multiple cancers including breast cancer, prostate cancer and pancreatic cancer (Sunters A, 2003; Roy SK, 2010;

Chen Q, 2010). Therefore, AKT acted as a cell survival factor through inhibiting cell apoptosis and cell cycle arrest.

Another important substrate of AKT is GSK3 (Glycogen synthase kinase 3), which is a multi-functional protein kinase and also is the first confirmed AKT substrate (Cross DA, 1995). GSK3 cooperates with distinct adapters or components and participates in many important signal pathways in cells, like the Wnt-β-catenin pathway and PI3K-AKT pathway (Ikeda S, 1998; Cross DA, 1995). GSK3 phosphorylates its substrates and then inhibits the function of substrates; these substrates of GSK3 are involved in many physiological processes, including cell survival, proliferation, migration and cell metabolism (Maurer U, 2006; Welcker M, 2004; Parker PJ, 1983; Zhou BP, 2004). For its namesake, GSK3 phosphorylated glycogen synthase, a key enzyme to convert glucose to glycogen, and thereby inhibited the production of glycogen (Fiol CJ, 1987). In the aspect of cell survival, GSK3 functions as an inhibitor for pro-survival proteins. For instance, the BCL-2 family member MCL-1 was a pro-survival protein and a substrate of GSK3 (Ding Q, 2007; Maurer U, 2006). The phosphorylation of MCL-1 by GSK3 facilitated the recognition of ubiquitin E3 ligase and subsequently induced MCL-1 to be degraded by the proteasome, which indicated that GSK3 suppressed cell survival through inhibiting MCL-1. (Ding Q, 2007). In the PI3K- AKT signal pathway, the kinase function of GSK3 is generally inhibited by the AKT-mediated phosphorylation, and AKT functions as a survival factor through inhibiting GSK3 (Kaidanovich-Beilin O, 2011). Furthermore, GSK3 negatively regulates cell proliferation through c-Myc (Welcker M, 2004). As an oncogenic transcription factor, c-Myc was a central regulator for promoting cell proliferation; GSK3 phosphorylated c-Myc at Thr58 and induced the degradation of c-Myc through ubiquitin-proteasome pathway (Sears R, 2000; Gregory MA, 2003). However, the phosphorylation of GSK3 by AKT repressed the kinase

activity of GSK3, which stabilized c-Myc and enhanced its oncogenic function on cell proliferation. In addition, GSK3 negatively regulates cell migration through inhibiting EMT-inducing transcription factor SNAIL (Zhou BP, 2004). SNAIL was highly associated with cancer metastasis and promoted the EMT (epithelial to mesenchymal transition) in which epithelial cells lost adherent and tight junctions and became migratory mesenchymal cells (Olmeda D, 2007; Wang Y, 2013); GSK3 inhibited SNAIL by phosphorylation and promoted its degradation (Zhou BP, 2004). Thus, the phosphorylation of GSK3 by AKT repressed GSK3 and enhanced cell migration and cancer metastasis. Therefore, AKT promoted cell proliferation and cell survival through inhibiting GSK3.

Furthermore, TSC2 (tuberous sclerosis complex 2, also Tuberin) is also a crucial substrate of AKT. TSC2, together with TSC1 and TBC, composes the TSC complex which is a negative regulator of mTORC1 (Huang J, 2008; Manning BD, 2017). The mTORC1 (mammalian targets of Rapamycin complex 1) is a protein complex that plays a central role in promoting anabolic processes, like the synthesis of proteins, nucleotides, lipids, which sequentially influence cell growth and proliferation (Laplante M, 2012; Saxton RA, 2017). The regulation of protein synthesis by AKT through mTORC1activation was illustrated for example. The phosphorylation of TSC2 by AKT resulted in the dissociation from RHEB (Ras homolog enriched in brain) and then RHEB loading with GTP activated the mTORC1; activated mTORC1 phosphorylated the transcription inhibitor 4EBP1 (eIF4E binding protein) and prevented 4EBP1 binding to eIF4E; eIF4E facilitated the translational initiation of HIF-1α which was involved in angiogenesis for tumor growth and metastasis (Brunn GJ, 1997; Gingras AC, 1999; Vogt PK, 2001; Dodd KM, 2015). And also mTORC1 activated S6 kinase through phosphorylation and the activation of S6 kinase up-regulated c-Myc translation, which promoted cancer cell growth and

proliferation (Holz MK, 2005; Csibi A, 2014). Moreover, mTORC1 was also activated by AKT through repressing the inhibitory effect of PRAS40, a regulatory component of mTORC1 (Sancak Y, 2007; Vander Haar E, 2007). Taken together, mTORC1 was activated by AKT through phosphorylating the mTORC1 regulators, which promoted cell growth and proliferation.

Finally, in addition to these three key substrates of AKT, AKT regulates other crucial substrates that play important roles in cell motility and cancer metastasis. AKT promotes cell motility and EMT and contributed to cell migration and cancer metastasis (Grille SJ, 2003). For instance, eNOS (endothelial nitric oxide synthase) is another AKT substrate that is involved in cell migration and angiogenesis. It was reported that AKT phosphorylated eNOS at Ser1177 and enhanced its catalytic activity to produce NO that was required for the migration of epithelial cell (Dimmeler S, 1999). Moreover, AKT regulates cell motility through the actin-binding protein Girdin (Enomoto A, 2005). Girdin was phosphorylated by AKT and bound to filamentous actin at the leading edge of migrating cells, which contributed to the formation of lamellipodia and cell motility (Enomoto A, 2005). In addition, AKT isoform AKT2 up-regulated β1-integrin expression and promoted the invasion and metastasis of breast and ovarian cancer cells (Arboleda MJ, 2003); knockdown of AKT2 inhibited the liver metastasis of cancer cell in xenograft mice (Rychahou PG, 2008). Therefore, AKT plays an important role in promoting cell migration and cancer metastasis.

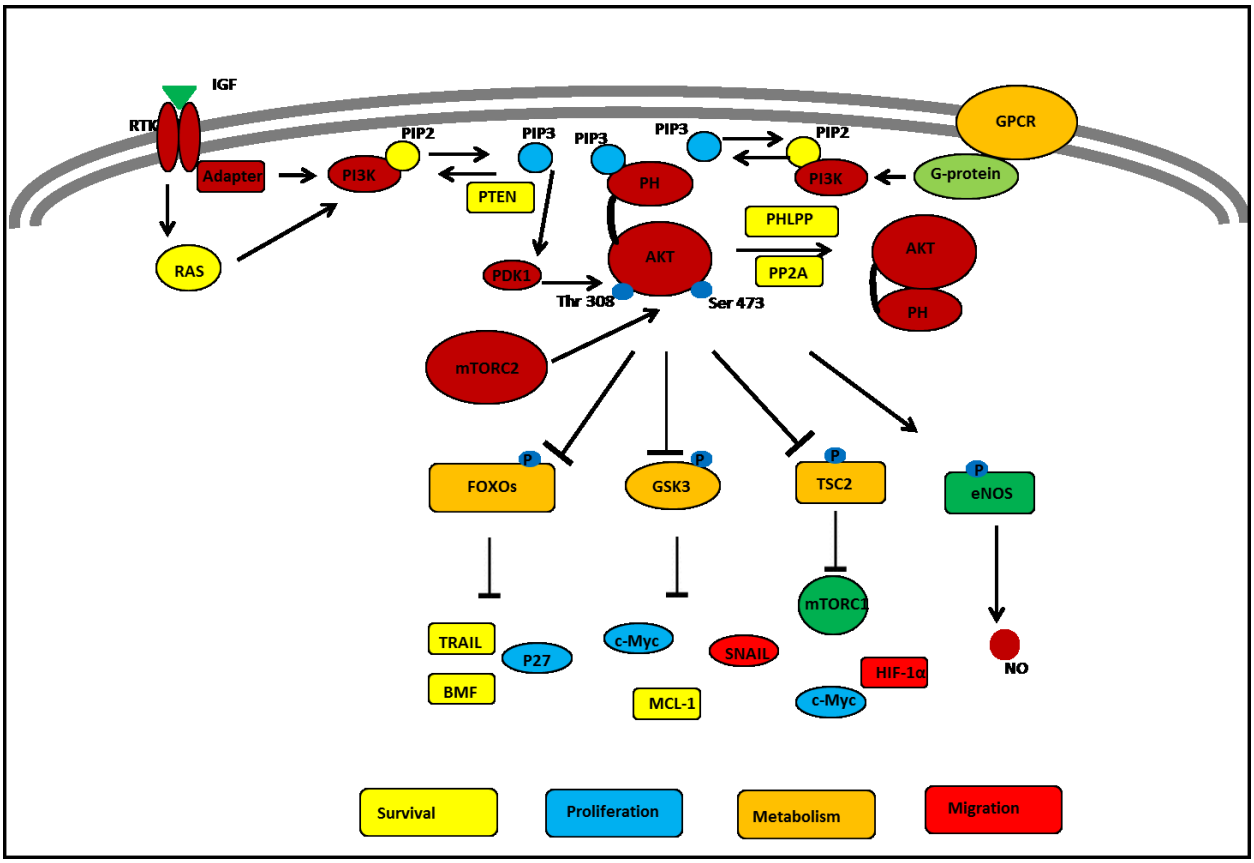


Figure 1. 2 The schematic of the PI3K-AKT pathway and its function.

The activation of the PI3K-AKT pathway is initiated by the stimulation of RTK or GPCR. RTK or GPCR associated with protein adapters recruits PI3K to the cytoplasm and produces second messenger PIP3 to activate AKT by phosphorylation at Thr 308 and Ser 473 of AKT. The activated AKT regulates the activity of its various downstream substrates (FOXOs, GSK3, TSC2 and eNOS) that are involved in cell survival, proliferation, metabolism and migration. The figure is developed based on the information from the publication by Manning BD et al. (Manning BD, 2014).

1.2.1.3 Regulation of PI3K-AKT pathway

The regulation of PI3K-AKT is controlled by regulators in many aspects. First, PI3K-AKT can be activated by extracellular signaling through the transmembrane receptor tyrosine kinases (RTK). The extracellular signal molecules (insulin or growth factor) bound to their receptors domains of RTK on cell membrane plasma and activated intracellular kinase function of these RTKs; the intracellular domains of these RTK with their scaffolding adaptors (IRS1/2) recruited PI3K to cytoplasm membrane and then promoted the producing of second messenger PIP3 (Vanhaesebroeck B, 2010). PIP3 recruited the inactivated AKT and interacted with PH domain of AKT, which facilitated the AKT activation through phosphorylation of AKT by PDK1 or mTORC2 (Manning BD, 2017). In addition, there are some G-protein–coupled receptors (GPCR) that can receive and transmit the external signaling and eventually activate AKT through RAS-dependent producing of PIP3 (Rodriguez-Viciana P,1994; Manning BD, 2017).

The most important activator for PI3K-AKT signal pathway is phosphoinositide 3-kinase (PI3K). The second messenger PIP3 is the mediator of the external signal and AKT activation, and PI3K controls the production of PIP3 (Hemmings BA, 2012). There are two types of PI3Ks that involve in the PI3K-AKT pathway and catalyze different substrates: class I PI3K and class II PI3K; the class I PI3K mediates the phosphorylation of PI(4,5)P2 and then produces PI(3,4,5)P3 at plasma membrane, while the class II PI3K catalyzes the phosphorylation of PI(4)P and then obtains PI(3,4)P2 at endomembrane. Both PI(3,4,5)P3 and PI(3,4)P2 can function as second messengers to recruit AKT and interact with the PH domain of inactivated AKT, then promote the activation of AKT (Hemmings BA, 2012; Manning BD, 2017).

The class I PI3Ks are protein complex that consists of the catalytic subunit and the regulatory subunit. The catalytic subunits include four isoforms: PI3K catalytic alpha polypeptide (p110-α, encoded by PIK3CA), PI3K catalytic beta polypeptide (p110-β, encoded by PIK3CB), PI3K catalytic gamma polypeptide (p110-γ, encoded by PIK3G) and PI3K catalytic delta polypeptide (p110-δ, encoded by PIK3D); the regulatory subunits include: p85-α, p85-β, p55-α, p150, p101 and p87 (Jean S, 2014). The catalytic subunit together with regulatory subunits composes a class I PI3K complex. The class II PI3Ks only have a catalytic unit like PI3KCα, PI3KCβ and PI3KCγ and catalyze the phosphorylation of PI and PI4P. The most frequent mutations on PI3Ks in breast cancer were reported in the catalytic subunit of p110-α and the regulatory subunit p85-α. The mutations in p110-α led to dismissing the inhibitory effect from regulatory subunit p85 and the constitutive activation of PI3K. Similarly, the mutations in p85α resulted in the loss of the inhibitory binding of p85-α to catalytic subunit p110-α which also led to the hyper-activation of p110-α. Eventually, all of these mutations upregulated the PI3K-AKT signal pathway and induced oncogenic transformation in breast cancers (Chen L, 2018). However, the mutation on PIK3CA was found in 8% of TNBC patients with basal-like features (3/38) in Martin's study (Martin V, 2012). Therefore, the mutations of PI3KCA were not the leading cause of over-activation of the PI3K-AKT pathway in TNBC.

As discussed above, the activation of PI3K-AKT requires second messenger PIP3 and the phosphorylation of AKT. Thus, the termination of the PI3K-AKT signal pathway is achieved in two different ways. Either the dephosphorylation of activated AKT or dephosphorylation of PIP3 contributes to the termination of the PI3K-AKT pathway. On the one hand, protein phosphatases contribute to the termination of PI3K-AKT through dephosphorylating AKT. PH domain leucine-rich repeat protein phosphatase (PHLPP) is a type of phosphatases that dephosphorylate

AKT at Ser473 and attenuate the activation of the PI3K-AKT pathway (Chen M, 2011). PHLPP had the function of a tumor suppressor and the reduced expression of PHLPP was observed in various cancers (Talantov D, 2005; Liu J, 2009; Chen M, 2011). Moreover, another AKT phosphatase PP2A (protein phosphatase 2A) was identified to dephosphorylate AKT at Thr308, which also resulted in the inactivation of the PI3K-AKT pathway (Kuo YC, 2008). On the other hand, lipid phosphatases that dephosphorylate PIP3 can also contribute to termination of PI3K-AKT signaling. PTEN is a dual phosphatase that dephosphorylates PIP3 to PIP2, which antagonizes the effect of PI3K and will be discussed in the following part (Maehama T, 1998). And the inositol polyphosphate-4-phosphatase type II (INPP4B) is also a lipid phosphatase that converts PI(3,4)P2 to PI3P; PI(3,4)P2 can also bind to the PH domain of AKT and activate AKT at endomembrane (Lopez SM, 2013). Therefore, INPP4B is a negative regulator of PI3K signaling. And it was found that low expression of INPP4B correlated with high tumor grade, larger tumor size, co-loss of PTEN and poor outcome in breast and ovarian cancer, which indicated that INPP4B acted as a tumor suppressor (Gewinner C, 2009). However, recent studies revealed a converse role of INPP4B. Loss of INPP4B led to the accumulation of PI(3,4)P2 but the decrease of p-AKT level in PTEN-null TNBC cells, which lowered the cell proliferation (Reed DE, 2017). These results suggest that the regulation of PI3K-AKT in cancers is a complicated process that requires further investigation.

Although the phosphorylation on Ser473 and Thr308 of AKT is crucial to the activation of AKT, other phosphorylation sites on AKT can also affect the stability or activity of AKT. Thr450 which was located in the turn motif of AKT was constitutively phosphorylated by mTORC2 and contributed to keeping the proper folding of AKT polypeptide and the stability of AKT (Facchineti V, 2008). Despite by mTORC2, Ser477 and Thr479 were also be

phosphorylated by cyclin A-CDK2 complex and helped the promotion of Akt activation (Liu P, 2014). By contrast, GSK3- mediated phosphorylation of Thr312 functioned as a negative signal to attenuate the AKT activity (Gulen MF, 2012). Thus, the phosphorylation on different sites of AKT exhibits the significance of the regulation of AKT.

In addition, the regulation of AKT is also performed by other post-translational modification. Since ubiquitination on multiple lysine residues was identified on AKT, ubiquitination was an important way to regulate the activity or the localization of AKT (Manning, BD, 2017). The AKT-specific E3 ligase TTC catalyzed poly-ubiquitination of AKT and promoted AKT degradation through 26S proteasome, which eventually inhibited the PI3K-AKT pathway (Suizu F, 2009). Conversely, the polyubiquitination of AKT mediated by E3 ligase TRAF6 contributed to the localization of AKT on the plasma membrane, which enhanced the activation of AKT by PI3K (Yang WL, 2009). However, this activation of AKT by ubiquitination was dismissed by the deubiquitination enzyme CYLD (Lim JH, 2012). Similarly, AKT was also reported to be sumoylated at Lys276 by SUMO E3 ligase PIAS, which was proposed to promote AKT activation and tumorigenesis (Lin CH, 2016). Finally, acetylation at Lys14 of AKT played a functionally important role on the AKT activation. Acetylation of Lys14 was required for the PH domain of AKT binding to PIP3 and helped the translocation of AKT to the plasma membrane for activation (Sundaresan NR, 2011).

1.2.2 Tumor suppressor PTEN

A tumor suppressor is a type of molecule that can control cell growth and prevent the formation of cancer cells; genomic alteration of tumor suppressors (gene mutation or loss) can

result in inactivity of the tumor suppressor genes and the functional failure of preventing pathological conversion from normal cells to cancer cells (Weinberg RA, 2014). Heretofore, some tumor suppressors have been identified in humans and one of these important tumor suppressors is *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) (Maehama T, 1998). The genomic alteration related to PTEN was first reported in the 1980s (Bigner SH, 1984). Homozygous deletion on chromosome 10 was found in patients with brain, prostate, and breast cancer (BCa), and further molecular studies revealed that *PTEN* (also named mutated in multiple advanced cancers [*MMAC1*]), was a novel gene on chromosome 10 and related to these cancers (Li J, 1997; Steck PA, 1997). Furthermore, germline mutations of *PTEN* were discovered in patients with the cancer predisposition syndrome, Cowden disease (Liaw D, 1997). Following that, the *Pten* knockout model by different research groups confirmed the essential role of PTEN on tumor suppression in multiple tissues (Di Cristofano A, 2001; Suzuki A, 1998; Podsypanina K, 1999). Therefore, *PTEN* was identified as a tumor suppressor and is frequently inactivated in multiple cancers or diseases.

As a tumor suppressor, *PTEN* exhibits the trait of haploinsufficiency for preventing cancer progression in multiple cancers (Di Cristofano A, 1999 & 2001). This means that the subtle downregulation of *PTEN* caused by the inactivation of one *PTEN* allele results in the loss of *PTEN* tumor-suppressing function. In the *Pten* knockdown mouse model, the functional consequence was enhanced cancer susceptibility and favoring of cancer progression due to the subtle change of PTEN protein level (Alimonti A, 2010). These observations indicate that PTEN is a crucial player in tumor suppression, and downregulated PTEN increases the risk of cancer.

PTEN is a dual-specificity phosphatase with 403 amino acids that catalyzes the dephosphorylation of both lipid substrates and protein substrates. PTEN dephosphorylates

protein substrates on tyrosine (Tyr), serine (Ser), and threonine (Thr) phosphorylated peptides. The substrates of PTEN protein phosphatase include focal adhesion kinase (FAK) and adapter protein SHC; the dephosphorylation by PTEN usually results in the inactivation of these substrates (Tamura M, 1999; Gu J, 1999). For example, PTEN interacts with FAK and results in the dephosphorylation of FAK at Tyr397, which increases the adhesion with extracellular matrix and inhibits cell migration (Tamura M, 1999).

However, the anti-tumor function of PTEN mostly relies on its lipid phosphatase activity. As an important cellular second messenger, PIP3 conveys the extracellular signal to activate PI3K–AKT signaling and PTEN can shut off this signal transmission by dephosphorylating PIP3 to PIP2. In detail, the extracellular signal insulin activates PI3K through IRS1, and then PI3K phosphorylates PIP2 to PIP3. PIP3 recruits a subset of downstream effectors (AKT) to the plasma membrane and promotes AKT activation through phosphorylation at Ser473 or Thr308. The activated phosphorylated (p)-AKT acts as a survival factor and promotes cell proliferation and cell metabolism by phosphorylating its downstream targets, including glycogen synthase kinase 3 (GSK3), forkhead box O 1 (FOXO1), BCL-2 antagonist of cell death (BAD), ubiquitin E3 ligase MDM2, and cell cycle proteins (Manning BD, 2017). Therefore, the anti-tumor function of PTEN it exerted on cell cycle progression, cell proliferation, and cell migration.

First, PTEN is involved in regulating cell cycle progression by inhibiting the PI3K–AKT pathway. PTEN inactivation results in the activation of AKT kinase, and then AKT inhibits GSK3 by phosphorylating it, leading to the accumulation of cyclin D1 and promoting cell cycle progression (Diehl JA, 1998). Moreover, PTEN abrogates the functional repression of FOXO1 by inhibiting AKT. FOXO1 functions as an important transcription factor and is involved in cell cycle arrest; FOXO1 promotes the transcription of cell cycle kinase inhibitor (CKI) p27kip1,

which is a negative regulator of the cell cycle (Medema RH, 2000). In addition, PTEN is also related to the G2–M checkpoint in the cell cycle. The loss of PTEN leads to the AKT-mediated phosphorylation of cell cycle regulator checkpoint kinase 1 (CHEK1) and inhibits the translocation of CHEK1 from the cytoplasm to the nucleus, which prevents its role in regulating the G2–M checkpoint and promotes the accumulation of double-strand breaks in tumor cells (Puc J, 2005a & 2005b).

Second, PTEN plays an important role in regulating cell motility and cell migration. It has been confirmed that, compared to PTEN-wt (wild-type) cells, PTEN-knockout cells exhibit increased rates of cell motility; reintroducing PTEN-wt, but not lipid phosphatase–deficient mutants PTEN G129E or PTEN C124S, reduced the enhanced cell motility of PTEN-knockout cells, which indicates that PTEN is a negative regulator of cell motility (Liliental J, 2000). *PTEN* deficiency is associated with cancer metastasis in multiple cancers (Chiang KC, 2016; Mulholland DJ, 2012; Dankort D, 2009). Moreover, PTEN is also related to cell polarity and epithelial-to-mesenchymal transition (EMT). Cytoplasmic PTEN localizes to the apical plasma membrane and catalyzes the conversion of PIP3 to PIP2 and recruits annexin 2, which attracts CDC42 to bind to the partition defective 6–atypical PKC (PAR6–aPKC) complex to establish cell polarity. However, PTEN inactivation leads to failed development of the apical surface and lumen, and then the loss of cell polarity (Martin-Belmonte F, 2007). Eventually, such cells lose the epithelial cell characteristics and obtain the properties of mesenchymal cells and accomplish EMT, which increases cell motility and invasiveness (Mulholland DJ, 2012; Song LB, 2009).

In addition, PTEN is a negative regulator of cell proliferation. As discussed above, the activated PI3K–AKT pathway can promote cell proliferation, and PTEN inhibits the activation of AKT. It has also been reported that PTEN acts as an inhibitor of cell proliferation and cell

survival by negatively regulating the p-AKT signaling pathway (Stambolic V, 1998, Datta SR, 1999). *PTEN* deficiency promotes tumorigenesis and malignant transformation in various cancers (Halvorsen OJ, 2003; Steck PA, 1997). In terms of BCa, the loss of *Pten* caused tumorigenesis in mouse mammary gland, and heterozygous deficiency of PTEN with Wnt1 accelerated the development of ductal carcinomas in the mammary gland (Kishimoto H, 2003; Li Y, 2001). Therefore, *PTEN* is a crucial tumor suppressor through negative regulation of the PI3K–AKT pathway.

The anti-tumor function of PTEN is not limited to its lipid dephosphorylation activity in the cytoplasm. When PTEN is translocated to the nucleus, it plays an important role in maintaining genomic stability (Baker SJ, 2007). There is convincing evidence to support the premise that the absence of PTEN in the nucleus is associated with the aggression of malignant cancers (Gimm O, 2000; Baker SJ, 2007; Milella M, 2017). For example, double-strand DNA repair is regulated positively by nuclear PTEN through the activation of RAD51, a key protein in double-strand repair; acting as a co-transcription factor, nuclear PTEN activates the transcription of RAD51 (Shen WH, 2007). This DNA repair function of PTEN does not require its lipid phosphatase activity. Moreover, in the nucleus, PTEN increases the E3 ligase activity of APC/C by enhancing the association of APC/C with its activator CDH1; this activation of APC/C promotes the degradation of oncoproteins polo-like protein 1 (PLK1) and Aurora kinases and consequently increases the anti-tumor activity of the APC/C–CDH1 complex (Song MS, 2011). In addition, PTEN deficiency activates the Tyr kinase SRC and enhances resistance to human epidermal growth factor receptor 2 (HER2) in BCa cells, which is independent of the lipid dephosphorylation activity of PTEN (Zhang S, 2011).

1.2.3 The regulation of PTEN

As PTEN plays a vital role in various biological events, its regulation has a dramatic influence on human cancers. To better understand the tumor suppressor function of PTEN, I will provide a simple clarification of the pathways or mechanisms involved in regulating PTEN. The tumor suppressor function of PTEN is affected by different mechanisms, such as genetic alteration, transcription activation or repression, post-transcription regulation, and protein interaction. These mechanisms exert their influence on PTEN separately or coordinately (Figure 1.3).

1.2.3.1 Genomic alteration of PTEN

The genetic alteration of *PTEN* is a very common phenomenon in multiple human tumors. Cowden Syndrome is a typical PTEN hamartoma tumor syndrome (PHTS), and it is related to germline mutation of the *PTEN* gene; approximately 80% of patients with Cowden Syndrome harbor a *PTEN* mutation (Liaw D, 1997). Forty percent of *PTEN* mutations are located in the catalytic domain of PTEN, which imparts the phosphatase activity in Cowden Syndrome; the remaining *PTEN* mutations alter the localization of PTEN protein (Orloff MS, 2008). In addition, heterozygous or homozygous deletions of *PTEN* have also been widely observed in multiple cancers, and result in the partial or total inactivation of PTEN (Dillon LM, 2014; Marsit CJ, 2005; Zhang HY, 2013). For example, the loss of *PTEN* alleles was observed in 50% of prostate cancer cases (Cairns P, 1997). The *PTEN* mutation rate was relatively low (4%, 2/54) in primary BCa and the *PTEN* expression rate in BCa (57.5%, 84/146) was much lower than that in normal breast

tissue (100%, 10/10) (Rhei E, 1997; Zhang HY, 2013). Despite taking into account *PTEN* genomic DNA mutations or deletions, PTEN expression was unexpectedly low in some patients with tumor with an intact *PTEN* genomic background, which indicates that there are alternative mechanisms regulating *PTEN* expression (Marsit CJ, 2005).

1.2.3.2 Transcriptional regulation of PTEN

As a tumor suppressor, the transcription of *PTEN* is negatively regulated by some oncogenic proteins or signaling pathways. For example, the proto-oncogenic protein c-Jun, a component of the AP-1 family of transcription factors, could bind to the 5′ upstream sequences of the *PTEN* promoter and repress *PTEN* transcription (Hettinger K, 2007). The ecotropic virus integration site 1 protein (EVI1), which is a nuclear protein and associated with leukemia, can reduce PTEN expression by directly repressing *PTEN* transcription in the hematopoietic system (Yoshimi A, 2011). In addition, the transcription factor BMI1, which is overexpressed in multiple cancers, can inhibit PTEN transcription (Song LB, 2009). As negative regulators of PTEN, the EMT transcription factor SNAI1 and the oncogenic factor ID1 can competitively bind to the promoter of *PTEN* and repress its transcription (Escriva M, 2008; Lee JY, 2009). On the other hand, PTEN upregulation at transcription level has also been identified. It has been established that the tumor suppressor p53 functions as a transcription factor to activate the expression of downstream genes. It has been confirmed that p53 binds to the promoter of *PTEN* and activated its transcription in vitro (Stambolic V, 2001). The activation of the ligand-activated nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) can upregulate *PTEN* transcription by binding to the upstream elements of the *PTEN* promoter in colorectal cancer

cells and BCa cells (Patel L, 2001). Moreover, NOTCH1 associates with C-repeat binding factor 1 (CBF1) to activate PTEN transcription (Whelan JT, 2007). Taken together, *PTEN* transcription is controlled by a complicated regulatory network, and its regulation is dependent on genetic background and ectopic activation of the signal pathway.

1.2.3.3 PTEN regulation on a post-transcription level by non-coding RNAs

The regulation of PTEN at transcription level has been illustrated above. However, there are other mechanisms control PTEN expression after its transcription, one of which is non-coding RNAs (Dillon LM, 2014). Non-coding RNAs are RNAs that do not directly produce proteins by translating their triple codon, but influence the expression of other proteins (Mattick JS, 2006; Pang KC, 2006). The non-coding RNAs include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small interfering RNAs (siRNAs), small nucleolar RNA (snoRNAs), competitive endogenous RNAs (ceRNAs), and microRNAs (Mattick JS, 2006; Pang KC, 2006).

MicroRNAs are non-coding RNAs that are 19–25 nucleotides in length; they can regulate the translation of their target mRNA by binding to the 3′ untranslated region (3′UTR) of a protein-encoding mRNA by RNA sequence base-pairing; microRNA binding to mRNA represses the expression of the target gene by inhibiting translation or promoting mRNA degradation (Han J, 2004; Mattick JS, 2006). MicroRNA-induced gene inactivation is one of the new mechanisms identified in the regulation of *PTEN* expression and activity (Pezzolesi MG, 2008; Huse JT, 2009). In previous studies, some microRNAs inactivated *PTEN* and acted as oncogenic players in multiple cancers (Dillon LM, 2014; Garofalo M, 2009). For example, miR-221 and miR-222 are overexpressed in non–small cell lung cancer and hepatocellular carcinoma

and promote tumorigenesis by targeting *PTEN* mRNA (Garofalo M, 2009); in ovarian cancer, miR-214 promotes cell survival and induces cisplatin resistance by targeting and inhibiting *PTEN* (Yang H, 2008); miR-21 was overexpressed in human hepatocellular cancer (HCC) tissues and cell lines, binding to *PTEN* mRNA and functioning as an oncogenic miR (Meng F, 2007); the upregulation of miR-144 in nasopharyngeal carcinoma enhanced cell proliferation, migration, and invasion by repressing *PTEN* (Zhang LY, 2013). Therefore, *PTEN*-targeting microRNAs act as negative regulators of *PTEN*, inactivating it.

Moreover, another non-coding RNA is involved in regulating PTEN. The ceRNA has the same binding sequence as its competing mRNA, which acts as a decoy to attract mRNA-targeting microRNAs and protects its competing mRNA from microRNA-induced translation repression. *PTENP1* is a cognate gene of *PTEN* that shares a homologous sequence with *PTEN* (Poliseno L, 2010). Some studies have revealed that the loss of *PTENP1* correlated positively with decreased expression levels of PTEN in endometrial, colon, and prostate cancer tissues (Haddadi N, 2018). These findings suggest that *PTENP1* functions as a guardian of *PTEN* and has a positive effect on the regulation of *PTEN* (Poliseno L, 2010). However, recent studies have reported the dual-role of *PTENP1* on *PTEN* regulation. *PTENP1* has three transcripts: one sense [PTENP1(S)] and two antisense [PTENP1(ASα) and PTENP1(ASβ)]; PTENP1(S) protects *PTEN* mRNA by competing with the mRNA to bind with microRNAs; PTENP1(ASβ) can interact with PTENP1(S) and stabilize it (Johnsson P, 2013). However, in the nucleus, PTENP1(ASα) acts as a negative regulator of *PTEN* transcription; it recruits the methyltransferase enhancer of zeste homolog 2 (EZH2) and DNA methyltransferase 3a (DNMT3a) to the *PTEN* promoter and represses *PTEN* transcription (Karreth FA, 2011). Therefore, non-coding RNAs play a complicated role in the regulation of *PTEN*.

1.2.3.4 Regulation of PTEN by post-translational modification

While *PTEN* translation may have been accomplished, there remain various mechanisms that control the stability or the activity of PTEN. Post-translational modification is a predominant means of regulating PTEN stability and activity; these modifications include phosphorylation, acetylation, oxidation, and ubiquitination (Bermúdez Brito M, 2015). The phosphorylation of PTEN has been identified at multiple residues of amino acids, such as Ser, Thr, and Tyr. The phosphorylation of PTEN at Ser380 and Ser385 at the C-terminal by casein kinase 2 (CK2) represses its phosphatase activity by changing the conformation of PTEN and inhibiting the interaction between PTEN and its substrates (Torres J, 2001; Bermúdez Brito M, 2015). Likewise, the alteration of conformation can also prevent its interaction between PTEN and the PTEN-associated transmembrane proteins and then block the localization of PTEN to the plasma membrane (Liang K, 2010). With the stimulation of chemoattractants, PTEN is also phosphorylated by RHOA-associated protein kinase (ROCK) at Ser229 and Thr321 in leukocytes; but the phosphorylation on these sites can activate PTEN and promote its localization to the plasma membrane, which has an inverse effect on PTEN compared to phosphorylation at Ser380 and Ser385 (Li Z, 2005). Moreover, PTEN phosphorylation at Tyr336 by the Tyr protein kinase FRK promotes the stability of PTEN by preventing the ubiquitination mediated by NEDD4-1 (neural precursor cell expressed developmentally downregulated protein 4-1) and the degradation by the proteasome (Yim EK, 2009). Taken together, these results suggested that the diverse phosphorylation of PTEN affected the regulation of PTEN.

Furthermore, ubiquitination is also important to the regulation of PTEN after translation. Ubiquitin is a small, 76–amino acid protein molecule and widely exists in most eukaryotic organisms; ubiquitination is a process where one or more ubiquitin molecules are linked to its substrate proteins through an isopeptide bond formed between the carboxyl group (COO−) of ubiquitin's glycine and the epsilon-amino group (ε-NH3+) of the substrate's lysine (Lys) (Pickart CM, 2004). The addition of a single ubiquitin is termed mono-ubiquitination, whereas a second or more ubiquitin can continuously be added to the former ubiquitin to construct a ubiquitin chain in a process termed poly-ubiquitination; this process is mediated and catalyzed in cascade by a series of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) (Pickart CM, 2004). Briefly, the ubiquitin molecule is activated by E1 and bound to the E1 cysteine sulfhydryl group. Then, E2 interacts with E1 and transfers ubiquitin to the active cysteine in E2. Lastly, E3 ligase recognizes the ubiquitin substrate and E2 with ubiquitin, and transfers the ubiquitin molecule to the substrate by creating an isopeptide bond between the carboxyl group (COO-) of the ubiquitin molecule's glycine and the epsilon-amino group (ε-NH3+) of the substrate's Lys (Lecker SH, 2006).

The function of ubiquitination is dramatically different between mono-ubiquitination and poly-ubiquitination. Although it is the initial step of the poly-ubiquitination of substrates, mono-ubiquitination also contributes to the nuclear–cytoplasmic shuttling of substrates and to endocytosis (Schnell JD, 2003). The mono-ubiquitination at Lys289 or Lys13 of PTEN is crucial to the nuclear importing of PTEN, which affects the tumor suppression function. In Cowden syndrome and cancers, mutations on these Lys residues contribute to the deficiency of PTEN shuttling from the cytoplasm to the nucleus. Conversely, the mono-ubiquitination of PTEN is reversed by the deubiquitinase HAUSP and thus the nuclear importing of PTEN is abolished and

tumor suppressor function of PTEN is inhibited (Colland F, 2009). Contrary to mono-ubiquitination, the poly-ubiquitination of substrates mainly acts a death tag and leads to proteasome degradation; PTEN with a Lys48-linked poly-ubiquitination chain is recognized and deconstructed by the 26S proteasome (Lecker SH, 2006). NEDD4-1 was first identified as the E3 ligase for PTEN ubiquitination (Wang X, 2007). NEDD4-1 expression correlates negatively with PTEN expression in bladder cancer, which proves that it is a negative regulator of PTEN through proteasome-dependent degradation (Wang X, 2007). However, it is still being debated whether the regulatory role of NEDD4-1 contributes to PTEN, as PTEN was unchanged in NEDD4-1 deficient mice (Fouladkou F, 2008). Following the discovery of NEDD4-1, other E3 ligases for PTEN ubiquitination have been observed. For example, XIAP, a RING domain E3 ligase, interacts with PTEN and promotes PTEN mono-ubiquitination in MCF-7 BCa cells, which contributes to the nuclear localization of PTEN (Van Themsche C, 2009); as an E3 ligase, MKRN1 can respond to the activation of EGFR and promote PTEN ubiquitination and degradation in cervical cancer (Lee MS, 2015). In sum, the ubiquitination of PTEN plays a vital role in the regulation of PTEN.

Finally, other post-translational modifications are also involved in the regulation of PTEN. The acetylation of PTEN takes place at Lys125 or Lys128 by PCAF; the acetylation at these sites enhances the interaction with proteins containing the PDZ domain and consequently reduces the catalytic activity of PTEN (Okumura K, 2006; Ikenoue T, 2008). Moreover, PTEN oxidation can be induced by reactive oxygen species (ROS). In primary T cell leukemia, a disulphide bond between cysteine (Cys)71 and Cys124 on PTEN formed under high H_2O_2 stress (Lee SR, 2002).

1.2.3.5 Regulation of PTEN activity by protein-protein interactions

It has been established that post-translational modifications can influence the stability and anti-tumor activity of PTEN through interaction with small molecules via covalent binding. In addition to these modifications, non-covalent interaction with other proteins can also regulate the stability and activity of PTEN. The interaction with proteins alters the conformation of PTEN and thus regulates its phosphatase activity; several positive regulators of PTEN have been demonstrated to enhance PTEN activity (Wu X, 2000; Cottor L, 2010). For example, the β-arrestins (β-arr1 and β-arr2), which are ubiquitously expressed in cells and can regulate G protein–associated receptor, acts as a scaffolding protein or directly binds to the C2 domain of PTEN, which enhances PTEN phosphatase activity and represses the PI3K–AKT pathway (Lima‐Fernandes E, 2011). Membrane-associated guanylate kinase inverted 2 (MAGI2), which is downstream of ROCK activation, can bind to the PDZ-binding domain of PTEN and elevate PTEN activity (Wu X, 2000). Another positive regulator of PTEN can interact with PTEN and improve its stability. In schwannoma, mammalian disks larger homologue 1 (DLG1) interacts with PTEN and thereby enhances its stability and phosphatase activity (Cottor L, 2010). Moreover, some proteins bind to PTEN and indirectly promote its activity. The motor protein myosin V directly binds to PTEN and improves its movement to the cytoplasmic membrane, which facilitates the dephosphorylation activity of PTEN on PIP3 (Van Diepen MT, 2009). In contrast, some negative regulators of PTEN exert their effect by interacting with PTEN. Recent studies have identified that, in BCa, PIP3-dependent RAC exchanger factor 2a (PREX2a) directly binds to PTEN and inhibits its phosphatase activity on PIP3; the overexpression of PREX2a resulted in the ectopic activation of AKT and thereby cell proliferation in a PTEN-dependent manner (Fine B, 2009). Moreover, shank-interacting protein-like 1 (SIPL1) interacts

with PTEN and inhibits its phosphatase activity in cervical cancer cells, which promotes tumorigenesis (He L, 2010). Therefore, these results indicate that protein interaction with PTEN also plays an important role in the regulation of PTEN activity.

Generally, the regulation of PTEN and its function by protein–protein interactions are complicated, and the effect of this regulation can be positive or negative for PTEN, mainly dependent on its role of interaction targets.

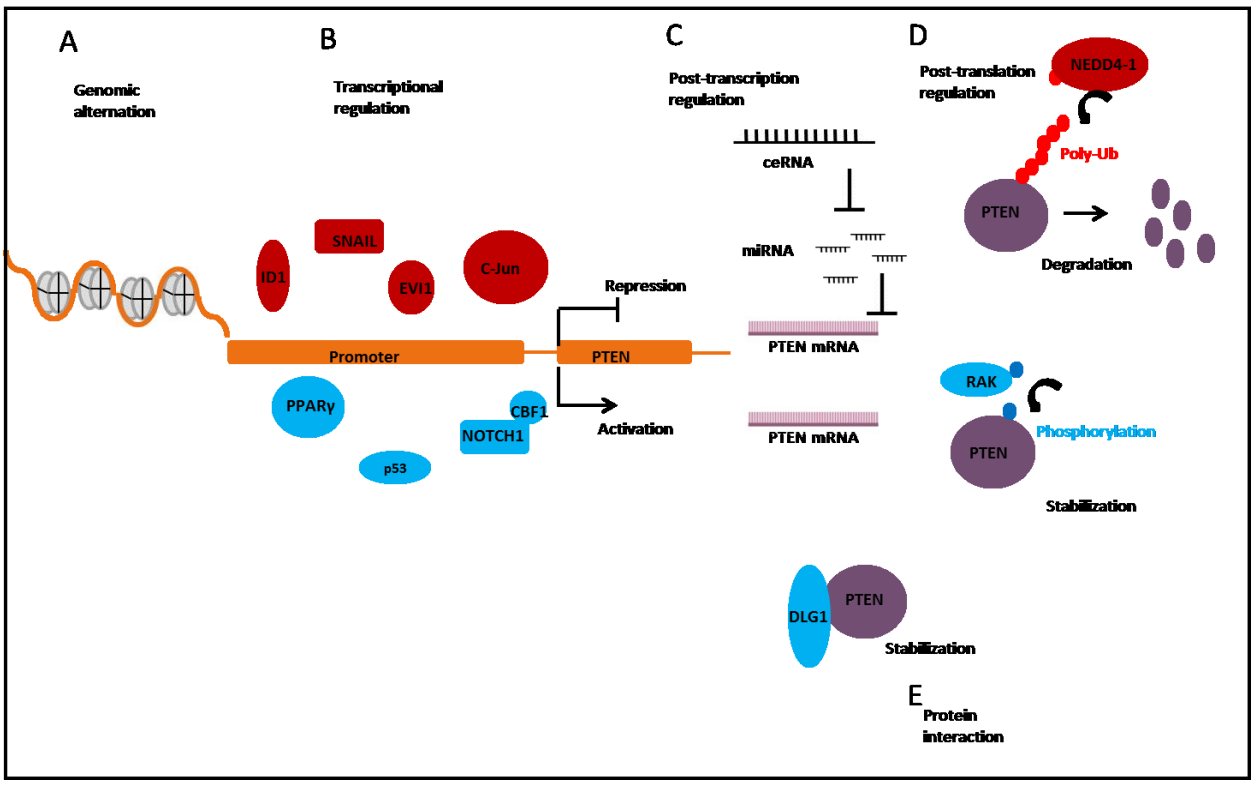


Figure 1. 3 The regulation of PTEN.

The regulation of PTEN is performed at multiple levels. A) The genetic alteration on PTEN (gene mutation or loss) results in the inactivity of PTEN and the functional failure as a tumor suppressor. B) Transcription activation or repression affects the expression of PTEN through a variety of transcription factors. C) Non-coding RNAs (miRNA or ceRNA) are involved in the regulation of PTEN. D) The post-translation modification of PTEN affects PTEN expression through stabilizing PTEN protein or promoting PTEN degradation. E) The interaction with other proteins stabilizes PTEN in the cytoplasm. This figure is created based on information from the publication by M Bermúdez Brito et al. (M Bermúdez Brito 2015).

1.3 MicroRNA in cancer

1.3.1 MicroRNA and its function

1.3.1.1MicroRNA biogenesis

MicroRNA (miRNA) is a type of small non-coding RNA which is a single strand RNA of 19-25 nucleotides (Mattick JS, 2006). The first miRNA lin-4 was found in Caenorhabditis. elegans in 1993 (Lee RC, 1993). Since then, numerous miRNAs have been identified and there are 38,589 miRNAs annotated in the database of miRbase in 2018. And these miRNAs are involved in the cellular processes including cell proliferation, migration, differentiation and apoptosis, through participating in the post-translational regulation of key proteins expressions such as enzymes, tumor suppressors and transcription factors (Hwang HW, 2006). Therefore, though not encoding proteins directly, miRNAs still play an important role in regulating these cellular processes through key protein targets.

Similar to other protein-coding genes, the biogenesis of miRNA is a multiple-step process and starts from gene transcription. A miRNA can be transcribed from an individual microRNA gene containing its own promoter, or from a spliced proportion of a protein-coding gene; the transcription of an individual miRNA gene is performed by the RNA polymerase II and an initial form of miRNA is named as primary miRNA (pri-miRNA), which contains ploy-A tail at 3' end and a 7-methylguanosine cap at 5' end; the pri-miRNA is recognized by Drosha ribonuclease and its protein partner DGCR8 (DiGeorge syndrome critical region 8) which is a double-stranded RNA binding protein and leads to the interaction with the stem-loop structure of pri-miRNA, and then pri-miRNA is processed to pre-miRNA by Drosha (Han J, 2004). The pre-

miRNA is a hairpin-structured RNA of approximately 70 nucleotides; some pre-miRNAs are directly from an intron proportion of other mRNA processed by mRNA splicing (MacFarlane LA, 2010). Subsequently, the pre-miRNA interacts with GTP-binding nuclear protein Ran (RanGTP)/exportin-5 (XPO5) complex and then is exported from nucleus to cytoplasm, in which pre-miRNA is further cleaved by Dicer complex composed by RNase III enzyme Dicer, transactivation-responsive RNA-binding protein 2 (TARBP2) and Argonaute protein (AGO) (MacFarlane LA, 2010). The cleaved product of pre-miRNA is a double-strand miRNA-miRNA duplex, and then the double-strand duplex separates and generates two single-strand miRNAs: the mature miRNA and the passenger miRNA; the major of mature miRNAs, are cooperated with AGO to form RNA induced silence complex (RISC) in cytoplasm and function as a regulator through binding with protein-coding mRNA or competitive endogenous RNA (ceRNA) which contain circular RNAs and pseudogenes mRNA s; some miRNAs are exported from cytoplasm via exosomes, and they enter neighboring cells where miRNAs bind to their intracellular targets or enter the circulation system (circulating miRNAs) (MacFarlane LA, 2010) (Figure 1.4).

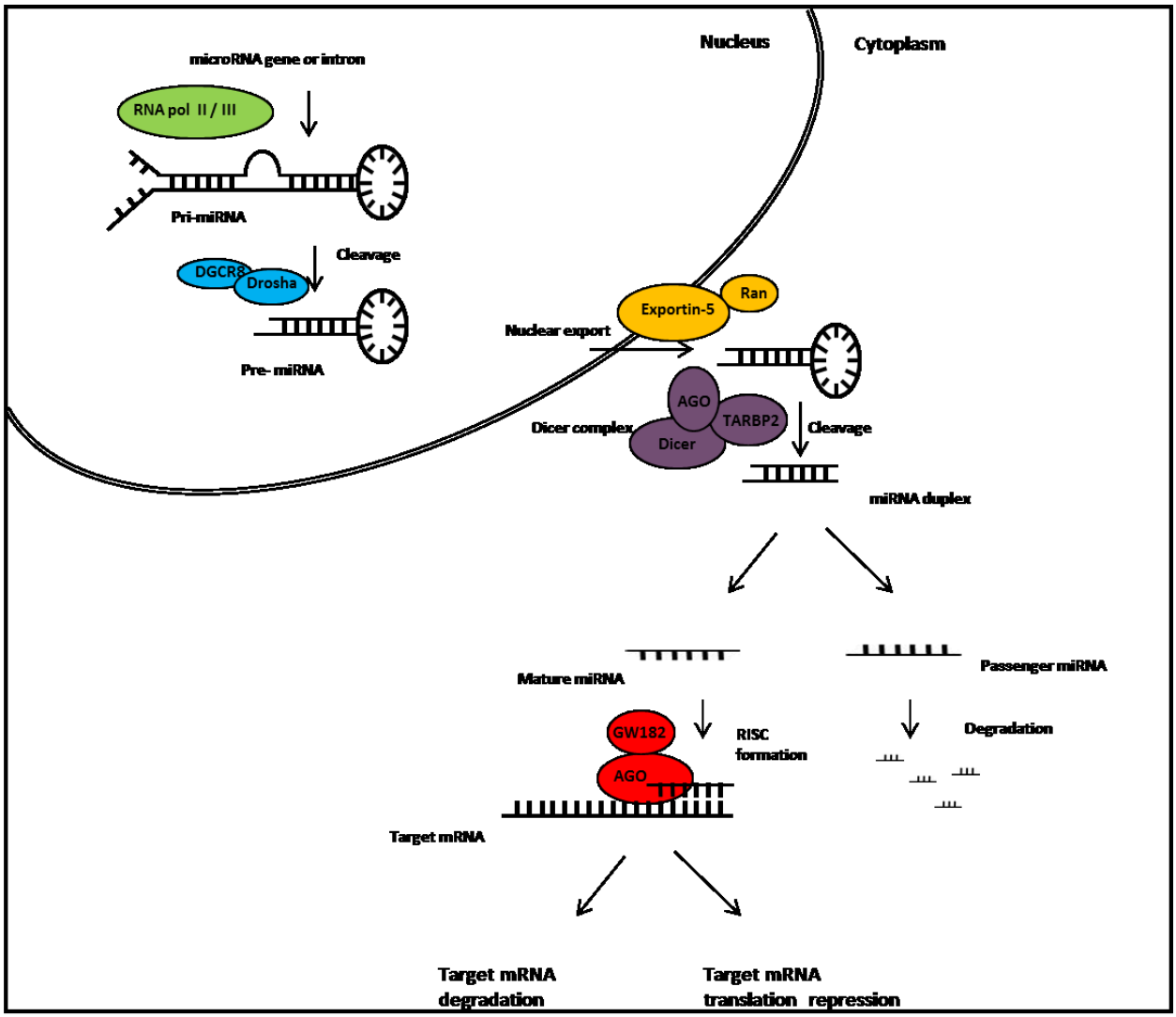


Figure 1. 4 The biogenesis of miRNA and its function.

The process of miRNA biogenesis is schematically represented. The pri-miRNA is transcribed from miRNA genes or intronic DNA; pri-miRNA is recognized by Drosha-DGCR8 complex and processed to pre-miRNA. The pre-miRNA is binding with XPO5-Ran complex and exported from nucleus to cytoplasm. Dicer complex cleaves the pre-miRNA into double-strand miRNA duplex. The double-strand miRNA duplex separates and single-strand mature miRNA is loaded into the RISC, which results in the target mRNA degradation or translation repression. This figure is based on information from the publication by Bertoli et al. (Bertoli G, 2015).

1.3.1.2 MicroRNA modification

After transcription of miRNA, the sequence of miRNA can be modified by several mechanisms, including miRNA editing, miRNA alternative cleavage and non-templated nucleotide addition, which give the diversity of miRNA and regulate the stability of miRNA (Krol J, 2010; Gebert LFR, 2019). The miRNA editing was recently found and denoted the nucleotide alteration on pri-miRNA sequence. The nucleotide alteration from adenosine to inosine was performed by double-stranded RNA-specific adenosine deaminase ADAR (adenosine deaminases acting on RNA). The conversion in pri-miRNA from adenosine to inosine made a nucleotide mismatch in double-strand pri-miRNA and affected the further step in miRNA maturation, which changed the sequence of pre-miRNA or promoted the degradation of pri-miRNA (Nishikura K, 2016). Moreover, alternative cleavage on miRNA precursor can also alter the sequence of mature miRNA and produce different miRNA isoforms (isomiRs) (Gebert LFR, 2019). Although transcribed from the same miRNA gene, there were different mature miRNA isoforms that exhibited slight alteration on their sequence. In the nucleus, the alternative cleavage of pri-miRNA was performed by Drosha and DGCR8 complex and pre-miRNA isoforms were generated; Drosha recognized the double-strand region of pri-miRNA and excised the redundant nucleotides on 3' end and 5'end of pri-miRNA to generate pre-miRNA; however, Drosha alternatively excised at different positions of nucleotide in pri-miRNA, which led to producing different pre-miRNA isoforms (Neilsen CT, 2012). And alternative cleavage also existed in the process of pre-miRNA. The pre-miRNA was the precursor of mature miRNA duplex and was processed by Dicer complex by cleavage; then isoforms of mature miRNA were generated through alternatively cleaved by Dicer complex, which resulted in the diversity of miRNA (Gebert LFR, 2018). Finally, non-templated nucleotide addition is a recently identified

mechanism to modify single strand miRNA mainly through adenylation or uridylation (Gebert LFR, 2019). The adenylation at 3' end of miRNA modulated miRNA stability; for instance, oncogenic miR-21 was adenylated by poly (A) RNA polymerase PAPD5 and its degradation was initiated (Boele J, 2014); while miR-122 in liver cancer cell was stabilized through mono-adenylation catalyzed by Gld2 (D'Ambrogio A, 2012). The uridylation of miRNA usually reduced miRNA stability and inhibited its function. The 3' uridylation mediated by terminal uridylyltransferase 4 (TUT4) promoted the degradation of miR-26b in lung carcinoma cell A549 and inhibited its function of repressing IL-6 (Jones MR, 2009).

1.3.1.3 MicroRNA function

Although not coding protein directly, miRNA is an important regulator of the translation of protein-coding mRNAs. The single strand of mature miRNA together with protein components assembled RNA induce silence complex (RISC), binds to its target mRNA through complete or incomplete pairing with complementary nucleotides on target mRNA and results in the translation repression or the degradation of target mRNA (Krol J, 2010). The binding site of miRNA is generally located at the 3' untranslated region (3' UTR) of target mRNA and is majorly determined by a 6-8-nucleotide sequence at the 5' end of miRNA, named as "seed" sequence (Czech B, 2011; Bartel DP, 2009). The seed sequence is utilized to recognize the potential binding site on mRNA through a complementary match with seed sequence; the seed sequence can bind to any complementary sequence of mRNA, but this binding mostly occurs at 3'UTR of mRNA (Matoulkova E, 2012; Orom UA, 2008; Qin W, 2010). Apart from the seed

sequence, the rest of the miRNA sequence is either completely or incompletely complementary match with target mRNA (Bartel DP, 2009).

Following the binding of miRNA to mRNA, mRNA degradation or translation repression is performed by RISC and a series of proteins recruited by RISC (MacFarlane LA, 2010; Bartel DP, 2009). Generally, mRNA degradation is initiated by AGO in RISC; AGO associates with miRNA, recruits the GW182 protein family PABPC (polyadenylate-binding protein complex) and promotes mRNA 3'UTR deadenylation; the deadenylation also promotes the decapping at 5'end of mRNA, which enhances the susceptibility of mRNA to rapid mRNA degradation (Czech B, 2011; Gebert LFR, 2019). The other hand, RISC also contributes to translation repression. RISC interferes the binding of eukaryotic initial factor 4 (eIF4) to mRNA, which disassembles the eIF4 translation initiation complex and inhibits ribosome scanning; then RISC recruits ATP-dependent RNA helicase DDX6 through CCR4-NOT complex, which also results in translation repression (Gebert LFR, 2019). Although both translation repression and mRNA degradation have the function of negative regulating on mRNA translation, the difference between them still exists; the translation repression is usually generated from incomplete match between miRNA and target mRNA and it can be rescued by another mRNA binding protein; while mRNA degradation is the result of complete match and is irreversible (Bhattacharyya SN, 2006; Krol J, 2010). Therefore, miRNA is generally a negative regulator of its target mRNA and decreases the expression of the target protein.

The role of miRNA in cancer is mainly dependent on the protein-coding mRNA it regulates. When binding to the mRNA of a tumor suppressor and reducing its protein expression, miRNAs can promote the development of cancer and play an oncogenic role (oncomiR) (MacFarlane LA, 2010). For instance, miR-21 was overexpressed in various cancers and was a

well-established oncomiR through binding to PTEN mRNA and reducing PTEN protein expression (Meng F, 2007). However, when binding to an oncogene and decreasing proto-oncogene expression, miRNAs could inhibit tumorigenesis and act as a tumor suppressor (tumor-suppressing miR); MiR-200 targeting the proto-oncogene VEGFR inhibited lung cancer cell invasion and metastasis (Roybal JD, 2011). Since miRNAs have such crucial targets, they play an important role in tumorigenesis, cancer cell proliferation, metastasis and apoptosis (Hwang HW, 2006). Moreover, one miRNA has the potential to bind to different mRNAs that contain its binding site and its inhibitory effect on mRNA translation is usually in a mild manner; meanwhile, multiple miRNAs can bind to the same mRNA simultaneously and lead to a cooperative repression on translation of target mRNA, which generally shuts off the translation of target mRNA (Bartel DP, 2009; MacFarlane LA, 2010). Thus, the regulatory role of miRNAs is highly important in cancer.

1.3.1.4 Dysregulation of miRNA in cancer

Aberrant miRNA expression is frequently observed in many cancers, which hints the dysregulation of miRNA expression in cancer. Considering numerous tumor-related genes are the targets of miRNAs, the dysregulation of miRNA has a significant impact on cancer; overexpression of oncomiR can reduce the expression of tumor suppressor and impair its function of inhibiting cell metastasis and cell proliferation, which facilitates tumorigenesis (MacFarlane LA, 2010). For example, oncogenic miR-21 is overexpressed in a variety of cancers and plays a crucial role in tumorigenesis (Ghorbanmehr N, 2018; Asangani IA, 2008; Anastasov N, 2012). High expression of miR-21inhibited tumor suppressor Pdcd4 and promoted cell

metastasis in colorectal cancer; and miR-21 overexpression enhanced cell proliferation through overcoming cell cycle arrest at the G2/M checkpoint in breast cancer cells (Asangani IA, 2008; Anastasov N, 2012). Apart from miR-21, overexpression of other miRNAs was validated in breast cancer, including miR-221/222, miR-181, miR-29a and miR-10b (Piva R, 2013; Christodoulatos GS, 2014). In contrast, less expression of tumor-suppressing miR is also observed in cancer and promotes tumorigenesis through the deficiency of their tumor-suppressing function (MacFarlane LA, 2010). For example, let-7 was demonstrated as a tumor-suppressing miRNA and inactivated in cancers (Bussing I, 2008; Roush S, 2008). Let-7 could inhibit cell proliferation and metastasis and induce apoptosis through negatively regulating its oncogenic targets RAS and Myc (Jiang R, 2014; Su JY, 2012; Kim SJ, 2012). Thus, downregulation of let-7 facilitated the initiation of cancer. In addition, the down-regulation of miR-200a, miR-93 and miR-195 was also observed in breast cancer and promoted tumorigenesis (Hu J, 2015; Zhao FL, 2014; Jang K, 2014).

Some mechanisms can influence miRNA expression and contribute to the dysregulation of miRNA in cancer. First, epigenetic mechanisms contributed to some aberrant expression of miRNAs in cancer. A large proportion of miRNA promoters sat in CpG islands (the DNA sequence with a high frequency of CpG sites), in which cytosine tended to be methylated; the high methylation of miRNA promoter sequence resulted in the inhibition of miRNA transcription and decreased miRNA expression (Lopez-Serra P, 2012). It was reported that the promoter sequence of miR-200, a tumor-suppressing miR targeting VEGFR1, was hypermethylated in BCa patients, which was related to tumor metastatic potential (Castilla MÁ, 2012). Furthermore, low expression of let7e-3p was related to a poor prognosis of BCa patients and the promoter of let7e-3p was identified to be hypermethylated in BCa cell lines and tissue samples (Aure MR,

2013). In addition, the altered histone acetylation was also involved in miRNA aberrant expression; the reduction of acetylated histones inhibited the expression of tumor-suppressing miRNA miR-27a/b in breast cancer cell line SKBR3 (Scott GK, 2006). Second, the transcription of miRNAs was repressed or activated by upstream proteins (Martinez NJ, 2009). A set of transcription factors worked cooperatively to regulate miRNA expressions such as p53, SMAD ataxia telangiectasia mutated (ATM) and Myc; and then miRNAs with these transcription factors could constitute a functional feedback loop in which transcription factors influenced miRNA expression and vice versa (Davis BN, 2010; Suzuki HI, 2009; Zhang Y, 2011; Li Y, 2014). For instance, proto-oncogenic Myc activated the transcription of miR-17-92 family and then these miRNA inhibited their target gene Btg1 and Sin3b to promote cell proliferation and cell cycle progression in the lymphomas (Li Y, 2014). Finally, the dysregulated expression of protein components in the miRNA biogenesis influences the expression of miRNA. Nucleolin that was a newly identified protein component in Drosh/DGCR8 complex and highly expressed in breast and gastric cancer tissues could promote the maturation of some miRNAs (miR-21, miR15a/16 and miR-103) which facilitated cancer metastasis (Qiu W, 2013; Pickering BF, 2011). And as Dicer was an important protein component in the process of miRNA maturation, the reduced expression of Dicer could decrease the level of tumor-suppressing miRNA; lower expression of Dicer was found in breast cancer and was associated with the triple-negative phenotype (Dedes KJ,2011; Passon N, 2012). Therefore, since microRNAs have multiple functions and the dysregulations of microRNAs are observed in a variety of cancers especially in breast cancer, further investigation on microRNAs in breast cancer is necessary.

1.3.2 Application of microRNAs in breast cancer

The aberrant miRNAs expression identified in cancers leads to the inhibition of tumor suppressor genes or overexpression of oncogenic genes. And the expression profile of miRNA usually reflects the embryonic or developmental origin of cancer type and this signature of miRNA expression can be utilized to identify different types of cancer. Therefore, these traits of miRNAs give rise to the possibility of developing new methods for cancer therapy, cancer diagnosis or prognosis (Bertoli G, 2015).

1.3.2.1 MicroRNA as a cancer diagnosis marker

The traditional diagnosis tools (mammography and ultrasound) are commonly used for the detection for the early stage of breast cancer; however, there are some technical limitations to acquire accurate diagnoses, such as breast density and calcification detection (Bertoli G, 2015). Some BCa-related miRNAs were identified as biomarkers for diagnosis, which required the biopsy to obtain tissue samples; a set of 13 miRNAs was used to differentiate breast cancer with normal breast tissues with 100% accuracy (Iorio MV, 2005). Furthermore, miRNAs could be used to distinguish the different subtypes of breast cancer; 309 miRNAs were identified in breast cancer and 9 of these miRNAs could be used as biomarkers to discriminate the subtype of luminal A BCa from luminal B BCa (Blenkiron C, 2007). Therefore, this type of miRNA biomarker is a useful tool for cancer diagnosis through screening specific microRNAs in biopsy samples of tissues.

Although the technology of biopsy has been improved, the unpleasant and invasive procedure of biopsy limits its application. Fortunately, circulating miRNAs secreted by tumor

cells can overcome the disadvantage of biopsy and have the potential to be used as novel biomarkers for cancer diagnosis (Heneghan HM, 2010). Circulating miRNAs revealed some exciting advantages to be as a biomarker. First, the expression level of these circulating miRNAs could be detected and quantified by high sensitive methods, including TaqMan-probe qRT-PCR, microarray and next-generation sequencing; second, as circulating miRNAs existed in serum, urine and saliva, these miRNAs were easier to acquire than biopsy; last, circulating miRNAs were relatively stable in their exosome even though miRNA sample experienced multiple freeze-thaw cycles or prolonged exposure to room temperature (Heneghan HM, 2010; Weber JA, 2010). Thus, single circulating miRNA or a set of circulating miRNAs are capable of being recognized as biomarkers for cancer diagnosis. For instance, compared to the serum of healthy controls, miR-155 in the serum of BCa patients was found to overexpress exclusively (Roth C, 2010). And the increased expression of miR-195 only existed in the blood of BCa patients (Heneghan HM, 2010). However, the investigation on the clinical use of circulating miRNAs is still progressing. The identification of expression profile of circulating miRNAs in serum, nipple aspirated fluid and tissues, is clinically performed to develop feasible miRNA markers for ductal in situ or invasive breast cancer patients (https://clinicaltrials.gov/ct2/show/study/NCT02127073). Therefore, circulating miRNAs are a type of promising biomarker for BCa diagnosis.

1.3.2.2 MicroRNA as biomarkers for predicting prognosis

Not only working as biomarkers for cancer diagnosing, but miRNAs can also be used to predict the therapy response or prognosis of cancer. The expression of single miRNA or a set of miRNAs is usually diverse in different subtypes of breast cancers and associated with the

inhibition of its target genes, which can be recognized as the miRNA signature of BCa subtypes (Andorfer CA, 2011). To a specific treatment, the miRNAs signature of an individual patient was distinct from other patients and this distinct miRNA signature was capable of being used as cancer therapy predictor to reflect the potential effect on them (Maillot G, 2009; Altman DG, 2012). For instance, Herceptin, a humanized monoclonal antibody against HER2 protein, was used to block the HER2-mediated activation of intracellular kinases and effectors; however, most of the patients with HER2+ metastatic breast tumor would develop the resistance to Herceptin within 12 months (Valabrega G,2007; Andorfer CA, 2011). MiR-125 was significantly down-regulated in HER2+ BCa patients and was negatively associated with the HER2 expression (Fassan M, 2013; Scott GK, 2007); BCa patients with high level of miR-125 would generally have a good therapeutic effect and low rate for the resistance to Herceptin (Valabrega G, 2007). Thus, miR-125 was capable of acting as a predictor of Herceptin therapeutic response and the high expression of miR-125 indicated a good therapeutic outcome. Some studies also revealed the application of circulating miRNAs in predicting therapy effect. The expression of single circulating miRNA or a set of circulating miRNAs was altered after treatments and could be a predictor of the treatments (Bertoli G, 2015). Circulating miR-210 was present in body fluids of BCa patients and was associated with Herceptin sensitivity, tumor presence and lymph node metastasis; the expression of circulating miR-210 was significantly higher in Herceptin-resistant, metastatic or pre-surgery HER2+ BCa patients, which suggested that circulating miR-210 was possibly used as a biomarker to predict the therapy resistance and the metastasis (Jung EJ, 2012). Furthermore, miR-155 was another circulating miRNA to monitor the effect of chemotherapy on BCa patients. The expression of miR-155 in serum was observed to be downregulated after

Taxane treatment, which reached the levels of healthy women (Sun Y, 2012). Therefore, miRNAs are capable of acting as a potential predictor for therapies response.

MiRNAs can also serve as prognosis markers for breast cancer patients. A prognosis marker should be relevant to the likely or predicted development of a disease and be capable of indicating its prospective outcome. Various studies have confirmed that miRNAs can be used as prognosis markers for BCa patients. Overexpression of miR-27a/b was associated with shorter overall survival (OS) of TNBC patients; while lower expression of miR-155 was indicated shorter OS of TNBC patients (Lü L, 2017). The down-regulated expression of miR-374a/b was associated with short disease-free survival (DFS) of TNBC patients (Liu Y, 2015; Lü L, 2017). And circulating miRNAs also exhibited their potential as prognosis markers. The expressions of miR-148b-3p and miR-652-3p in serum were significantly lower in two independent cohorts of BCa patients; whereas higher expression of miR-10b-5p in serum was associated with poor prognosis of BCa patients (Mangolini A, 2015). A set of four circulating miRNAs (miR-18b, miR-103, miR-107, and miR-652) could predict the recurrence and overall survival of TNBC patients (Sahlberg KK, 2015). High expression of these four miRNAs in serum was associated with the short relapse-free survival and the overall survival (Sahlberg KK, 2015). These results suggested that miRNAs could function as prognosis markers to predict the prospective outcome of BCa patients.

1.3.2.3 MicroRNA as a cancer therapy target

In addition to being used as diagnosis biomarkers and prognosis predictors, miRNAs are potential targets to be directly used in cancer therapy. In the context of oncogenic miRNAs

overexpression or tumor-suppressing miRNAs inhibition in specific breast cancer patients, anti-miRNA oligonucleotides or miRNA mimics can be delivered to tumor cells directly or indirectly via circulation system, in which aberrant miRNA expression will be rescued and its oncogenic effect will be diminished. Some promising results have been obtained in pre-clinical experiments. For instance, due to its downregulated expression in multiple cancers, miR-145 mimic was introduced to suppress its oncogenic targets in breast cancer cells or xenograft mice (Tang L, 2016; Inamoto T, 2015). And because oncogenic miR-21 was overexpressed in breast cancer cells and tissues, anti-miR-21 oligonucleotides was used to reduce miR-21 expression and inhibit tumor growth in xenograft mice (Yan LX, 2011). Furthermore, miRNAs are potential to regulate the chemo-resistance and miRNA inhibitors can improve the therapeutic effect of other therapies. Overexpression of miR-195 by transfection could increase the sensitivity of ADR-resistant MCF-7 BCa cells to the drug, which promoted apoptosis through down-regulating Raf-1 and Bcl-2 (Yang G, 2013). It was also reported that miR-100 mediated chemo-resistance in non-small cell lung cancer through down-regulating HOXA1 and miR-100 inhibitors enhanced the drug sensitivity in chemotherapy (Xiao F, 2014). Based on the results in the above studies, miRNAs could work as therapeutic targets and exhibit its potential in cancer treatment.

The clinical applications of miRNAs in cancer therapy are still investigated. The tumor-suppressing miR-34a that down-regulated the expression of multiple oncogenes such as Myc, was generally lost or under-expressed in multiple cancers (Wei JS, 2008; Slabáková E, 2017). MRX34 (Mirna Therapeutics, Inc), a liposomal formulation of miR-34a mimic entered the clinical phase I trial in 2013 and was given to 47 patients with multiple cancers by an intravenous method. MRX34 exhibited evidence of anti-tumor activity in a subset of patients with refractory advanced solid tumors, including hepatocellular carcinoma and breast cancer

(Beg MS, 2017). However, five immune-related serious adverse responds were identified and the phase I trial was terminated in 2017, which indicated that further study should be performed on reducing immune response of microRNA-based therapies. Furthermore, therapeutic anti-miRNA oligonucleotides anti-miR-221 and anti-miR-10b are still in development by Regulus Therapeutics for the hepatocellular carcinoma treatment and the glioblastoma treatment, respectively (Nguyen DD, 2017). Obviously, though miRNAs have the potential in developing novel cancer therapies, the side effects of miRNA therapies and the stability of miRNAs are the top issues that restrict its clinical use. Thus, more investigation on miRNA is required for a comprehensive understanding of miRNA therapies before its widespread clinical application.

1.4 Hypothesis and objectives

The aim of my project is to identify the regulation of tumor suppressor PTEN by predicted microRNAs in cancer cells and determine the function of individual microRNA (miR-498) in breast cancer. Breast cancer (BCa) is the most common cancer in Canada and triple negative breast cancer (TNBC) is a subtype of BCa with the highest mortality (Carey LA, 2011). As a lipid and protein phosphatase, PTEN is a well-known tumor suppressor that mainly relies on its function of negatively regulating PI3K-AKT signal pathway, which is usually inactivated in multiple cancers, e.g. breast cancer, lung cancer, brain cancer and prostate cancer (Steck, PA, 1997; Li J, 1997; Marsit CJ, 2005). The expression rate of PTEN in BCa tissues (57.5%, 84/146) is significantly lower than that in normal breast tissues (100%, 10/10), and the mutations of PTEN have also been observed in BCa (~5%) (Zhang HY, 2013; Rhei E, 1997). However, this cannot explain the observation that PTEN is repressed in some types of cancers with intact

PTEN genes. For instance, reduced PTEN protein expression is seen in 38% of invasive breast cancers and in 11% of in situ breast cancers, which indicates that other mechanisms regulating PTEN must exist (Bose S, 2002; Marsit CJ, 2005). MicroRNA-induced gene inactivation is one of the new mechanisms identified in the regulation of PTEN expression and activity (Pezzolesi MG, 2008; Huse JT, 2009). MicroRNAs are a type of small non-coding RNA with the function of repressing the translation of protein-coding mRNAs (Olsen PH, 1999; Pang KC, 2007; Pillai RS, 2005). In prior studies, some miRNAs were observed to inactivate PTEN and acted as oncogenic players in multiple cancers. MiR-221and miR-222 were over-expressed in NSCLC and hepatocellular carcinoma and promoted tumorigenesis by targeting PTEN (Garofalo M, 2009). However, studies of PTEN regulation by miRNAs in breast cancer are limited and the identification of novel miRNAs that results in the PTEN inactivation is of great importance to improving the breast cancer treatment. Therefore, in this study, I aim to identify oncogenic microRNA(s) targeting PTEN in breast cancer and the hypothesis is as follows:

Oncogenic microRNA(s) can negatively regulate PTEN by targeting PTEN mRNA and when overexpressed in breast cancer cells, they promote cancer cell proliferation and metastasis.

There were three objectives designed to test this hypothesis:

1. To examine the regulation of PTEN protein by predicted PTEN-targeting microRNAs and determine the binding site and clinical impact of PTEN-targeting microRNA candidate.

2. To investigate the expression of PTEN and microRNA candidate in TNBC cells and the oncogenic effects of microRNA candidate on cell proliferation and metastasis.

3. To inhibit the oncogenic effects of microRNA candidate by a microRNA decoy.

Chapter 2 Materials and Methods

2.01 MicroRNA target prediction

Online microRNA databases were used to predict the potential miRNAs that could bind to PTEN mRNA in this project. Five microRNA databases (Miranda, rna22, miTarget, Pictar and Targetscan) were applied to predict the potential microRNAs that bind to PTEN mRNA. The predicted microRNAs targeting PTEN mRNA were preliminarily selected from the pool of microRNAs that were at least shown twice in prediction from these five microRNA databases. Regarding the publications on reporting PTEN-targeting microRNAs and the analysis of microRNAs expression level in microRNA microarray, nineteen predicted microRNAs targeting PTEN were selected in this study to prepare the microRNA-expressing plasmids and perform further experiments.

2.02 PCR and agarose gel electrophoresis

Polymerase Chain Reactions (PCR) were used to get all the genomic DNA of predicted miRNAs (Table 2.1). The sequence of each miRNA was acquired from the OriGene website (http://www.origene.com/MicroRNA/). The genomic DNA used as PCR template was extracted from human cell line HEK293. The cloning primers for these miRNAs were designed by Primer Premier 5 and synthesised by Integrated DNA Technologies (IDT). Working primer (10 μM) was used for PCR. The total volume of each PCR Reaction was 50 μL and was prepared as following: 10x PFU Buffer (5 μL, Stratagene, USA), 10 mM dNTP (1 μL), 2.5 mg/μL of Gelatin to reduce background (2 μL), Primer 1 (1 μL), Primer 2 (1 μL), genomic DNA at 200 ng/ μL (2

μL), the PFU polymerase (1 μL, Stratagene, USA) and ddH_2O (37μL) was added together. PCR reaction samples were vortexed gently to mix constituents, spun down quickly, and PCR was run in a GeneAmp PCR System 9700 thermal cycler (Applied Biosciences, USA) with the following conditions: One initial denaturation cycle at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 1 minute, annealing (50-55°C) for 0.5 minutes, and extension at 72°C for 1 minute; 1 cycle of final extension at 72°C for 5 minutes, and then finally rest at 4°C.

Table 2.1 - List of microRNAs and their cloning primers used in this study

microRNA name	Cloning primer name	Primer sequence
miR-19a	5' primer	**CGGGATCC**ATGTCAAAGTGCTTACAGTGCAGGT
	3' primer	**CGGAATTC**GCATGGATTTGCACAGCAGAATAT
miR-29a	5' primer	**CGGGATCC**ATACTACACCATTTTCTATCAGAGA
	3' primer	**CGGAATTC**CCAGGAGTGTTTCTAGGTATC
miR-29c	5' primer	**CGGGATCC**TCTGTTGACTCCTAGCAGCCAT
	3' primer	**CGGAATTC**GCATGAATAGACTCTTCTCAACAGA
miR-22	5' primer	**CGGGATCC**AGGGTGAGAGCAGGACTGGGTTTC
	3' primer	**CGGAATTC**GATACGGAAGGAGGGTCAAGAAGGA
miR-198	5' primer	**CGGGATCC**AAGGGTGCCTTAGACCGAAGC
	3' primer	**CGGAATTC**ACCTCCCCAAGTCACAGTTTTAG
miR-214	5' primer	**CGGGATCC**AATTGGACTTAAGAATAAACCTTGTG
	3' primer	**CGGAATTC**AGGTCAAGGGTAGTGATTTGCTGA
miR-338	5' primer	**CGGGATCC**GCAGGTTTAGACGAAGAGATGCGTA
	3' primer	**CGGAATTC**CTGCTGGCTTTGAACCCTGCC
miR-301a	5' primer	**CGGGATCC**CCAATCCTAAAGTATTAACAATACCC
	3' primer	**CGGAATTC**CCTAAAGTTTGACAGAACCTATCCA
miR-301b	5' primer	**CGGGATCC**TGAGTGCGGGTTCGTCTTTGTGTGT
	3' primer	**CGGAATTC**GTGCAACAGGGAAAGAGTGTCGGG
miR-421	5' primer	**CGGGATCC**CAGGTTGTATTATCATTGT
	3' primer	**CGGAATTC**GACGACTCCCAGCCTCTT
miR-425	5' primer	**CGGGATCC**TGGAGGTGATGGGTGTAGACGTG
	3' primer	**CGGAATTC**AGGCAACAGCCATTTACGGGC
miR-454	5' primer	**CGGGATCC**AGATCAGTATAAAACTTTTCCCCT
	3' primer	**CGGAATTC**GTATCACTAACCAGCTATGTGTC
miR-486	5' primer	**CGGGATCC**TGTGAAACACGTTGCTCCCT
	3' primer	**CGGAATTC**ACACGGTGACCTCCTTAAGCT
miR-498	5' primer	**CGGGATCC**TTGCATTTTATGGTGGTGATTGA
	3' primer	**CGGAATTC**GAGCTGTGAACATGACACTGCAC
miR-374a	5' primer	**CGGGATCC**CATCTAATAAACATTTACTGAGGCC
	3' primer	**CGGAATTC**ATGAGCTATTACTTACTGCTTTTTG
miR-374b	5' primer	**CGGAATTC**TGCATTAGACTTTGTCAAAACTGGA
	3' primer	**CCGCTCGAG**GCACTACACAGAAAAGTCCTCAACA
miR-543	5' primer	**CGGGATCC**GCAAAATCACTGTTTCTGTGCCATT
	3' primer	**CGGAATTC**AATTGCAGTGGCAAAGGAGAAGAG
miR-569	5' primer	**CGGGATCC**ATGGAAGCCAACTTTAAGACCCT
	3' primer	**CGGAATTC**GCAGCTTGCTGACCTCAAAGC
miR1297	5' primer	**CGGGATCC**ATCCTTTGACCTAAGCTAACTA
	3' primer	**CGGAATTC**CTTTGGAACCTATGATTCATTC
miR-1-1	5' primer	**CGGGATCC**TGCCTTTCTGGATCGTGTGAGTGTG
	3' primer	**CGGAATTC**CGACCCCCCAGCCCTCACCAG

2.03 Construction of microRNA-expressing plasmids

Generally, PCR products of genomic DNA of predicted microRNAs were cloned into the BamHI-EcoR I restriction site of the pcDNA3.1 vector (Stratagene, USA), carrying the Ampicillin resistance gene. Extraction of DNA digestion samples was performed using the Qiagen II Agarose Gel Extraction Protocol (QiaexII handbook, Qiagen, USA). The pcDNA3.1 was digested by BamHI & EcoR I and the digestion reaction was prepared by mixing 10x Fast Digest buffer (20 µL) with BamHI (3 µL), EcoR I (3 µL), ddH_2O (168 µL), and 3 µg of pcDNA3.1 (6µL). The PCR products were also digested by BamHI & EcoR I and the digestion reaction was prepared by mixing 10 x Fast Digest buffer (20 µL) with BamHI (3 µL), EcoR I (3 µL), ddH_2O (74 µL), and PCR product (100 µL). The reaction mix was vortexed and spun down quickly, and incubated at 37°C water bath for 1 hour. Each sample (200 µL) was mixed with 20 µL sodium acetate and 600 µL isopropanol, and stored for 30 minutes at -20°C, then spun at high speed (15, 000 x g) for 15 minutes at 4°C (in a cold room). Purification of DNA was done as follows: DNA precipitate was washed with 75% ethanol and vortexed briefly, and then spun down for 2 minutes at 15, 000 x g. Ethanol was gently removed, and the DNA digestion samples were quickly spun down. The residual ethanol was removed using small pipette tips, and DNA digestion samples were air-dried for 10-15 minutes before being dissolved in 30 µL of Tris buffer (pH = 8.0) (10 mM). DNA digestion products were stored at -20°C for further usage. For DNA ligation, 8 µL of ddH_2O was added to a microfuge tube followed by 4 µL of 5x buffer, 5 µL of digested PCR product, 1.5 µL of a plasmid vector, and finally 1.5 µL of T4 DNA ligase for a total of 20 µL. Samples of DNA ligation were vortexed, spun down quickly at 15, 000 x g, and incubated at 20°C for 1 hour. DNA ligation products were stored at -20°C for further use or used immediately.

2.04 DNA transformation and plasmid extraction

DH5α competent cell aliquots were taken from -80°C and put on ice for DNA transformation. DNA ligation products were incubated on ice for 5 minutes alongside DH5α. DH5α aliquot (50 μL) was transferred to a 15 mL reaction tube. In a 15 mL culture tube, for each transformation reaction, 3 μL of DNA ligation product was added to 50 μL of DH5α aliquot (one aliquot per sample), and the reaction mix was kept on ice for 1 hour. Rest of ligation products was stored at -20°C. The recombined plasmid vector had the resistance to Ampicillin or Kanamycin (Sigma Aldrich, USA). The reaction mix was incubated in a 42°C water bath for 1.5 minutes and then kept on ice for 3-5 minutes. Then the reaction tube was added 1 mL of LB Broth (Lennox) (Cat# LBL405.1, Bioshop Canada Inc., CA) medium to and placed in a MaxQ 4000 rocker (Barnstead, USA) and incubated for 1.5 hours at 37°C. The reaction mix together with LB medium was transferred into EP tubes and spun down at 5, 000 x g for 1 minute. All medium was removed and the DH5α cell pellet was aspirated by 50 μL fresh LB Broth medium using a pipette. Then DH5α cells were transferred onto the plate medium of LB Broth with agar (Lennox) (Bioshop CA Inc., CA) for bacterial culture, which were perfused over the dish of LB Broth with agar using a sterile glass pipette. Ampicillin or Kanamycin agar plates were prepared for culturing bacterial colonies containing plasmid constructs. Plate medium was made using LB Broth with agar (Lennox) at a concentration of 35 mg/L, with 50 μg/mL antibiotic (Ampicillin or Kanamycin). Bacterial plates were incubated at 37°C for 18 hours, before being stored at 4°C. Bacteria colonies were picked from LB Broth agar plates and seeded to 5 mL of LB Broth medium (Sigma Aldrich, USA) with either Ampicillin or Kanamycin (50 μg/mL). From each plate, three bacteria samples (taken from three colonies) were prepared. Tubes containing each bacteria sample were incubated in a shaker overnight (18-20 hours) at 37°C. The next day, mini-

preparation of DNA plasmid was conducted using GeneJet Plasmid Mini-prep Kit (Thermo Scientific, Ottawa Canada). Plasmids were digested using the BamH I and EcoR I restriction enzymes, and plasmid digestion was examined by electrophoresis using 1% agarose gels and visualized under an Ultraviolet Trans-illuminator (UVP, USA). A positive plasmid for each construct was kept in -20°C fridge or used for maximum preparation of DNA.

In order to prepare a sufficient amount of plasmid DNA, a maximum preparation of DNA was performed. For max prep DNA, firstly, a positive plasmid was transformed into DH5α cells. 1 µL of DNA was introduced to 15 µL of DH5α cells in 10 mL culture tubes on ice, and DH5α cells were kept on ice for 1 hour. DH5α cells were thermally shocked for DNA uptake by dipping tubes for 1.5 minutes in a 42°C water bath and then putting them on ice for 5 minutes. 1 mL of LB Broth medium was added to each tube, and tubes were placed in a MaxQ 4000 rocker set to 37°C for 1.5 hours. 50 µL of sample was added aseptically to LB Broth agar plates with antibiotic (depending on the construct used) and kept in a bacterial incubator at 37°C for 18 hours. The next day, colonies on LB Broth agar plates were transferred into 300 mL of LB Broth medium with antibiotics and cultured at 37°C for 20 hours. The plasmid DNA was prepared using the GeneJet Maxiprep Kit (Thermo Scientific, Ottawa CA) protocol. Extracted DNA was stored in TE buffer at pH 8.0 (1 mM Tris.CL, and 0.1 mM EDTA) (Sigma Aldrich, USA), and DNA concentration was measured using a DU40 UV spectrophotometer (Beckman Coulter, USA). The concentration of diluted plasmid DNA (10 µL of DNA sample was added to 990 µL of ddH_2O, with the blank control of a 10 µL TE buffer/ 990 µL ddH_2O) was measured using DNA nucleotide analysis on the DU40 UV Spectrophotometer. The absorbance ratios (260 nm/280 nm) of plasmid DNA were between 1.8 and 2.0, which suggested good DNA integrity and purity for transfection experiments.

2.05 Cell culture and cell lines

All cell lines used in this study were purchased from the American Type Culture Collection (ATCC, USA) and stored in liquid nitrogen. These cell lines included the human embryonic kidney cell lines HEK293 (ATCC® CRL-1573™) and HEK293T (ATCC® CRL-11268™); the breast epithelial cell line MCF-10A (ATCC® CRL-10317™）; the breast carcinoma cell lines Hs 578T (ATCC® HTB-126™), MDA-MB-157 (ATCC® HTB-24™), MDA-MB-231(ATCC® HTB-26™), BT-549 (ATCC® HTB-122™) and MCF-7 (ATCC® HTB-22™) All cell lines were cultured in α-MEM (Minimal Essential Medium alpha) (Gibco Life Technologies Corp, USA) with 8% FBS (Fetal Bovine Serum – Sigma Aldrich, USA) in a humidified Forma Steri-Cycle Incubator (Thermo Fischer, USA) at 37°C with 5% CO_2. Medium was changed every three days until cells reached 80-100% confluence, and then cells were sub-cultured by removing medium, briefly washing the cells with PBS (Phosphate Buffered Saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 at pH 7.4) (Sigma Aldrich, USA) and then detaching cells from the cell dishes by incubating in 1.3% Trypsin-EDTA (Sigma Aldrich, USA) for 3 minutes at 37°C. Detached cells were spun down into pellets, and the old medium was removed before cells were re-suspended in fresh medium and subdivided into new dishes.

2.06 Transient transfection

In order to overexpress microRNAs in human cells, transfection was performed using the Calcium Phosphate method. Calcium phosphate precipitates formed when $CaCl_2$ and HEPES

buffer were mixed and then plasmid DNA in these precipitates was taken in by cells to express genes on plasmid DNA (Kingston R, 2003). To perform this transfection, plasmid DNA solution was prepared by ddH_2O, plasmid DNA and $CaCl_2$. The total DNA did not exceed 30 µg; 63 µL of 2 M $CaCl_2$ was added to DNA solution; ddH_2O was added to bring up the total volume to 500 µL. Aliquots of HEPES buffer (500 µL) were prepared with the following reagents: 280 mM NaCl, 1.5 mM $Na_2HPO_4*2H_2O$, 12 mM dextrose, and 50 mM HEPES (Sigma Aldrich, USA) at pH 7.05. Aliquot of 500 µL HEPES buffer was mixed with 500 µL DNA solutions drop-wise in the presence of bubbling. Mixed transfection solution was incubated at 20°C for 30 minutes. Cells with 50-70% confluence were chosen for transfection and the medium was refreshed prior to transfection. Approximately 10 minutes before the addition of samples, 10 µL of a transfection efficiency-increasing solution "QI" (Roche, USA) was added to each cell dish. After the incubation of 30 minutes, the transfection solution was added drop-wise into cell culture dishes. Cells were incubated with transfection buffer for 16 hours and then cells were washed by PBS twice and refilled by fresh medium. After the incubation of 24 hours (40 hours after transfection), transfected cells were harvested by scratching.

2.07 Viral transduction

The genomic DNA sequence of miR-498 (~700bp) was inserted into retroviral vector pBABE-Ires-GFP through BamH I and EcoR I (2.5 U/µL). The pBABE-miR-498-GFP and pCL-Ampho retrovirus packaging vector were co-transfected into HEK293T cells by Calcium Phosphate method with the ratio of 1:1 and total DNA amount of 30 µg. 48 hours after transfection, the supernatant medium of viral vector transfection containing virus was collected

and filtered by a 0.45 μm filter to remove any cells or particles. All aliquots of virus supernatant were kept in -80°C for following experiments. For retrovirus transduction, cells were seeded in tissue culture dishes and transduced by virus supernatant when reaching 30%-40% confluence. After removal old medium in the dishes, 4 mL of fresh medium was added into each cell dish and kept in 37°C cell culture incubator before virus transduction. Virus supernatant expressing miR-498 ($1x10^7$ PFU) was added to cell dishes with 8 μg/mL Polybrene for enhancing virus transduction. The empty vector of pBABE-Ires-GFP was used for making virus supernatant of control miRNA. And 6-8 hours later, 6 mL fresh medium was added to each dish. Then 40 hours later, miR-498-transduced cells were harvested by scratching or trypsinized for following experiments (Nolan G, Nolan lab retroviral transduction protocols).

2.08 Polyacrylamide gel electrophoresis and proteins transfer

In order to distinguish the proteins in whole cell lysis for immunoblotting, polyacrylamide gel electrophoresis (PAGE) was performed (Weber K, 1969). The proteins in whole cell lysis were separated by an acrylamide gel according to molecular weight (electrophoresis). Regarding the molecule weight of proteins, 10-12% polyacrylamide gels were prepared (using 40% acrylamide stock, 1.5 M Tris-8.8, ddH_2O, 10% SDS, 10% APS) (Sigma Aldrich, USA) and combined with a stacking gel composed of 5.3% acrylamide. The combined gel was immersed in gel tanks filled with 1 x Running Buffer (25 mM Tris, 192 mM Glycine, and 1% SDS) (Sigma Aldrich, USA). Before being loaded into gel wells, 50 μg of protein samples was mixed in 1.5 mL EP tube with an equal volume of 2 x loading buffer (0.1 M Tris pH 6.8, 20% Glycerol, 4% SDS, and 0.2% Bromophenol Blue) (Sigma Aldrich, USA) and kept in boiled water bath for 10 minutes. The protein samples and 3 μL of BLUeye Prestained

Protein Ladder (GeneDirex, USA) were loaded into gel wells and ran at 60 Volts until the dye reached the lower edge of the gel (PowerPac 300, Bio-Rad, USA).

Proteins were transferred from gels to Immobilon Nitrocellulose membranes (Millipore, USA) using a Transblot Semi-Dry Transfer Cell (Bio-Rad, USA). Two thick filter sheets were soaked in Anion Buffer 1 (0.3 M Tris, 15% methanol in ddH_2O). One thin sheet was soaked in Anion Buffer 2 (0.025 M Tris, 15% methanol in ddH_2O) and stacked on the thick sheets. A section of PVDF membrane which was cut to match the size of the acrylamide gel was soaked in methanol and Anion Buffer 2, and placed on the thin filter sheet. Then the acrylamide gel itself was soaked in Cation Buffer (0.025 M Tris, 15% methanol, 0.04 M Amino-N-Caproic Acid in ddH_2O) (Sigma Aldrich, USA) and put on top of polyvinylidene difluoride (PVDF) membrane. Bubbles were removed by gently wiping the membrane with a glass rod. Finally, one thin filter sheet and two thick filter sheets were sequentially soaked in Cation Buffer 3 and placed on top of the acrylamide gel. The transfer machine was plugged into the PS 500XT DC Power Supply (Hoeffer Scientific, USA), and the process of transferring ran for 1.5 hours at 75 milliamps for one gel. PVDF membranes with transferred proteins were dried at 4°C for 4-18 hours for subsequent immunoblotting use.

2.09 Immunoblotting

In order to examine protein levels in cell samples, immunoblotting was performed. PVDF membranes were briefly immersed in methanol and then washed 5 minutes by 10mL TBST (0.1 M TBS with 1% Tween20) for 3 times (Uniqema Americas LLC, USA). Next, PVDF

membranes were immersed in 5 % milk-TBST solution (wt/vol, Instant Skim Milk Powder – Smucker Foods, CA) for 1 hour at 20°C. The milk-TBST solution was removed and membranes were washed in TBST for 5 minutes by three times to remove milk residue, before being incubated with 10 mL of the primary antibody/TBS solution for 1.5 hours at 20°C (Table 2.2). After incubation of primary antibody, membranes were washed with TBST for 5 minutes by three times to remove excess primary antibody and then incubated in 10 mL of the secondary antibody solution for 1 hour (1 μL of the secondary antibody, 1% powdered milk and TBST). Subsequently, membranes were washed with TBST for 5 minutes by three times to remove secondary antibody residues, brought into a dark room and soaked in ECL solution for 3 minutes (Western Lightning TM Plus-ECL – GE Healthcare, UK). Excessive ECL solution on membranes was removed, and then membranes were wrapped in Saran wrap and enclosed in X-ray film cassettes. In dark room, Super RX-N X-Ray film (Fuji Film Corporation, Japan) was placed over membranes, and exposed for 10 seconds to 10 minutes, before being developed in an Optimax X-Ray Film Producer (Protec, USA). The protein bands on films were determined by matching up with the visible gel protein ladder on the membrane, and then films were scanned at 600 dpi into .tiff files. The quantitative analysis of protein bands was performed by software ImageJ (National Institutes of Health, USA) and the detail process was shown as followed: First, opened the immunoblotting image by ImageJ and used the "rectangular" tool to select the interested band area; pressed "Ctrl"+ "3" to show the histogram of the intensity of this band and then select "draw line" tool to draw a line that enclosed the bottom of the histogram; used the "magic wand" tool to click any area of the enclosed histogram and the intensity of the band as a numerical value was shown in a new window; recorded this value in an Excel file and repeated

for another protein band; collected the intensity values of all protein bands to quantify the protein bands; the intensity values of interested proteins were normalized to its β-actin.

For multiple immunoblotting on the same membrane, used membranes were stripped of old antibodies by washing in TBST for 5 min and incubating membranes at 50°C in glass tubes containing 50 mL of stripping buffer (mercaptoethanol, 10 mL of SDS, Tris pH 6.8, and ddH_2O – Sigma Aldrich, USA). Membranes were rinsed in ddH_2O and washed in TBST for 5 minutes by three times before next immunoblotting which started from the step of milk blocking. The protein β-actin was used as a loading control for this assay to normalize the amount of protein between samples.

Table 2.2 - Primary Antibodies

List of antibodies used in this study, including the antibody name, the protein target, company that made them, the working concentration used in immunoblotting, and the target of the secondary antibody used alongside it during immunoblotting analysis.

Antibody Name	Protein Target	Company	Dilution	Secondary Target
Sc-7974	PTEN	Santa-Cruz	1:200	Mouse Monoclonal
F2922	FLAG epitope	Sigma Aldrich	1:1000	Mouse Monoclonal
Sc-9996	GFP	Santa-Cruz	1:1000	Mouse Monoclonal
Sc-5298	AKT	Santa-Cruz	1:200	Mouse Monoclonal
A3854	β-actin	Sigma Aldrich	1:10000	Mouse Monoclonal
Sc-7985-R	p-AKT(Ser473)	Santa-Cruz	1:200	Rabbit Monoclonal

2.10 Luciferase assay

To examine the binding sites of microRNA on PTEN mRNA 3'UTR, a luciferase reporter assay was used (Wang B, 2017). The pMIR-REPORTER miRNA Expression Reporter Vector System consisted of a firefly luciferase reporter vector and a beta-gal reporter control vector. The pMIR-PTEN mRNA 3'UTR luciferase reporter was constructed by myself, which contained a firefly luciferase reporter gene followed by the DNA sequence of PTEN mRNA 3' UTR. The three luciferase reporters pMIR-PTEN 3'UTR part A, part B and part C were constructed by inserting the predicted miR-498 targeting sequences of PTEN mRNA 3'UTR which followed the luciferase gene. These luciferase reporters could be used to mimic PTEN mRNA as the target of miR-498. The beta-gal reporter vector containing a beta-galactosidase gene was used as transfection control. The expression of beta-galactosidase from this control vector was used to normalize the differences in cell viability and transfection efficiency. The luciferase catalyzed an oxidative reaction in which luciferase substrate luciferin was converted to oxyluciferin and then fluorescence signal was emitted. The fluorescence signal was measured by luciferase assay instrument (Lumat LB 9507, Berthold Technologies, Germany) to reflect the luciferase activity and luciferase transcription.

Next, 5 μg of pMIR- PTEN 3' UTR reporter and 2 μg of beta-galactosidase vector were co-transfected with control miRNA or miR-498 into HEK293 cells. 40 hours later, transfected cells were harvested and aliquots of transfected cells were made for further experiments. The 1x cell culture lysis buffer was prepared by diluting 5x cell culture lysis buffer (Cat. #E153, Promega, USA). Each of aliquots was added 500 μL 1x cell culture lysis buffer, mixed by vortex and placed on ice for 30 minutes in a 1.5 mL EP tube. The cell lysate was centrifuged for

12,000g, 10 minutes at 4^{O}C and the supernatant was transferred to a new 1.5 mL EP tube and kept on ice. The luciferase assay agent was prepared by adding Luciferase Assay Buffer (Cat. #E152A, Promega, USA) to the vial of lyophilized Luciferase Assay substrate (Cat. #E151A, Promega, USA). The luciferase assay agent was loaded to the luciferase assay instrument (Lumat LB 9507, Berthold Technologies, Germany). Next, turned on the luciferase assay instrument, selected the sample tunnel: INJ1, the inject volume: 100 μL and the measure-time: 10 seconds, then inserted an empty sample tube and measured 4 to 6 times to drain out water and drain in luciferase substrate. Next, 2 μL cell lysate was added to a new sample tube and fluorescence signal (relative light unit, RLU) was measured by luciferase assay instrument. The old sample tube was removed and a new sample tube with another sample was added and measured. After all samples were measured, ddH_2O was loaded to wash out substrate residue. To determine the activity of beta-galactosidase, 50 μL of cell lysate and 100 μL lysis buffer was added into a 1.5 mL EP tube along with 150 μL 2x beta-galactosidase assay buffer. Then the EP tubes were kept in 37^{O}C water bath for 15 to 30 minutes. After water bath, 500 μL Na_2CO_3 was added to terminate the beta-galactosidase reaction and OD values at 420 nm were measured by DU40 spectrophotometer to evaluate the activity of beta-galactosidase. The beta-galactosidase was used as transfection control. The RLU value of each sample was normalized to the OD 420 values of the beta-galactosidase assay to get the final luciferase activity. This final luciferase activity was used to reflect the regulation of luciferase translation by miRNA.

2.11 RNA extraction

To examine the miR-498 expression in transfected cells or transduced cells, total RNA was extracted by Trizol (Invitrogen, USA). Cells were washed twice by pre-cold PBS, scratched and harvested by 1mL PBS in a 1.5 mL EP tube, then spun down and the supernatant was discarded. 1 mL Trizol was added to each tube and the cell pellet was dispersed by vortex for 1 min then incubated in 20°C for 5 minutes. Next, 0.2 mL chloroform was added and mixed completely by severely vortexed. After incubation for 5 minutes and centrifugation at 12000g at 4°C for 15 minutes, three phases were observed in EP tubes and 0.3 mL of the supernatant phase was transferred to a new EP tube along with the equal volume of isopropanol, then EP tubes were kept in -80°C overnight. EP tubes containing total RNA were centrifuged at 12000g at 4°C for 15 minutes and RNA pellets were obtained. The supernatant was removed and RNA pellets were suspended and washed twice by 75% ethanol which was prepared by DEPC-treated ddH_2O. After 75% ethanol was removed completely and RNA pellets were air-dried for 5 minutes, 100 μL DEPC-treated ddH_2O was added to resolve RNA. Until RNA pellets were totally resolved, 10 μL of RNA solution was taken and diluted to 1000 μL for measuring the concentration of RNA by the D40 UV Spectrophotometer (Beckman Coulter, USA).

2.12 qRT-PCR

In the purpose of confirming and quantifying the expression of miR-498, the qRT-PCR assay was performed using TaqMan MicroRNA probe (miR-498 assay ID: 001044; U6snRNA assay ID: 001973, Thermo Scientific, Ottawa CA). The TaqMan MicroRNA Reverse Transcription Kit was used to get cDNA samples from total RNA. The reverse transcription (RT) master mix was prepared in an EP tube as follows:

Component	Volume per reaction
100mM dNTPs mix	0.15 μL
Reverse Transcriptase (50 U/μL)	1.00 μL
10x Reverse Transcription Buffer	1.50 μL
RNase Inhibitor (20 U/μL)	0.19 μL
Nuclease-free water	4.16 μL
Total mix volume	7.00 μL

The RT master was mixed gently, centrifuged to bring down all solution to the bottom of the EP tube and placed on ice. Next, 10 ng of RNA (5 μL), 3 μL 5x RT primers and 7 μL RT master mix were added to a 200 μL PCR tube, gently mixed, quickly centrifuged and incubated in ice for 5 minutes before loading into the thermal cycler. PCR tubes were loaded into thermal

cycler with the cycler condition as follows: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C kept.

RT products and the components of TaqMan qPCR kit were gently mixed and then placed on ice. The TaqMan qPCR mix of three replicates was prepared in an EP tube as follows:

Component	Volume per master mix
TaqMan® Universal PCR Master Mix II	33.00 µL
Nuclease-free water	25.30 µL
TaqMan® Small RNA Assay (20x)	3.30 µL
Product from RT reaction	4.40 µL
Total volume	66.00 µL

All qPCR components were added into an eppendorf (EP) tube, gently mixed by inversion and briefly centrifuged. Then 20 µL of the qPCR reaction mix was transferred to one tube of MicroAmp Fast Reaction Strip Tubes (Applied Biosystems, Thermo Fisher scientific Ottawa CA), and all tubes were sealed by optical caps and spun down gently. All tubes were loaded into real-time PCR instrument StepOne plus (Applied Biosystems, Thermo Fisher

scientific Ottawa CA) and it ran the program as follows: 50°C for 2 minutes; 95°C for 10 minutes; 95°C for 15 seconds, 60°C for 60 seconds, 40 cycles; 4°C forever. The qPCR results were analyzed by StepOne software. U6 snRNA was used as endogenous control and the expression of miR-498 was normalized to the expression of U6 snRNA by the comparative Ct method.

To quantify the expression of PTEN mRNA, the STBR Green qRT-PCR was performed using PowerUp SYBR Green Master Mix (Part No. 100031508, Applied Biosystems, Thermo Fisher scientific Thermo Fisher scientific Ottawa CA). The MLV Reverse Transcription Kit was used to get cDNA samples from total RNA. The reverse transcription (RT) master mix for each RT reaction was prepared in an EP tube as follows:

Component	Volume per reaction
10mM dNTPs mix	2 μL
MLV Reverse Transcriptase (200 U/μL)	1 μL
5x Reverse Transcription Buffer	4 μL
RNase Inhibitor (10 U/μL)	1 μL
Nuclease-free water	1 μL
Total mix volume	9 μL

The RT master mix was gently centrifuged to bring down all solution to the bottom of the EP tube and placed on ice. Next, 1000 ng of total RNA (10μL), 1μL Random Hexamer Pd(N)6

(0.2 μg/ μL) and 9 μL RT master mix were added to a 200 μL PCR tube, gently mixed, quickly centrifuged and incubated in ice for 5 minutes before loading into the thermal cycler. PCR tubes were loaded into thermal cycler with the cycler condition as follows: 25°C for 10 minutes, 37°C for 60 minutes, 95°C for 10 minutes and 4°C kept.

RT products and the components of SYBR Green master mix were gently mixed and then placed on ice. The qPCR primers of PTEN and GAPDH were obtained from OriGene (PTEN CAT#: HP200295; GAPDH, CAT#: HP205798). The SYBR Green qPCR reaction mix of three replicates was prepared in an EP tube as follows:

Component	Volume per master mix
2xSYBR Green Master Mix II	33.00 μL
Nuclease-free water	19.20 μL
Primer Forward (10 pm/ μL)	3.60 μL
Primer Reverse (10 pm/ μL)	3.60 μL
Product from RT reaction	6.60μL
Total volume	66.00 μL

All qPCR components were added into an EP tube, gently mixed by inversion and briefly centrifuged. Then 20 μL of the qPCR reaction mix was transferred to one tube of MicroAmp Fast Reaction Strip Tubes (Applied Biosystems, Thermo Fisher scientific Ottawa CA), and all tubes were sealed by optical caps and spun down gently by mini centrifuge (C1012-E, Benchmark Scientific, USA). All tubes were loaded into real-time PCR instrument StepOne plus (Applied Biosystems,Thermo Fisher scientific Ottawa CA) and ran the program as following: 50°C for 2 minutes; 95°C for 2 minutes; 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 60 seconds, 40 cycles; 4°C kept. The qPCR results were analyzed by StepOne software. GAPDH was used as endogenous control and the expression of PTEN was normalized to the expression of GAPDH by the comparative Ct method.

2.13 Cell cycle analysis

In order to examine the oncogenic effect of miR-498 on cell cycle progression, cells were stained by propidium iodide (PI) and examined by Flowcytometry (Wang B, 2017). Flow cytometry was used to sort cells undergoing different cell cycle phases by quantifying how much DNA was being stained by PI (G2/M cells have twice the DNA as G1 cells, and S phase cells are in-between G1 and G2 DNA amounts). Cells were transduced with cont.miR or miR-498. Then transduced cells were harvested using trypsin (3 minutes at 37°C) and re-suspended in cold (4°C) FCS (flow cytometry staining) wash buffer (0.5% FBS, 1 mM EDTA, and 0.05% sodium azide in PBS). The cell number of each cell sample aliquot was counted using a Bright Line Counting Chamber 3200 (Hausser Scientific, USA), and suspended cells from each aliquot were

transferred into a 15 mL tube at a density of 1 million cells / mL. Pre-cold 70% ethanol was prepared prior to the experiment, and 3 mL of 70% ethanol was added drop-wise to each cell tube. Cells were fixed in 70% ethanol overnight at -20°C. Once cells were fixed, they were centrifuged at 500 x g for 5 minutes and then re-suspended in fresh FCS wash buffer. Cells were washed by FCS buffer twice and this process was gently operated. Once washing was done, 1 mL of propidium iodide dye (50 µg/ml PI, 3.8 mM sodium citrate) (Sigma Aldrich, USA) was added to EP tubes for staining cellular DNA. A stock solution of RNase A (10 µg/ml RNase A, Worthington Biochemicals, USA) was prepared prior to these experiments by boiling solution for 5 minutes and storing aliquots at -20°C, and 40 µL of RNase A stock solution was added to each EP tube alongside the propidium iodide (PI) dye in order to eliminate RNA material. These cells were incubated with the dye solution overnight in 4°C fridge to ensure maximum dye staining efficiency. Once incubation was complete, cells were centrifuged at 800 x g for 5 minutes and washed by FCS buffer twice. Cell samples were re-suspended in 500 µL of FSC buffer in 1.5 mL EP tubes and stored at 4°C in the dark until flow cytometry was operated. Once flow cytometry was done, data readouts were analyzed by FlowJo software and the PI absorbance values of cell populations in G1, S, and G2/M phases were calculated. Average of absorbance values were calculated for each triplicate and plotted in Microsoft Excel (Microsoft, USA); error bars indicated the standard deviation of the mean, and statistical significance was analyzed by an unpaired Student's T-Test without assuming equal variance.

2.14 Introduction of point mutations by PCR

To confirm the predicted binding site of miR-498 on PTEN mRNA 3' UTR, point mutation on predicted binding site was made by PCR using QuickChange II site-directed Mutagenesis Kit(Cat#:200523, Agilent Technologies, USA). The luciferase reporter plasmid containing PTEN mRNA 3'UTR was used as PCR template. The mutation primers were designed by online Agilent QuickChange Primer Design program and synthesized by IDT. The PCR reaction was prepared as follows:

10x reaction buffer	5 μL
Template plasmid (10 ng/ μL)	2 μL
Primer 1 (50 ng/ μL)	2.5 μL
Primer 2 (50 ng/ μL)	2.5 μL
dNTP (10 mM/ μL)	1 μL
pfu polymerase (2.5U/ μL)	1 μL
ddH_2O	31 μL
Total volume	50 μL

PCR tubes were loaded into thermal cycler and the cycle was set as follows: 95°C for 30 seconds; 95°C for 30 seconds, 5°C for 1 minute and 68°C for 8 minutes, 18 cycles. After PCR was done, 1 μL Dpn I was added to PCR reaction product to digest parental plasmid template and PCR tubes were kept in 37°C for 1 hour. Next, 2 μL of digested PCR product was used to add into competent bacteria cells for transformation. Five colonies of transfected bacteria were chosen to test the point mutations by sequencing.

The mutation primers (Primer 1and Primer 2) for preparing mutation at the binding site of miR-498 on luciferase reporter were as follow:

Primer 1: 5'-CTCTAATTTCCTTTTTAACACAAATGTCCTTTTTAATCGCAACATT

ATATGTATTCAGTATTCAAGTAAAATTCCCT-3'

Primer 2: 5'-AGGGAATTTTACTTGAATACTGAATACATATAATGTTGCGATTAA

AAAGGACATTTGTGTTAAAAAGGAAATTAGAG-3'

2.15 Scratch healing assay

Scratch healing assay was performed to determine the effect of microRNAs on cell migration (Liang C, 2007). Cells were transduced with interested gene or microRNA (PTEN ORF, PTEN siRNA or miR-498 or miR-498 decoy) and then transduced cells were seeded into 24-well plates. Before scratching was performed, these transduced cells were treated with the serum starvation of 16 hours. Subsequently, the wound scratches were made by sterile P10 micropipette tips at the monolayer of cells in each well. The scratched monolayer of cells was gently washed twice by sterile PBS and the proper volume of medium was gently added into each well. The 24-well plate was put into a chamber where the lens of microscopy was inside and the environment was set the same as the cell culture incubator. The position parameters of a scratch area in cell monolayer of each well were inputted to microscopy controlling software (Metamorph); the images of these scratch areas were recorded by a digital camera through microscopy lens. The scratch areas were continually taken photo every 30 minutes for 24 hours

or the images of scratch areas were recorded at time points of 0-hour, 24-hour and 48-hour. Cell migration was evaluated by two methods. One was to track the movement of cells through the camera of phase contrast microscopy. The number of translocated cells that were detected to change location during the time-lapse imaging and the average distance of cell migration was both counted by itrack4U and used to evaluate the capability of cell migration (Cordelières FP, 2013). The other one was to analyze the relative blank wound area. The wound area was calculated by ImageJ with MRI wound healing tool (Baecker V, 2012) and statistical analysis of the relative wound area was also used to evaluate the capability of cell migration.

2.16 Clonogenic assay

The clonogenic assay was performed to determine the effect of miR-498 on cell proliferation and viability. miR-498 was transduced into cells. 250 cells or 500 transduced cells were seeded into the 60 mm dishes and incubated for 14 days. Then the medium in cell dishes was removed and cell dishes were washed twice by PBS. The cell colonies were fixed by 10% (vol/vol) formaldehyde for 1 hour and stained by 0.5% (wt/vol) crystal violet for 1 hour. The ratio of colony formation (N/n) was calculated by the number of colonies (N) divided by the numbers of seeded cells (n). Colonies with over 50 cells were counted to determine the colony number ratio. The ratio of cell colony was used to evaluate the capability of cell proliferation and viability.

2.17 MTT assay

To determine the oncogenic effect of miR-498 or PTEN siRNA in TNBC cells and breast epithelial cells, the cell proliferation and viability was examined by MTT assay. TNBC cells were transduced with miR-498 or PTEN siRNA and transduced cells were seeded in 96-well plates. After the incubation of 48 hours, 50 ul MTT (50 μg /ml) was added to cell medium in each well and cell plates were incubated in 37°C for 3 hours. Next, the cell medium was gently removed and 150 μL MTT solvent was added to each well. After 30 minutes the value of optical density (OD) at wavelength of 550 nm of each well was measured by iMark ^{TM} microplate absorbance spectrophotometer (Bio-Rad, USA) and normalized to the absorption value of control well. The MTT assay results were used to evaluate the capability of cell viability and proliferation.

2.18 Statistical analysis

Data are presented as the means ± standard deviation (SD). Statistical comparisons were performed using the unpaired two-tailed Student's t-tests.

Chapter 3 Results

3.1 Screening and identifying microRNAs that regulate PTEN

The tumor suppressor PTEN plays a crucial role in suppressing tumor progression and is one of the most inactivated tumor suppressors in various cancers, e.g. breast cancer, lung cancer, brain cancer and prostate cancer (Steck, P.A, 1997; Li J, 1997; Marsit CJ, 2005). PTEN inactivity is associated with large tumor sizes, considerable lymph node metastasis and an aggressive triple-negative phenotype in breast cancer (BCa) (Li S, 2017). PTEN expression is much lower in BCa tissue (57.5%, 84/117) than in the normal tissue (100%, 10/10); however, PTEN mutation rates are approximately 5% in BCa, which indicates that other mechanisms negatively regulate PTEN expression (Rhei E, 1997; Zhang HY, 2013). MicroRNA-induced gene inactivation is one of the new mechanisms identified in the regulation of PTEN expression and activity (Pezzolesi MG, 2008; Huse JT, 2009). MicroRNA is one type of non-coding RNA and is one of the mechanisms regulating the protein expression, which usually results in the down-regulation of its target protein (Pang KC, 2007). The inactivation or down-regulation of PTEN by microRNAs has been identified by many research groups in various cancers. Specifically, miR-221 and miR-222 negatively regulated PTEN in non-small cell lung cancer and hepatocellular carcinoma and promoted tumorigenesis by directly binding to the mRNA of PTEN and suppressing PTEN expression (Garofalo M, 2009); miR-214 promoted cell survival and induced cisplatin resistance by targeting and inhibiting PTEN in ovarian cancer (Yang H, 2008); miR-21 was overexpressed in tissues and cell lines of human hepatocellular cancer (HCC) where it bound to PTEN mRNA and functioned as an oncogenic miR (Meng F, 2007). However, except miR-21that is a well-established oncogenic miR (Gong C, 2011; Wang ZX, 2011), the number of identified PTEN-targeting microRNAs in BCa is still limited and the identification of novel miRNAs that results in the PTEN inactivation is of great importance to improve the breast

cancer treatment. Thus, it is necessary to identify the oncogenic PTEN-targeting microRNAs in BCa and clarify the relationship between the PTEN inactivation and microRNAs in BCa.

In this section, I used online databases to predict the PTEN-targeting microRNAs and screened their effect of predicted microRNAs on PTEN protein level in cancer cell line MCF-7 and A549. Next, the luciferase reporter assay was applied to further examine whether predicted microRNAs were capable of binding to the PTEN mRNA 3'UTR and the mutagenesis was used to define the binding site on PTEN mRNA. The regulation of exogenous and endogenous PTEN by the confirmed PTEN-targeting microRNA was screened by immunoblotting in HEK293 cell and cancer cell lines MCF-7 and A549. Finally, the clinical impact of PTEN and the confirmed PTEN-targeting microRNAs were investigated.

3.1.1 Prediction of microRNAs targeting PTEN

To investigate microRNAs that target *PTEN* and downregulate PTEN protein levels, microRNA databases (MiRanda, RNA22, miTarget, picTar, TargetScan) were used to predict the potential microRNAs that bind to *PTEN* mRNA. The microRNA databases predict the potential microRNAs targeting PTEN mRNA and can be used to evaluate the binding sites of the microRNAs. The predicted microRNAs targeting *PTEN* mRNA were preliminarily selected from a pool of microRNAs where at least two of five microRNA databases predicted binding. Nineteen predicted microRNAs targeting *PTEN* were selected as potential microRNAs to prepare the expression plasmids and for performing further confirmation by experiments (Figure 3.1.1).

The genomic DNA sequences of the 19 microRNAs were obtained from the OriGene TrueClone database, and the cloning primers for each of the 19 microRNAs were personally designed by Primer Premier 5. The genomic DNA used as PCR template was extracted from HEK293 cells. The sequences of microRNAs (approximately 700 bp) were acquired through PCR and inserted into the plasmid vector pcDNA3.1 to make plasmids that could express microRNA in human cancer cells. The nineteen microRNA-expressing plasmids were tested and confirmed by restriction enzyme digestion and the microRNA sequences within these plasmids were confirmed by DNA sequencing before the next experiments (The digestion of some plasmids was shown in Figure 3.1.2).

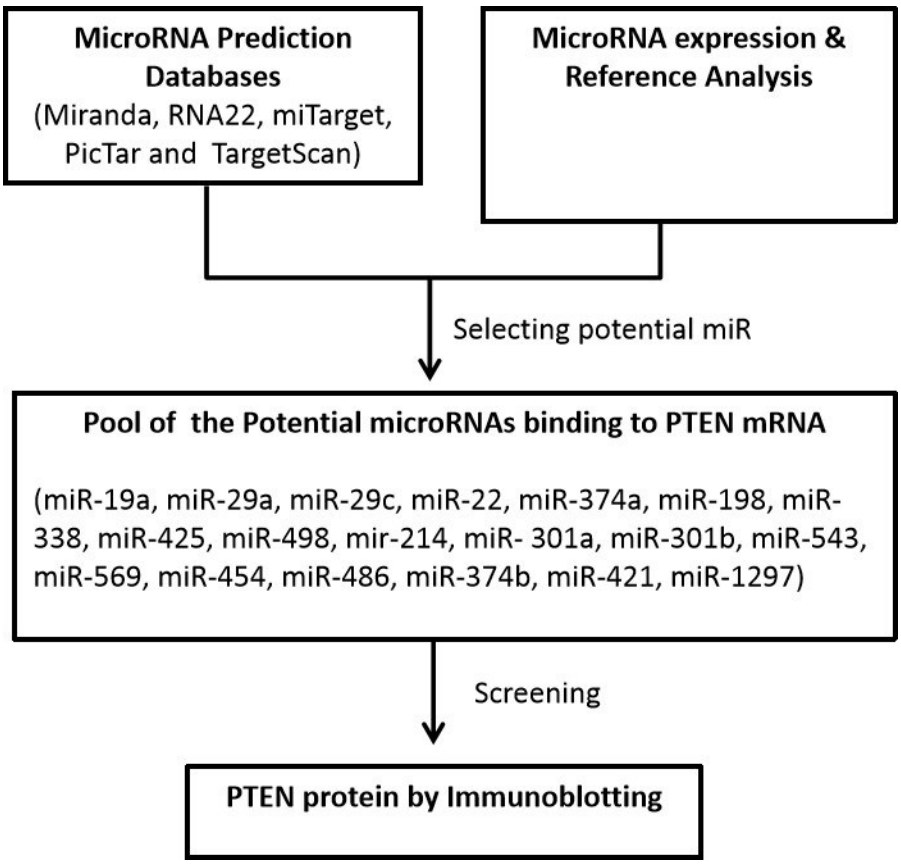


Figure 3.1.1 Summary of the screening for microRNAs that regulates PTEN protein level.

To find the potential microRNAs that bind to PTEN mRNA and regulate PTEN, five online databases (Miranda, RNA22, miTarget, PicTar and TargetScan) were utilized to predict the potential microRNAs. MicroRNAs which were predicted by at least two databases were chosen as the potential PTEN-targeting microRNA. The microRNA expression constructs were made based on the pcDNA3 plasmid and were transfected into cancer cells. After 48 hours of microRNA transfection, the cells were collected and lysed and then change of endogenous PTEN was examined by immunoblotting to screen the microRNAs that regulate PTEN.

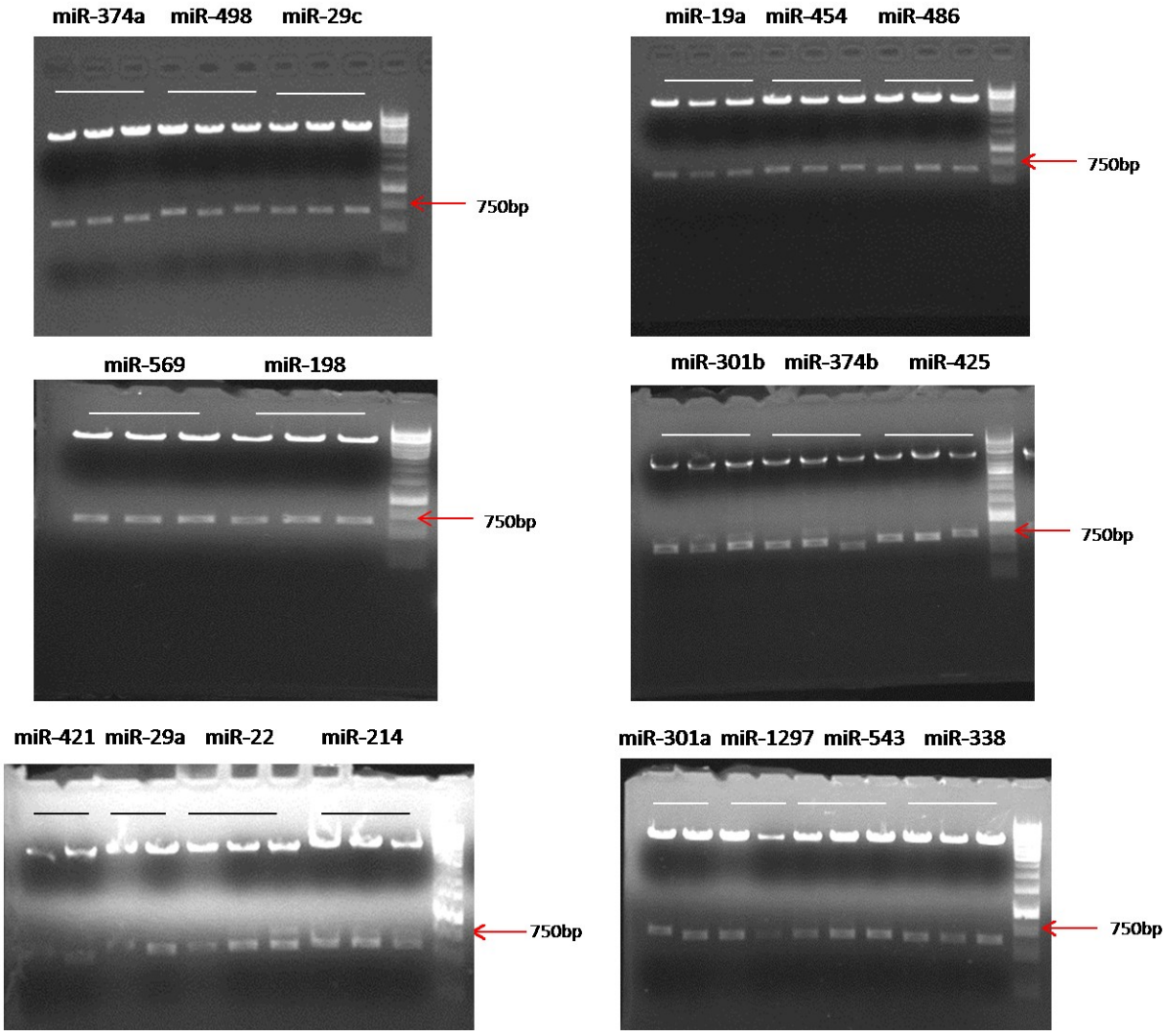


Figure 3.1.2 Constructs of microRNA plasmids tested by restriction digestion.

The digestion of microRNA expression constructs was examined by electrophoresis and the digestion results of these constructs were shown in Figure 3.1.2. The sequences of genomic DNA (~700bp) which contained the potential microRNAs were acquired from Origene and the PCR primers were designed by Primer Premier 5. The genomic DNA was amplified by the polymerase chain reaction (PCR) using the genomic DNA of HEK293 cell as template. The microRNA constructs from positive clones were digested by restriction digestion and examined by electrophoresis. The microRNA sequences in positive clones were confirmed by DNA sequencing.

3.1.2 Screening PTEN -targeting microRNAs by immunoblotting and luciferase assay

The 19 microRNAs were transfected into MCF-7 cells and A549 lung cancer cells for examining the change of endogenous PTEN protein level. These two cell lines exhibited moderate expression of wt PTEN, and the calcium phosphate method showed that they were of high transfection efficiency. The immunoblotting results indicated that miR-425, miR-454, miR-301b, miR-498, and miR-374b reduced >50% of endogenous PTEN protein in the MCF-7 cells (Figure 3.1.3A); miR-22, miR-425, miR-29a, miR-374b, miR-454, miR-301b, miR-498, and miR-29c reduced >50% of endogenous of PTEN in the A549 cells (Figure 3.1.3B). Based on the preliminary screening results, miR-498, miR-425, miR-374b, miR-454, miR-301b, and miR-22 remained in the pool of candidate *PTEN*-targeting microRNA. As microRNAs (miR-22 and miR-425) have been shown to target *PTEN* (Ma J, 2014; Bar N, 2010), the remaining microRNAs were chosen for further confirmation.

Subsequently, I used the luciferase reporter assay to confirm the binding of four microRNAs to *PTEN* mRNA. The luciferase reporter was constructed by inserting the 3′UTR of *PTEN* mRNA into the pMIR-REPORT luciferase vector following the luciferase gene. Each of the four microRNAs (miR-498, miR-454, miR-374b, miR-301b) was co-transfected with the *PTEN* mRNA 3′UTR luciferase reporter plasmid into HEK 293 cells to confirm their binding to the *PTEN* mRNA 3′UTR. pMIR REPORT beta-galactosidase was used as the transfection control. If the microRNA bound to the *PTEN* mRNA 3′UTR in the luciferase reporter, the luciferase translation would be inhibited, and then luciferase activity would be downregulated. The relative light unit (RLU) was recorded and indicated the luciferase activity. The RLU values were normalized to the optical density (OD) 420 nm value of the beta-galactosidase assay. Of the

four microRNAs, miR-498 decreased luciferase activity significantly compared to the control miR, which indicated that miR-498 can bind to the *PTEN* mRNA 3′UTR (Figure 3.1.4). Among the remaining three microRNAs, miR-454 and miR-301b showed only a slight reduction of luciferase activity, and miR-374b did not decrease luciferase activity significantly compared to the control miR. Based on these results, miR-498 was selected as a *PTEN*-targeting microRNA to investigate its role in regulating *PTEN* and its function.

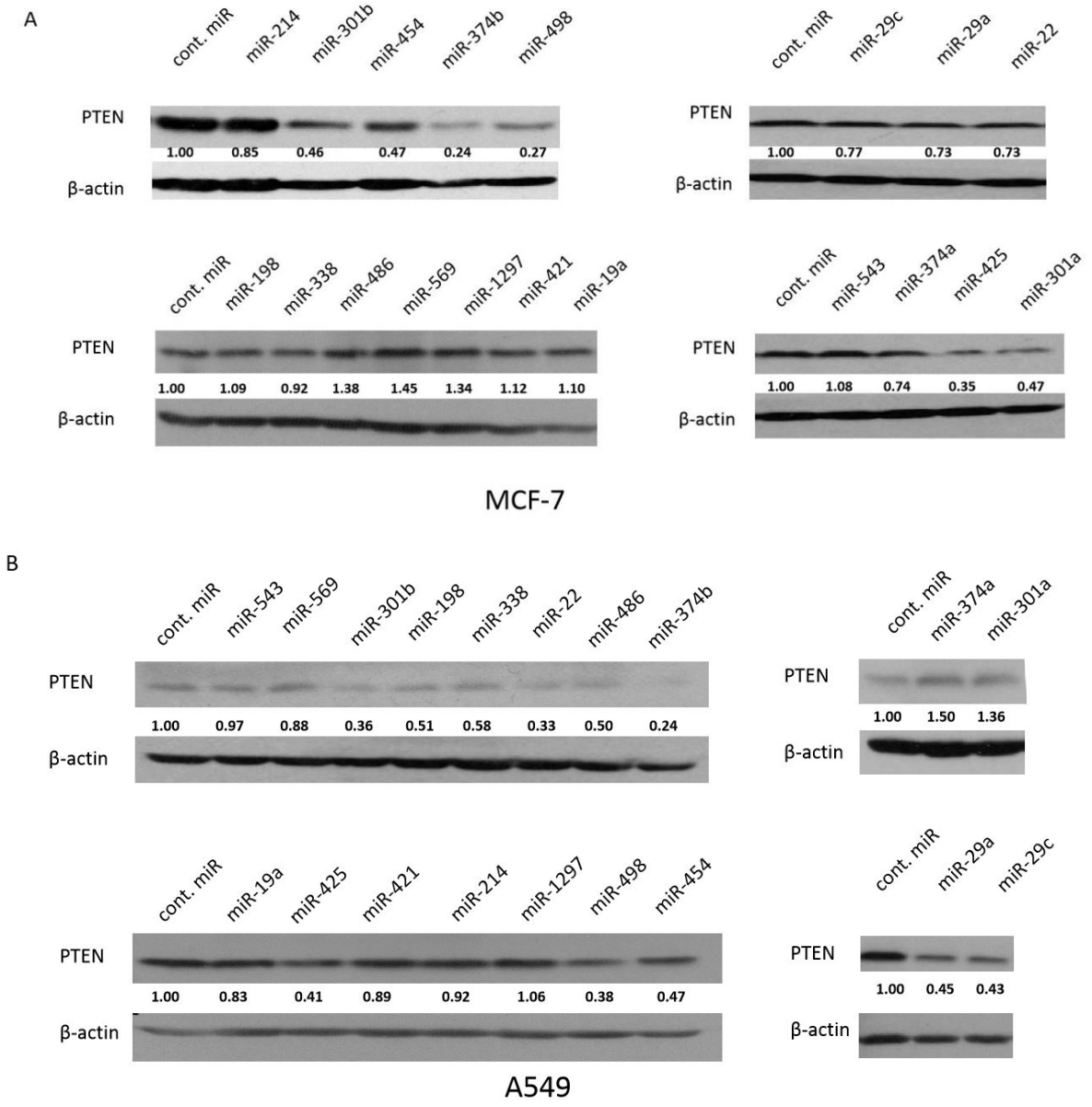


Figure 3.1.3 Screening of PTEN-targeting microRNAs by immunoblotting.

Individual microRNA was transfected into the breast cancer cell line MCF-7 (A) and the lung cancer cell line A549 (B) to examine the change on protein level of endogenous PTEN. After 48 hours of transfection, transfected cells were collected and lysed and then 50 μg of total protein was loaded for SDS-PAGE electrophoresis. PTEN antibody was used to detect the endogenous PTEN protein.

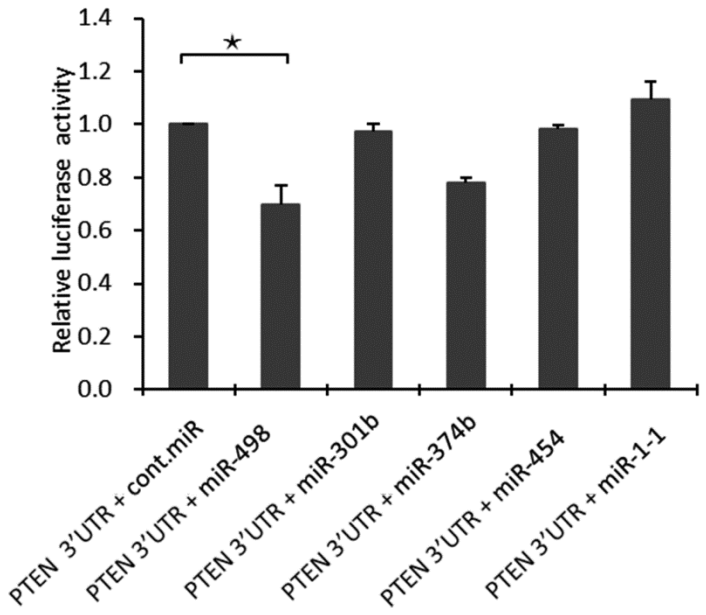


Figure 3.1.4 Screening of microRNAs binding to PTEN mRNA 3'UTR.

Four potential PTEN-targeting microRNAs (miR-498, miR-301b, miR-374b and miR-454) screened by previous immunoblotting and one non-PTEN targeting microRNA (miR-1-1) were examined in this luciferase assay. The plasmid of beta-galactosidase was also co-transfected with the luciferase reporter and microRNA to evaluate the efficiency of transfection. The relative luciferase activity of PTEN-targeting microRNA was normalized to the control miR. The results were based on two independent experiments, $\star$ $p<0.05$.

3.1.3 Identification the binding site of miR-498 on PTEN mRNA

The earlier results confirmed that PTEN protein was negatively regulated by miR-498 and miR-498 bound to the *PTEN* mRNA 3′UTR. However, the binding sites of miR-498 on *PTEN* mRNA are not clearly identified. Therefore, to identify the miR-498 binding sites on the *PTEN* mRNA 3′UTR, the database microRNA.org was used to find the putative binding sites. Although microRNA.org predicted five putative binding sites of miR-498 on *PTEN* mRNA, three binding sites with mirSVR scores ≤ -0.05 were chosen for further examination. mirSVR is an algorithm used for scoring and ranking the efficiency of microRNA target sites, and the mirSVR score is used for evaluating the targeting of mRNA and downregulation of PTEN. A microRNA binding site with a mirSVR score of less than -0.05 indicates a higher probability of microRNA binding. Three binding sites were identified: site A, site B, and site C in 26–48 bp, 833–855 bp, and 1242–1269 bp, respectively, of the *PTEN* mRNA 3′UTR. The DNA sequence containing each of the three binding sites was cloned and inserted to a luciferase reporter. These luciferase reporters were designated *PTEN* mRNA 3′UTR part A, part B, and part C, respectively. Then, each luciferase reporter was co-transfected with miR-498 into HEK 293 cells, with transfection of beta-galactosidase as a transfection control. After 48 hours, transfected cells were lysed and luciferase activity was measured. It was expected that luciferase activity would decrease once miR-498 bound to the *PTEN* mRNA 3′UTR sequence. The luciferase activity was measured and normalized to beta-galactosidase activity. The results indicated that compared to the control miR, miR-498 could not downregulate the luciferase activity of the *PTEN* mRNA 3′UTR part A reporter or the part C reporter, which meant that miR-498 did not bind to two predicted binding sites (Figure 3.1.5A & C). However, miR-498 reduced the luciferase activity

of the *PTEN* mRNA 3′UTR part B reporter significantly, which confirmed that miR-498 can bind to the *PTEN* mRNA 3′UTR at site B (Figure 3.1.5B).

To confirm this finding, miR-498 binding site B on the *PTEN* mRNA 3′UTR part B reporter was mutated by four nucleotides (Figure 3.1.6A). Then, miR-498 was co-transfected with mutated *PTEN* mRNA 3′UTR part B reporter or wt *PTEN* mRNA 3′UTR part B reporter. It was expected that the mutated binding site on the *PTEN* mRNA 3′UTR part B reporter would lose the ability to bind miR-498 and thus miR-498 would not reduce its luciferase activity. Consistent with the expectation, miR-498 reduced the luciferase activity of the wt part B reporter significantly, but did not alter the luciferase activity of the mutated part B reporter (Figure 3.1.6B & D). The results demonstrate that binding site B (833–855 bp on *PTEN* mRNA 3′UTR) is the functional binding site for regulating *PTEN* through miR-498. Simultaneously, miR-498 expression was measured using TaqMan Probe qPCR, and U6 snRNA was used as the PCR control. The expression of miR-498 was normalized to U6 snRNA expression by the comparative threshold cycle (Ct) method. The TaqMan qPCR assay results confirmed that miR-498 expression was >100-fold higher in miR-498–transfected cells compared to that in control miR–transfected cells (Figure 3.1.6C & E).

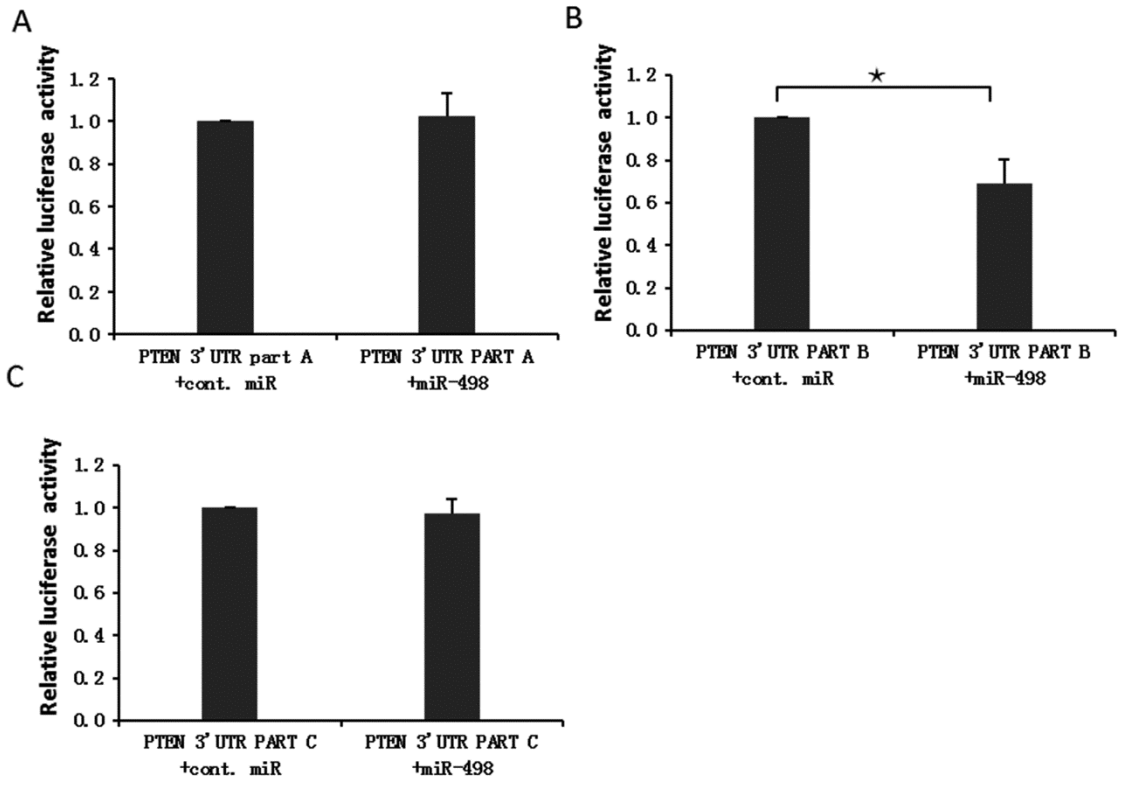


Figure 3.1.5 Identification of miR-498 binding sites on PTEN mRNA by luciferase assay.

To identify the binding sites of miR-498 to PTEN mRNA 3' UTR, the luciferase reporter which contained the predicted binding site of miR-498 was constructed and co-transfected with miR-498 into HEK 293 cells. (A) The binding site A was not a functional one for miR-498 binding. The luciferase activity did not decrease, which indicated that the predicted binding site A of miR-498 was not functional for the binding of miR-498. (B) The binding site B was a confirmed binding site for miR-498 binding. The results showed that miR-498 significantly reduce the activity of luciferase compared with the cont. miR, which indicated miR-498 could bind to the 3'UTR of PTEN. (C) The binding site C was not a functional one for miR-498 binding. The luciferase activity did not decrease significantly, which indicated that the predicted binding site C of miR-498 was not functional for the binding of miR-498. The plasmid of beta-galactosidase was also co-transfected with the luciferase reporter to evaluate the efficiency of transfection. The relative luciferase activity of miR-498 transfection was obtained by normalizing to the control miR transfection. The results were based on three independent experiments, ⋆ $p<0.05$.

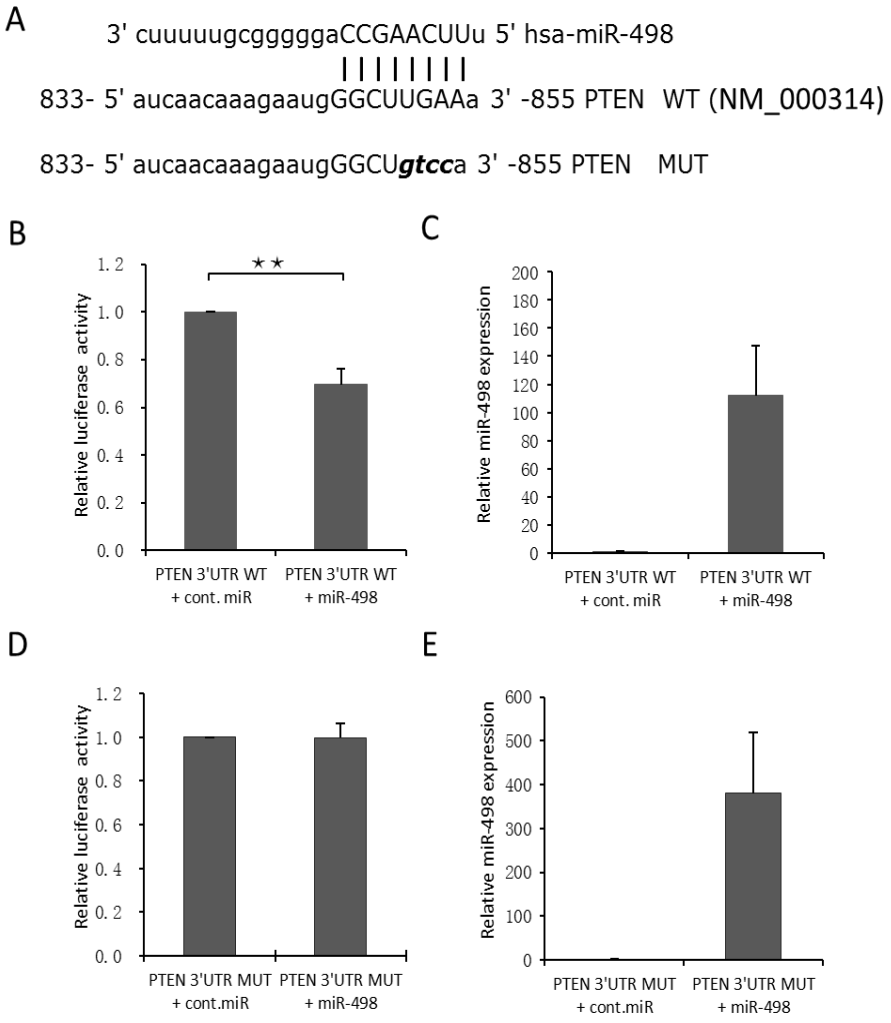


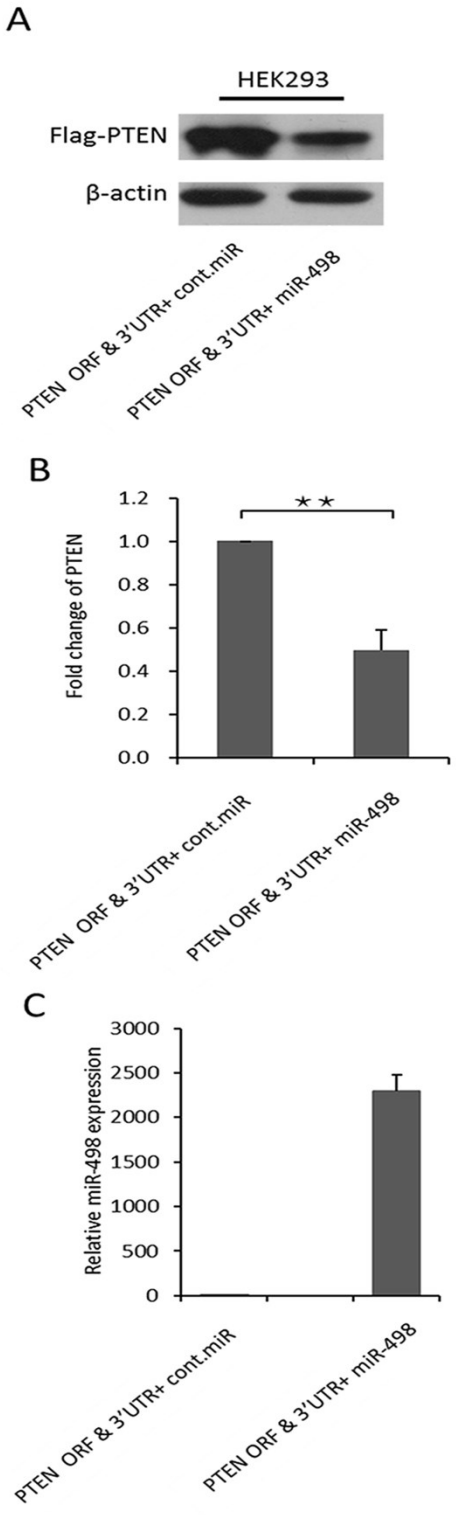
Figure 3.1.6 Determination the binding site of miR-498 on PTEN mRNA by mutagenesis

The binding site B of miR-498 on PTEN mRNA was further investigated by non-functional mutation for miR-498 binding. (A) The wild-type or mutated sequence of binding site B of miR-498 was shown. The wild-type binding site (WT) and the mutated binding site (MUT) on PTEN mRNA 3' UTR were aligned as indicated. (B) miR-498 binding to the wild-type site on PTEN mRNA was determined by luciferase assay. A plasmid expressing miR-498 or control miR was co-transfected into HEK293 cells with a luciferase reporter containing the wild-type binding site. (C) Levels of miR-498 expression were determined by TaqMan probe qRT-PCR. The expression of miR-498 was normalized to the expression of U6 snRNA by the comparative Ct method. (D)

miR-498 binding to the mutated 3'UTR of PTEN mRNA was denied by luciferase assay. (E) Levels of miR-498 expression were determined by TaqMan probe qRT-PCR. The results were based on three independent experiments, ⋆⋆ $p<0.01$.

3.1.4 Protein expression of PTEN repressed by miR-498

Although the luciferase assay results indicated that miR-498 bound to the *PTEN* mRNA 3′UTR, the effect of miR-498 on the exogenous protein expression of PTEN was not directly determined. To confirm the effect of miR-498 on PTEN protein expression, an exogenous protein expressing plasmid was used and the DNA sequence of the *PTEN* mRNA open reading frame (ORF) and 3′UTR was inserted into the plasmid following Flag-tagging. The transcript product of the plasmid could be used to mimic *PTEN* mRNA as the target of miR-498 and to examine the effect of miR-498 on PTEN protein. The PTEN-expressing plasmid was co-transfected with miR-498 or control miR into HEK 293 cells. The transfected cells were lysed 48 hours later and the expression of exogenous PTEN was examined by immunoblotting. The immunoblotting showed that miR-498 reduced Flag-PTEN significantly compared to the control miR (Figure 3.1.7A), which indicated that the binding of miR-498 to the *PTEN* mRNA 3′UTR repressed the expression of PTEN protein. The protein band of Flag-PTEN was quantified by ImageJ and normalized to β-actin. Repression of exogenous PTEN by miR-498 was evaluated by the fold change of Flag-PTEN as compared to the control miR. The experiment was repeated three times and miR-498 reduced the exogenous PTEN protein level by 50% compared to that of the control miR (Figure 3.1.7B). Furthermore, TaqMan qPCR assay confirmed the overexpression of miR-498, and the miR-498 expression level in cells transfected with miR-498 was >2000-fold higher than that in control miR–transfected cells (Figure 3.1.7C).


A
HEK293
Flag-PTEN
β-actin
PTEN ORF & 3'UTR+ cont.miR
PTEN ORF & 3'UTR+ miR-498
B
Fold change of PTEN
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
PTEN ORF & 3'UTR+ cont.miR
PTEN ORF & 3'UTR+ miR-498
C
Relative miR-498 expression
3000
2500
2000
1500
1000
500
0
PTEN ORF & 3'UTR+ cont.miR
PTEN ORF & 3'UTR+ miR-498

3.1.7 Exogenous PTEN repressed by miR-498.

Exogenous PTEN was repressed by miR-498. (A) The protein level of exogenous PTEN was examined by miR-498. To examine the effect of miR-498 on the exogenous expression of PTEN, the plasmid containing Flag-tagged PTEN ORF & 3'UTR was co-transfected into HEK293 cells with miR-498 or control miR. After 48 hours, cells were lysed and exogenous PTEN was examined by immunoblotting. (B) The protein change of exogenous PTEN in HEK293 cells was statistically significant based on the quantification of protein bands of immunoblotting. The results were based on three independent experiments (n=3), ⋆⋆ $p<0.01$. (C) miR-498 was overexpressed in the HEK 293 cells that were transfected with miR-498 and PTEN ORF & 3'UTR. The miR-498 expression was measured by TaqMan probe qRT-PCR and normalized to U6 snRNA.

To confirm the effect of miR-498 on the suppression of endogenous PTEN in multiple cancer cells, miR-498 was overexpressed in MCF-7 BCa cells and A549 lung cancer cells. These cell lines both have the wt *PTEN* gene, and PTEN protein expression is moderate and detectable by immunoblotting. The cells were transfected with miR-498–expressing plasmid. In the MCF-7 cells, miR-498 decreased the endogenous PTEN protein level significantly compared to the control miR, which indicated that miR-498 suppressed PTEN protein expression (Figure 3.1.8A). In the A549 cells, consistent with the results in the MCF-7 cells, miR-498 reduced the endogenous PTEN protein level significantly compared with the control miR (Figure 3.1.8B). The expression of PTEN protein was quantified and normalized to that of β-actin. PTEN suppression by miR-498 was quantified as the fold change of PTEN expression. The results were based on three independent experiments (Figure 3.1.8C & D). Simultaneously, miR-498 expression was measured by TaqMan Probe qPCR, and U6 snRNA was used as the PCR control. Moreover, miR-498 expression was normalized to that of U6 snRNA by the comparative Ct method. The qPCR results indicated that miR-498 was overexpressed in miR-498–transfected cells compared to control miR–transfected cells (Figure 3.1.8E & F).

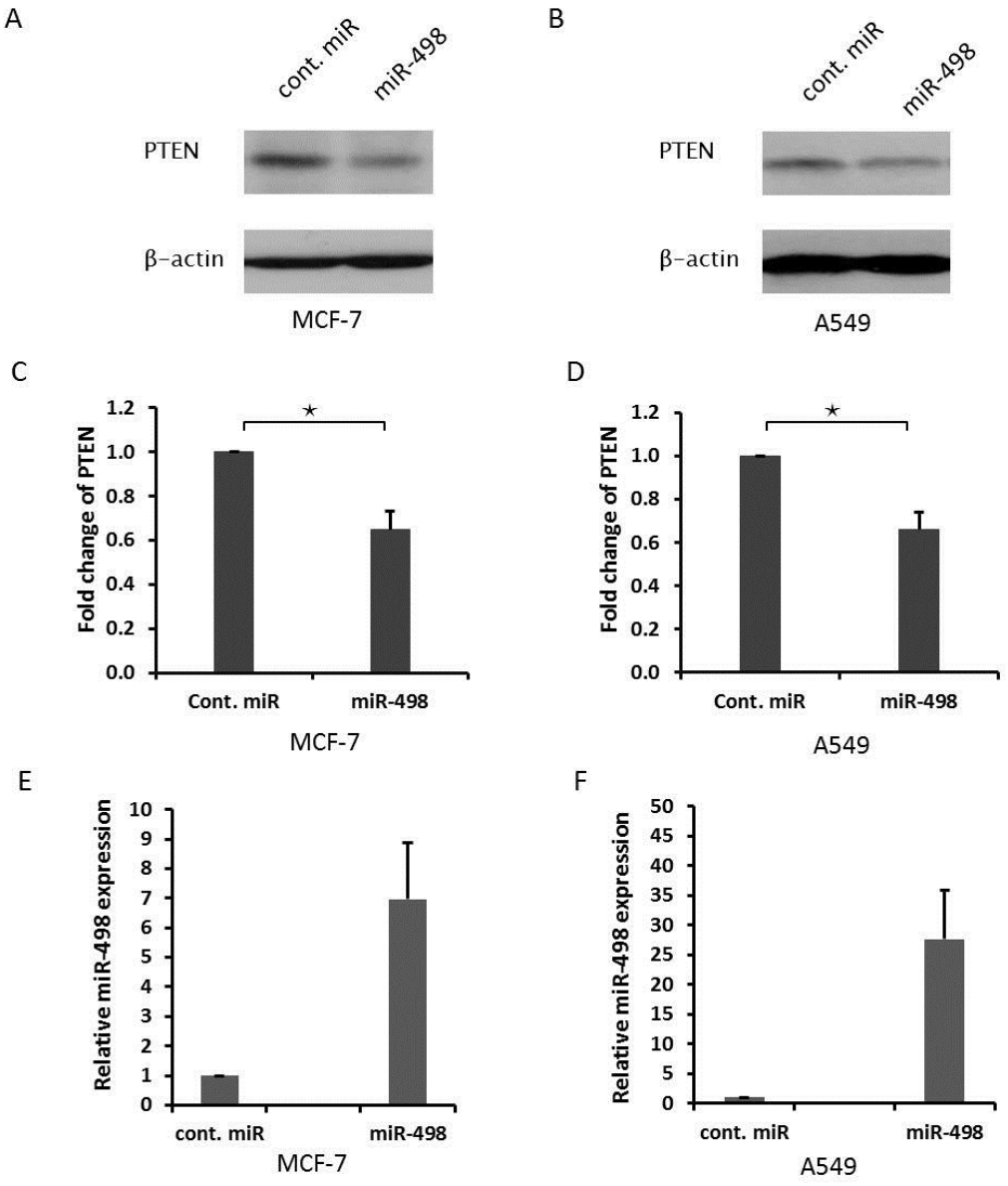


Figure 3.1.8 Endogenous PTEN suppressed by miR-498.

Endogenous PTEN was suppressed by miR-498 in MCF-7 and A549 cells. (A) The endogenous PTEN in MCF-7 breast cancer cells by miR-498 transfection. The miR-498 or control miR was transfected into MCF-7 cells. PTEN levels were detected by immunoblotting with PTEN antibody. (B) The endogenous PTEN in A549 lung cancer cells by miR-498 transfection. The miR-498 or control miR was transfected into A549 cells. (C) The protein change of endogenous PTEN in MCF-7 cells was statistically significant based on the quantification of immunoblotting. Fold change of PTEN protein level by miR-498 in MCF-7 was calculated based on three

experiments (n=3, ⋆ $p<0.05$). (D) The protein change of endogenous PTEN in A549 cells was statistically significant based on the quantification of immunoblotting. Fold change of PTEN protein level by miR-498 in A549 was calculated based on three experiments (n=3, ⋆ $p<0.05$). (E) MiR-498 overexpressed in MCF-7 cells transfected with miR-498. The expression of miR-498 was examined by TaqMan qRT-PCR and U6 snRNA was used as PCR control. And the expression of miR-498 was normalized to the expression of U6 snRNA by the comparative Ct method. (F) MiR-498 overexpressed in A549 cells transfected with miR-498.

3.1.5 Clinical impact of PTEN and miR-498 in cancer

The prior experiments demonstrated that miR-498 is a *PTEN*-targeting microRNA and a negative regulator of the tumor suppressor PTEN. In the context of the tumor-suppressing function of wt PTEN, I hypothesized that miR-498 could play a pro-oncogenic role. However, the role miR-498 plays in BCa remained unclear. Therefore, I intended to investigate the expression of PTEN in breast and lung cancer tissues and the relation of miR-498 with the overall survival time of patients with BCa.

First, online microarray databases were used to investigate the expression of PTEN in BCa tissues. Oncomine is an online cancer microarray database where researchers can assess gene expression in cancer tissues based on published microarray data. As shown in Figure 3.1.9A, in the Finak breast dataset, PTEN expression in BCa tissue was significantly lower compared to that in normal breast tissue. The ratio of PTEN in BCa tissue versus normal tissue was 1:8.940. Similarly, in the Cancer Genome Atlas (TCGA) breast dataset, PTEN expression in BCa tissue was significantly lower than that in normal breast tissue, with a ratio of 1:2.376 (Figure 3.1.9B). These results indicate that as a tumor suppressor, PTEN is inhibited in BCa tissues when compared to normal breast tissues. However, while PTEN expression in lung cancer tissues was also downregulated compared with that in normal lung tissues, the reduction was not significant. Specifically, in the Okayama lung dataset, the ratio of PTEN expression in lung cancer tissue versus normal tissue was 1:1.308; in the Selamat lung dataset, this ratio was 1:1.272 (Figure 3.1.10). As there was no significant difference in PTEN expression between lung cancer tissues and normal tissues, I chose to focus on BCa and investigate the clinical impact of miR-498 in BCa.

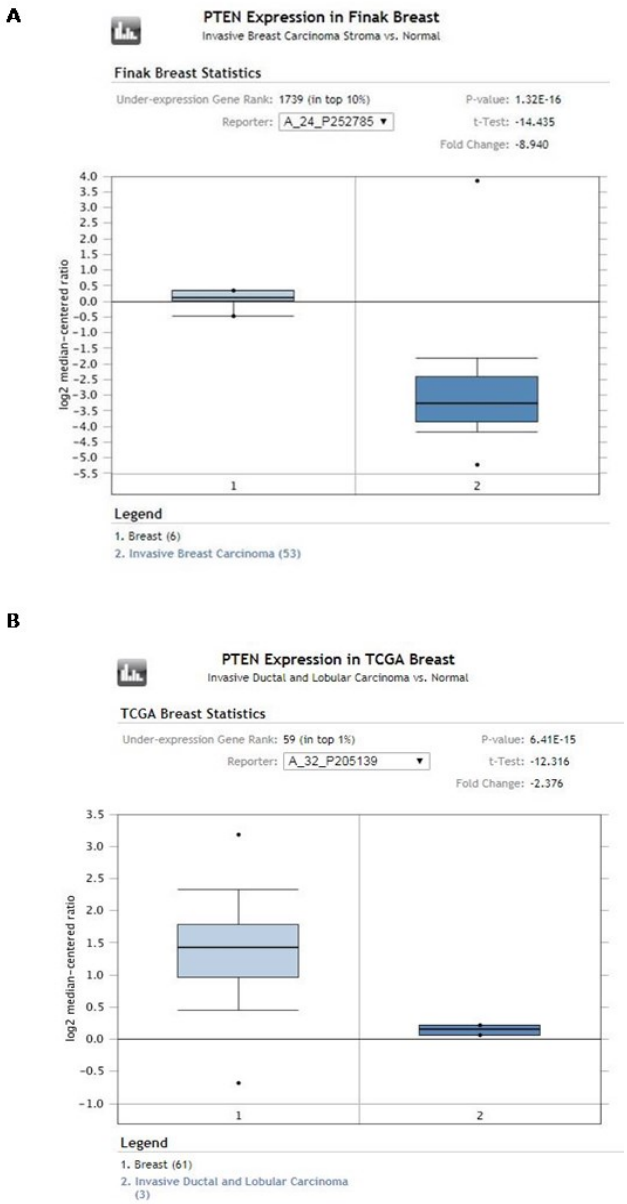


Figure 3.1.9 PTEN suppressed in the tissues of breast cancer patients.

PTEN expression in breast cancer patients. (A) The PTEN expression level in tissue samples of breast cancer patients compared to the level in non-tumor breast tissues. (B) The PTEN expression in the invasive breast cancer compared to the PTEN expression in non-tumor breast tissues. The figures of PTEN mRNA expression in breast cancer patients were acquired from the Oncomine database.

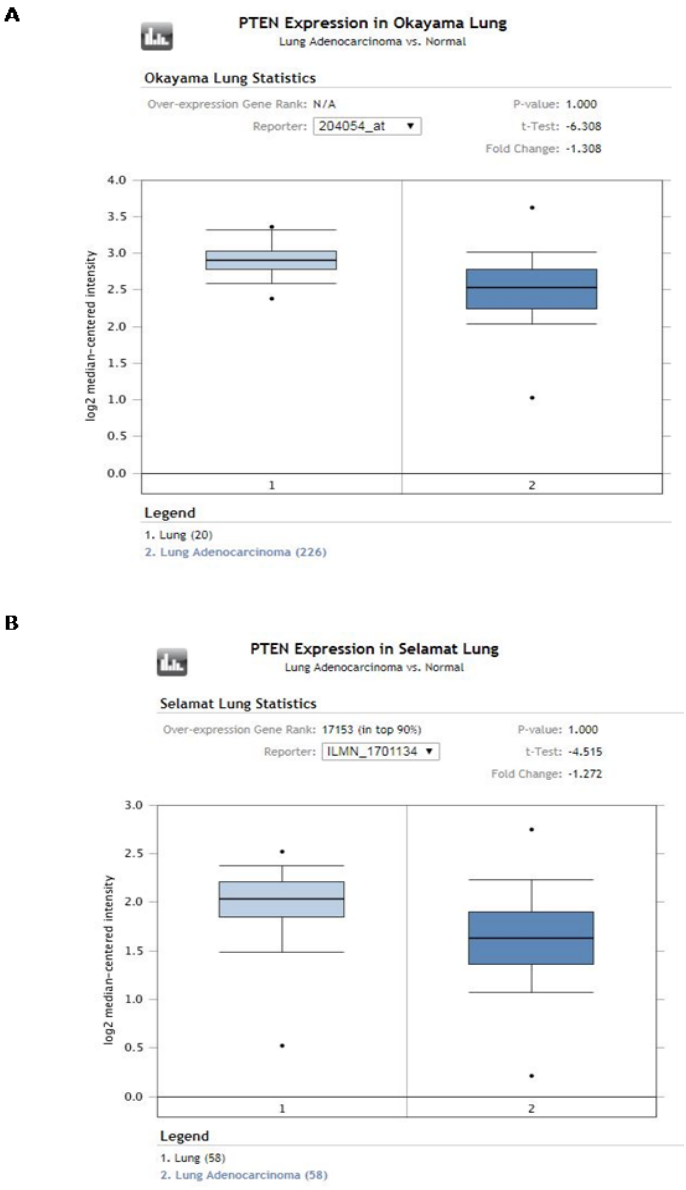


Figure 3.1.10 PTEN suppressed in the tissues of lung cancer patients.

PTEN expression in lung cancer patients. (A) The PTEN expression level in tissue samples of lung adenocarcinoma patients compared to the level in non-tumor lung tissues. (B) The PTEN expression in the lung adenocarcinoma tissues was reduced but not significantly, compared to the PTEN expression in non-tumor lung tissues. The figures of PTEN mRNA expression in lung cancer patients were acquired from the Oncomine database.

To identify the clinical impact of miR-498 in BCa, I investigated the relation of miR-498 and the overall survival of patients with BCa through Kaplan–Meier analysis. The Kaplan–Meier survival curves indicated that miR-498 expression was negatively associated with the overall survival of patients with BCa. Based on TCGA datasets, patients with BCa with high levels of miR-498 (n = 571) had significantly lower overall survival time (P = 1.6e-4) than patients with low levels of miR-498 (n = 490), which possibly suggested a negative relation between miR-498 expression and the overall survival in BCa (Figure 3.1.11A). Similarly, another dataset, GSE40267, with fewer patient samples, also showed a trend of the negative relation between miR-498 expression and overall survival in BCa, although the difference in overall survival was not significant (P = 0.063) between the upper quartile (n = 21) and the lower quartile (n = 21) of patients (Figure 3.1.11B). Thus, these results indicate that high miR-498 expression levels are associated with shorter overall survival time of patients with BCa, which hint that miR-498 probably plays an oncogenic role in BCa.

Next, I examined miR-498 expression levels in patients with BCa in online databases or publications. Unfortunately, there is no information on miR-498 expression in BCa in multiple databases, including Oncomine, TCGA, miRCancerdb, and miRmine. However, some previously published work examined miR-498 expression in BCa tissue samples. Matamala et al. indicated that miR-498 overexpression was identified in tissue samples of triple-negative BCa (TNBC), a subtype of BCa; microarray data indicated that 19 of 31 TNBC tissue samples exhibited significantly higher levels of miR-498 (61.3%) than normal breast tissues (Matamala N, 2016). These results further hint that miR-498 probably plays an oncogenic role in TNBC. Unfortunately, the relation between miR-498 overexpression and *PTEN* inactivation in TNBC

was unclear. Therefore, in the following section, I examine whether miR-498 overexpression leads to *PTEN* inactivation in TNBC.

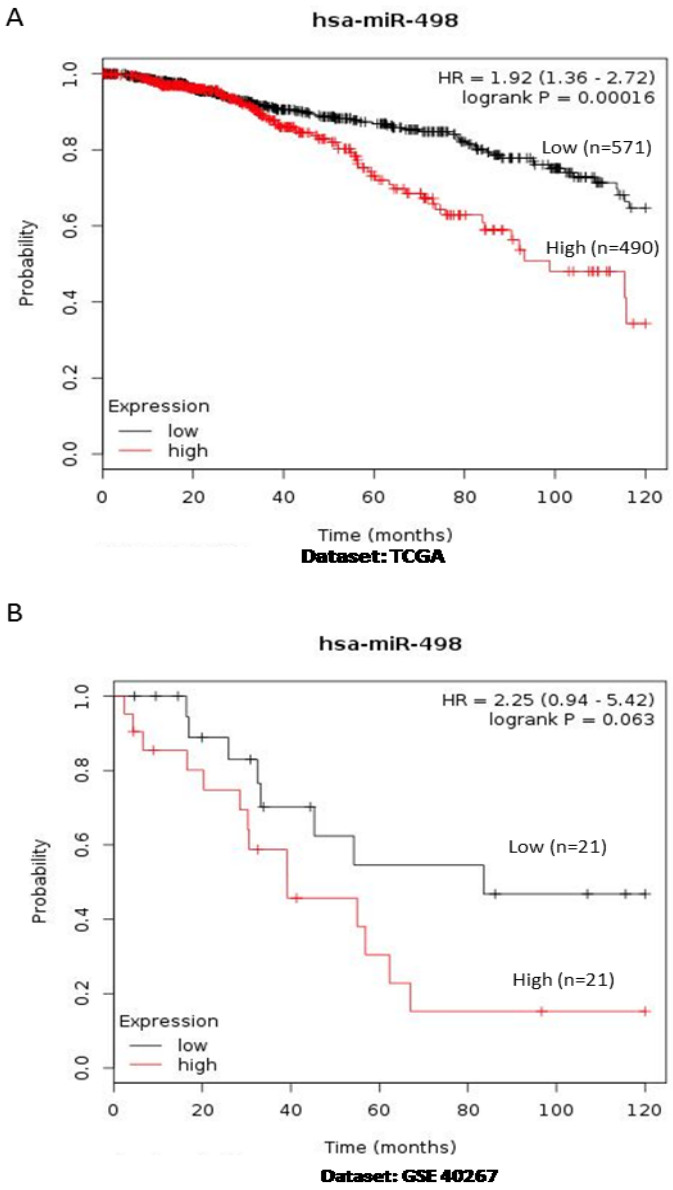


Figure 3.1.11 miR-498 expression negatively associated with the overall survival time of breast cancer (BCa) patients.

The miR-498 expression with the overall survival time of BCa patients. (A) The dataset TCGA for BCa patients with high miR-498 expression versus BCa patients with low miR-498 expression (P=1.6e-4). (B) The dataset GSE 40267 for BCa patients with high miR-498 expression versus BCa patients with low miR-498 expression. The patients were split by quartiles. These figures were acquired from http://kmplot.com/analysis/index.php?p=service&cancer=breast_mirna.

Summarily, in this section, I have screened 19 predicted PTEN-targeting microRNAs by immunoblotting in the breast cancer cell line MCF-7 and the lung cancer cell line A549. After preliminary screening, four microRNAs (miR-454, miR-498, miR-301b and miR-374b) were selected for further study. Luciferase assay results indicated that miR-498 was capable of binding to the site of 833-855bp on PTEN mRNA 3' UTR. I also confirmed the regulation of exogenous and endogenous PTEN by miR-498 in HEK293 cell, cancer cells MCF-7 and A549. Finally, the clinical impact of PTEN and miR-498 was investigated. PTEN expression in breast cancer tissues was significantly lower than that in normal tissues; high miR-498 expression was correlated with shorter overall survival time and the expression of miR-498 in TNBC tissues was significantly higher than that in normal tissues.

3.2 Regulation of PTEN by miR-498 and its oncogenic effects in TNBC cells

Triple negative breast cancer (TNBC) is a molecular subtype of breast cancer, in which PR, ER and HER2 are absent (Rakha EA, 2006; Nielsen TO, 2004). Due to this trait, the TNBC is the most lethal BCa subtype and traditional BCa therapies like the hormone replacement therapy are not effective to TNBC patients (Perou CM, Nature, 2000; Sørlie T, PNAS, 2001). Therefore, the study to investigate new therapeutic targets or methods to repress TNBC is very urgent and necessary. Recent studies reported that the PI3K-AKT pathway was over-activated in a subset of TNBC patients (Mentero, JC, 2012; Kriegsmann M, 2014); the activated AKT functioned as a survival factor and promoted cell proliferation and metabolism, enhanced cell migration and inhibited apoptosis (Manning BD, 2017). As an inhibitor of PI3K-AKT pathway, tumor suppressor PTEN was found to be down-regulated in the tumor samples of breast cancer patients and it negatively correlated with the large tumor sizes, lymph node metastasis and aggressive triple-negative phenotype (Li S, 2017); the loss of PTEN expression was associated with the triple-negative ($p = 0.0024$) breast ductal cancers and promoted cell proliferation by activating AKT and mTORC1 (Khan F, 2018); 48% of metastatic TNBC patients had a lower expression of tumor suppressor PTEN, which indicated the correlation between TNBC metastasis and PTEN (Kim SB, 2017; Craig DW, 2013). Therefore, PTEN is a crucial tumor suppressor for TNBC and negatively control cell proliferation and migration.

Based on my previous results in Chapter 3.1, I have already demonstrated that miR-498 binds to the 3' UTR of PTEN mRNA and down-regulates the protein level of PTEN. Previous

studies indicated that miR-498 was overexpressed in TNBC tissue samples (19/31) (Matamala N, 2016); and the results of Kaplan Meier analysis revealed that the expression levels of miR-498 in breast cancer patients were negatively related with their overall survival time (Lánczky A, 2016). Since tumor suppressor PTEN was a crucial player in the development of triple negative breast cancer, I hypothesized that miR-498 played an important role in triple negative breast cancer through down-regulating PTEN.

Therefore, in this section, the role of miR-498 in triple negative breast cancer cells was investigated. First, the expression levels of miR-498 in four cell lines of triple negative breast cancer were examined using miR-498-specific TaqMan probe by qRT-PCR. Simultaneously, the expression of tumor suppressor PTEN, the target of miR-498, was screened both in protein level and in mRNA level in triple negative breast cancer cell lines. Second, to investigate the regulation of PTEN by miR-498 on the protein level, miR-498 was overexpressed in the TNBC cell lines which had a lower expression of miR-498. Third, the oncogenic effects of miR-498 on cell proliferation, cell cycle progression and cell migration were examined by clonogenic assay, flow cytometry and scratch wound healing assay, respectively. Finally, to further confirm that the oncogenic effects of miR-498 were dependent on PTEN suppression, exogenous PTEN was introduced to TNBC cells in which miR-498 was overexpressed. And also siPTEN was introduced to TNBC cells, comparing with miR-498 transduced cells. The effect of siPTEN in TNBC cells was compared with the effect of miR-498. Based on the results of the experiments above, the oncogenic role of miR-498 in TNBC cells was illustrated.

3.2.1 The relation between miR-498 expression and PTEN expression in TNBC cells

Previous studies have indicated that miR-498 is overexpressed in TNBC tissue samples (19/31) (Matamala N, 2016). Moreover, Kaplan–Meier analysis has shown that miR-498 correlates negatively with the overall survival time of patients with BCa. PTEN inactivity is associated with the aggressive triple-negative phenotype (Li S, 2017). However, the relation between miR-498 and *PTEN* expression in TNBC cells has not been clearly illustrated.

To identify the relationship between miR-498 and *PTEN* expression in TNBC cells, I first examined the expression levels of miR-498 in four TNBC cell lines (MDA-MB-157, MDA-MB-231, Hs578T, BT-549) qRT-PCR using TaqMan probes. A non-tumorigenic breast epithelial cell line (MCF-10A) was used as a control for comparison with the TNBC cells. miR-498 was overexpressed in three TNBC cell lines. Specifically, compared with MCF-10A cells, Hs578T cells showed 6-fold increased miR-498 expression, MDA-MB-231 cells showed 1.64-fold increased miR-498 expression, and BT-549 cells showed 1.23-fold increased miR-498 expression (Figure 3.2.1A). This result indicates that miR-498 expression is upregulated in these TNBC cell lines compared to that in the non-tumorigenic breast epithelial cell line MCF-10A. Moreover, according to the clinical data from the previous study by Matamala (Matamala N, 2016), the miR-498 expression was significantly higher in the tissues of TNBC patients than in normal breast tissues (Figure 3.2.1B). These results indicated that miR-498 indeed was overexpressed in TNBC tissues.

Next, PTEN protein levels in the four TNBC cell lines was examined by immunoblotting with PTEN endogenous antibody. Compared to the non-tumorigenic MCF-10A cells, the four

TNBC cell lines had lower PTEN protein levels. In particular, the Hs578T and MDA-MB-231 cells had reduced PTEN expression, and only a slight decrease in PTEN was detected in the MDA-MB-157 cells. This indicates that PTEN protein is downregulated in TNBC cells (Figure 3.2.2A). Furthermore, the *PTEN* mRNA level was screened by qPCR, and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as the endogenous control gene to normalize the *PTEN* expression in each cell line. The mRNA expression of *PTEN* in each cell line was compared to that in the MCF-10A cells. Consistent with the PTEN protein levels, the *PTEN* mRNA in the Hs578T cells was the lowest of the four TNBC cell lines and was only 28% of that of the MCF-10A cells; likewise, the *PTEN* mRNA level of MDA-MB-231 was reduced to 43% of that of the MCF-10A cells (Figure 3.2.2B). Considering miR-498 was overexpressed in TNBC cell lines and tissues and the PTEN low expression in TNBC cells, it was speculated that is a possible inverse relationship between *PTEN* expression and miR-498 in TNBC, which is consistent with the hypothesis of an oncogenic role of miR-498 in TNBC. Therefore, I used TNBC cells as models to analyze the effect of miR-498 on endogenous PTEN and its biological functions.

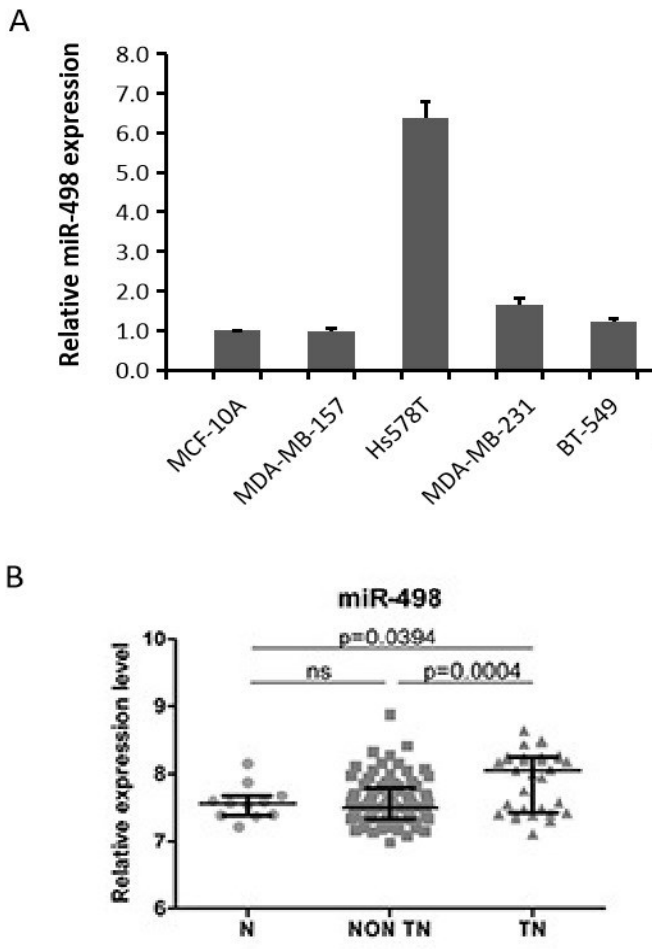


Figure 3.2.1 miR-498 over-expressed in TNBC cells and tissues.

(A) The expression levels of miR-498 in four TNBC cell lines and one non-tumorigenic breast epithelial cell line were examined by qPCR. The result was the mean of two independent experiments. Error bar indicated the SD. (B) miR-498 expression in the tissues of TNBC patients. The figure 3.2.1B is reproduced with original authors' permission and cited from Figure 3A of the published article on Oncotarget. 2016; 7:20068-20079 by Matamala N. N: normal tissue, NON TN: non-TNBC tissue, TN: TNBC tissue.

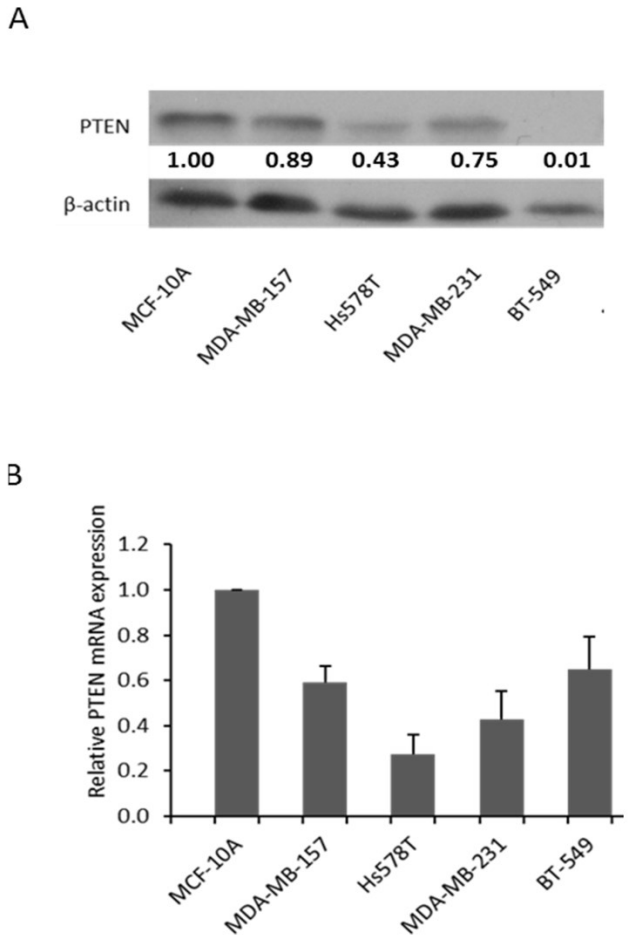


Figure 3.2.2 PTEN expression inhibited in TNBC cells.

The expression of PTEN was screened and analyzed by immunoblotting and qPCR in four TNBC cell lines (MDA-MB-157, Hs578T, MDA-MB-231 and BT-549) and one non-tumorigenic breast epithelial cell line (MCF-10A). (A) PTEN protein expression in TNBC cell lines, compared to the non-tumorigenic cell MCF-10A. (B) The mRNA levels of PTEN in four TNBC cells. The PTEN mRNA level was screened by qPCR and GAPDH was used as the endogenous control gene to normalize the PTEN expression level in each cell line. The expression of PTEN in each cell line was compared to the one in the MCF-10A.

3.2.2 miR-498 suppressed PTEN in TNBC cells

There was an inverse relation between miR-498 expression and *PTEN* expression in TNBC cell lines. In particular, Hs578T cells had high expression of miR-498 and PTEN was inhibited at both protein and mRNA level. However, in MDA-MB-157 cells, miR-498 expression was relatively low and PTEN was relatively highly expressed. MDA-MB-231 cells had moderate levels of miR-498 and are a widely used cell model of TNBC. Therefore, the MDA-MB-157 and MDA-MB-231 cells were chosen to introduce exogenous miR-498 and to observe the effect of miR-498 on PTEN protein.

miR-498 was transduced into the MDA-MB-231 or MDA-MB-157 TNBC cells by retroviral expression vector, and the transduced cells were collected and lysed for immunoblotting. In MDA-MB-231 cells, miR-498 significantly reduced the protein level of PTEN as compared with the control miR (Figure 3.2.3A). I also quantified PTEN protein in immunoblotting films, and showed the fold change of PTEN. Statistically, miR-498 decreased 50% of endogenous PTEN compared to the control miR (Figure 3.2.3B). To confirm the overexpression of miR-498, miR-498 expression levels were measured by qRT-PCR using an miR-498 TaqMan probe. The qRT-PCR results demonstrated that miR-498 levels in miR-498–transduced cells was >600-fold higher than that in the control miR–transduced cells (Figure 3.2.3C). In MDA-MB-157 cells, consistent with the results in the MDA-MB-231 cells, miR-498 reduced PTEN protein levels significantly in miR-498–transduced cells compared to cells transduced with the control miR (Figure 3.2.3D, E & F). These results suggest that miR-498 overexpression can suppress PTEN protein levels in TNBC cell lines.

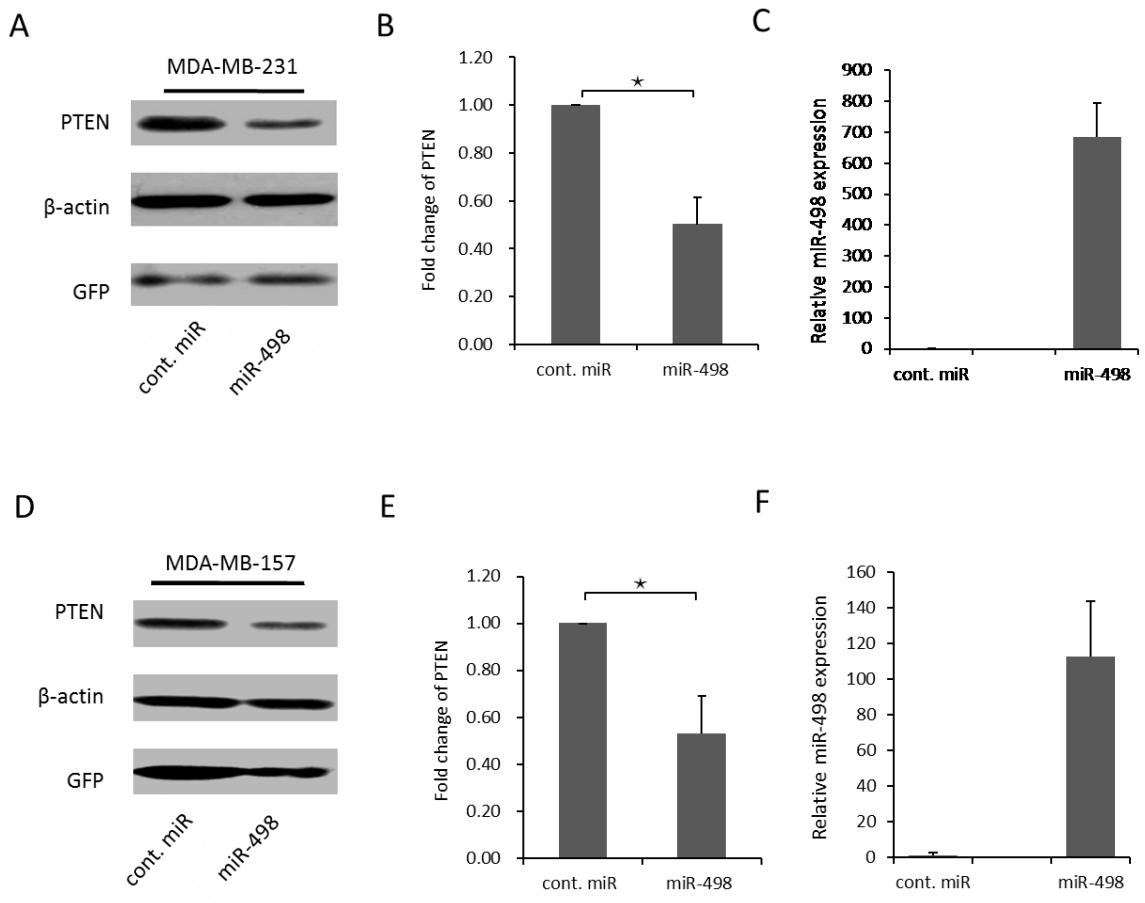


Figure 3.2.3 miR-498 suppressed PTEN in TNBC cells.

The miR-498 suppressed PTEN in TNBC cells MDA-MB-231 and MDA-MB-157. (A) PTEN protein in MDA-MB-231 cells by miR-498. The miR-498 or control miR was transduced into TNBC cells by the retroviral expression vector. Then 48 hours later the transduced cells were collected and lysed for immunoblotting. Endogenous PTEN was detected by immunoblotting. GFP was used as a marker for retroviral transduction efficiency. (B) The fold change of PTEN protein by miR-498 overexpression was shown. (C) The miR-498 expression in transduced MDA-MB-231 cells was examined by qRT-PCR. (D) PTEN protein in MDA-MB-157 cells by miR-498. Same as (A), except MDA-MB-157 cells were used. (E) The fold change of PTEN protein by miR-498 overexpression in MDA-MB-157 cells was shown. (F) The miR-498 expression in transduced MDA-MB-157 cells was examined by qRT-PCR. Error bars indicated the SD, ⋆$P<0.05$ (n=3); the results were from three independent experiments.

3.2.3 PTEN suppression enhanced phosphorylation of AKT by miR-498

Although PTEN in TNBC cells was suppressed by miR-498 overexpression, the effects of miR-498 on the downstream substrates of PTEN in the cells were still unclear. p-AKT is the most important downstream substrate of the phosphatase PTEN and PTEN inhibits the activity of p-AKT, which affects numerous substrates of the PI3K–AKT pathways (Stambolic V, 1998; Datta SR, 1999). Therefore, following PTEN suppression by miR-498, whether p-AKT was activated by the miR-498–induced PTEN suppression in TNBC cells was investigated.

The TNBC cell lines MDA-MB-231 and BT-549 were used for these experiments. MDA-MB-231 cells express wt PTEN protein while BT-549 is a PTEN-null cell line with a deletion at 822 bp of the *PTEN* mRNA ORF that leads to the termination of protein translation. BT-549 cells were used as a negative control for observing the change in p-AKT after introducing miR-498. After miR-498 was transduced into MDA-MB-231 (PTEN-Wt) and BT-549 cells (PTEN-null), I examined the protein levels of PTEN and p-AKT Ser473 in the cells by immunoblotting, and detected the expression of miR-498 by qRT-PCR to confirm the overexpression of miR-498 by retroviral transduction. It was expected that miR-498 would reduce PTEN protein but increase p-AKT in the MDA-MB-231 cells while not affecting p-AKT in the BT-549 cells. As expected, in the PTEN-Wt MDA-MB-231 cells, immunoblotting indicated that, compared with control miR, miR-498 enhanced the protein level of p-AKT due to PTEN suppression (Figure 3.2.4A). The transduction of miR-498 increased miR-498 expression in the MDA-MB-231 cells compared to the transduction of control miR, leading to PTEN suppression (Figure 3.2.4B). In contrast, in BT-549 cells (PTEN-null), miR-498 did not increase p-AKT levels, which indicated that the increase in p-AKT levels by miR-498 requires the presence of PTEN (Figure 3.2.4C). Moreover, the miR-498 expression level was examined using qRT-PCR to confirm its

overexpression by retroviral transduction (Figure 3.2.4D). Based on the results, miR-498 acts as a positive regulator of p-AKT in TNBC cells with wt PTEN, but had no effect on TNBC cells with PTEN deletion.

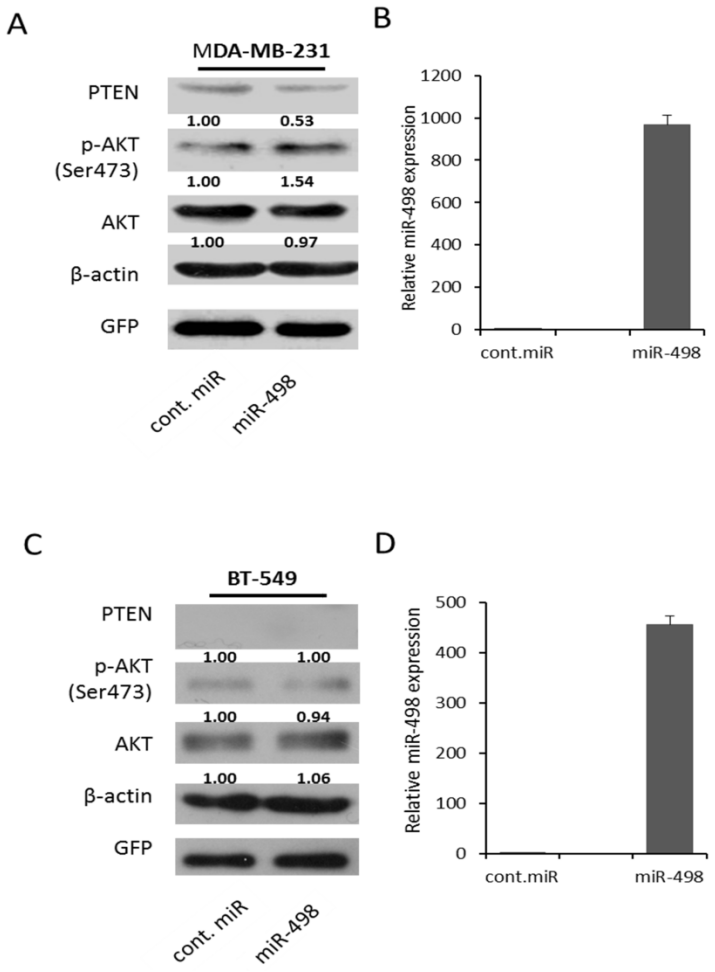


Figure 3.2.4 PTEN suppression by miR-498 enhanced the phosphorylation of AKT in PTEN-Wt TNBC cell.

PTEN suppression by miR-498 enhanced the phosphorylation of AKT in TNBC PTEN-Wt cell MDA-MB-231but not in PTEN-null cell BT-549. (A) The endogenous PTEN and p-AKT levels in MDA-MB-231 cells (PTEN-Wt). (B) The overexpression of miR-498 in MDA-MB-231, compared to the transduction of control miR. (C) The p-AKT level in BT-549 cells (PTEN-null). (D) The overexpression of miR-498 in BT-549, compared to the transduction of control miR. The endogenous PTEN and p-AKT were examined by immunoblotting. GFP was used as a marker of retroviral transduction efficiency. The expression of miR-498 was confirmed by qRT-PCR using TaqMan probe and U6 snRNA was used as the control to normalized miR-498 expression.

3.2.4 Effect of miR-498 on cell proliferation and survival

It has been reported that PTEN acts as an inhibitor of cell proliferation and cell survival by negatively regulating the PI3K–AKT signaling pathway (Stambolic V, 1998; Datta SR, 1999). Therefore, as a negative regulator of *PTEN*, miR-498 was expected to promote cell proliferation and cell survival by downregulating *PTEN* in TNBC cells. To confirm this role of miR-498, a clonogenic assay was performed to test the oncogenic effect of miR-498 on proliferation and survival in MDA-MB-231 (PTEN-Wt) and BT-549 (PTEN-null) cells.

TNBC cells were transduced with miR-498 or control miR, and the number of cell colonies was used to evaluate the cell proliferation and cell survival capability. Figure 3.2.5A shows that miR-498 significantly increased the MDA-MB-231 cell colony numbers by 63% compared to the control miR. This suggests that miR-498 enhances cell proliferation and survival in the PTEN-Wt MDA-MB-231 cells. However, miR-498 did not increase the BT-549 (PTEN-null) cell colony numbers compared to the control miR (Figure 3.2.5B). Taken together with the result of PTEN suppression by miR-498 in Figure 3.2.4, these results suggest that miR-498 promotes cell proliferation and survival by suppressing *PTEN*, and confirmed that miR-498 plays an oncogenic role in TNBC cells.

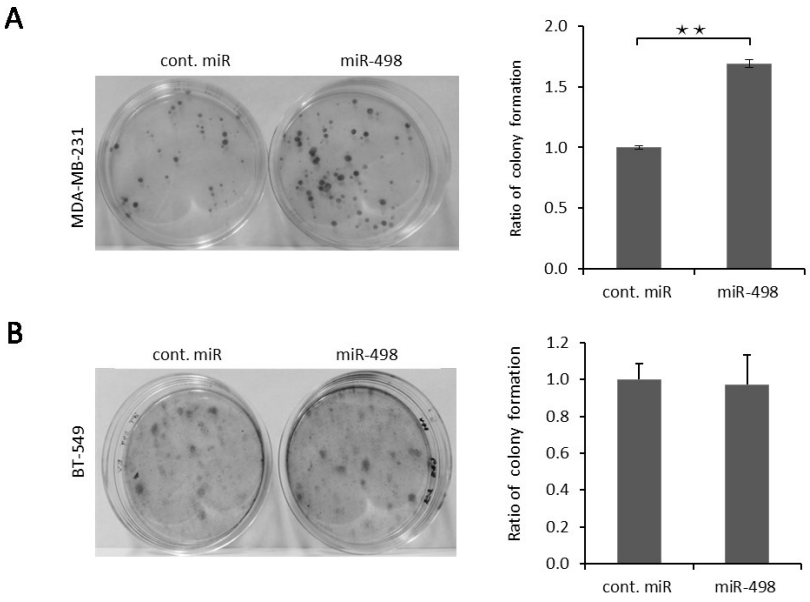


Figure 3.2.5 miR-498 enhanced cell proliferation and survival.

The miR-498 enhanced cell proliferation and survival in TNBC PTEN-Wt cell line MDA-MB-231but not in PTEN-null cell line BT-549. (A) The effect of miR-498 on cell colony numbers of MDA-MB-231. This suggested that miR-498 could enhance cell proliferation. (B) The effect of miR-498 on cell colony numbers of BT-549. The effect of miR-498 on cell proliferation was examined by clonogenic assay. MiR-498 was transduced into TNBC cells MDA-MB-231(PTEN-Wt) and BT-549 (PTEN-null). Transduced cells were seeded into the 60 mm dishes and incubated for 14 days. The ratio of colony formation (N/n) was calculated by the number of colonies (N) divided by the numbers of seeded cells (n). Colonies with over 50 cells were counted to determine the colony number ratio. Error bars indicated the SD, ⋆⋆ $P<0.01$; the results were from three independent experiments.

3.2.5 Effect of miR-498 on cell cycle progression

The tumor-suppressing role of PTEN involves not only inhibition of cell proliferation and cell survival, but also inhibition of cell cycle progression. The cell cycle checkpoints are crucial to cell cycle progression, and cells that can pass the checkpoints start the process of cell division (Malumbres M, 2009). PTEN has been reported to regulate cell cycle progression (Li DM, 1998; Sun H, 1999). Specifically, PTEN induces cell cycle arrest at the G1 phase by inhibiting the PI3K–AKT signaling pathway (Sun H, 1999). As the earlier results confirmed that miR-498 suppresses PTEN protein levels in TNBC cells, I examined the effect of miR-498 on cell cycle progression by suppressing PTEN.

The effect of miR-498 on cell cycle progression was examined by flow cytometry. In MDA-MB-231 cells (PTEN-Wt), miR-498 significantly increased the proportion of cells in the S phase and simultaneously reduced the proportion of cells in the G1 phase (Figure 3.2.6A). Compared to the control miR, miR-498 decreased the G1/S ratio of MDA-MB-231 cells from 2.06 to 1.18, which suggests that miR-498 promotes cell division by overpassing the G1/S checkpoint. However, miR-498 did not change the proportion of cells in G2 or M phase, which means that miR-498 only contributes to helping MDA-MB-231 cells pass the G1/S checkpoint instead of the G2/M checkpoint. This result indicates that miR-498 can promote cell cycle progression. Furthermore, miR-498 did not significantly increase the proportion of BT-549 (PTEN-null) cells in the S phase or did not decrease the G1/S ratio (Figure 3.2.6B). Interestingly, miR-498 reduced the proportion of cells in the G2 or M phase, which suggests that miR-498 has an effect on the cell cycle through other potential targets other than *PTEN*. Taken together with the results of *PTEN* suppression by miR-498 in Figure 3.2.4, these results indicate that miR-498 promotes G1–S transition and advances cell cycle progression by suppressing *PTEN*.

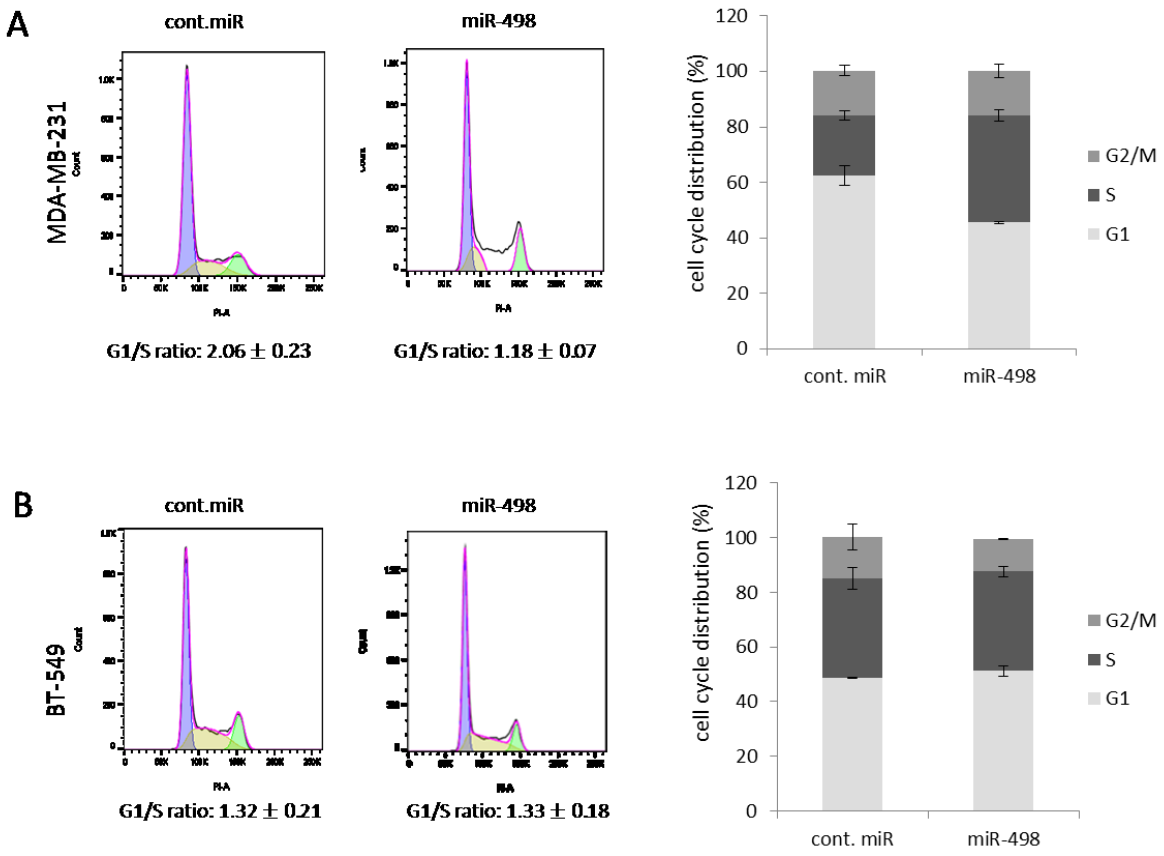


Figure 3.2.6 miR-498 promoted cell cycle progression.

The miR-498 promoted cell cycle progression in TNBCPTEN-Wt cell MDA-MB-231but not in PTEN-null cell BT-549. (A) The effect of miR-498 on the proportions of cells in MDA-MB-231 cells. (B) The effect of miR-498 on the proportions of cells in BT-549 cells. The cells were analyzed by flow cytometry and the data was analyzed using FlowJo software. Error bars indicated the SD; the results were the mean value of three independent experiments.

3.2.6 Effect of miR-498 on cell migration

Cell migration is crucial for tumor metastasis, leading to the translocation of tumor cells from the primary site or organ to other organs and to the formation of secondary tumor colonies. Thus, tumor metastasis is one of the most urgent issues for controlling tumor development. As it has been reported that PTEN inhibits tumor cell migration, I investigated whether miR-498 would enhance the migration of TNBC cells by suppressing PTEN (Raftopoulou M, 2004; Marino S, 2002).

To determine the effect of miR-498 on cell migration, the scratch healing assay was performed. MDA-MB-231 cells were transduced with miR-498 or control miR, and the migration of transduced cells was observed under phase contrast microscopy. The cell-free wound area was defined as the blank area and was used to evaluate the speed of cell migration; the relative blank area was obtained by quantifying the cell-free area at each timepoint and then normalizing it to the quantified blank area of 0 hours. At 24 hours after scratching, the blank wound area of miR-498–transduced cells was obviously less than that of control miR–transduced cells (Figure 3.2.7A). Specifically, >70% of the blank wound area was covered by miR-498–transduced cells, whereas only 30% of the blank wound area was covered by control miR–transduced cells (Figure 3.2.7B). In addition, I used itrack4U software to track the movement of the cells by analyzing time-lapse images. I found that there were significantly more migrated miR-498–transduced cells than migrated control miR–transduced cells, which suggested that miR-498 increases the number of migrated cells (Figure 3.2.7C). The distance covered by the migrated cells was also examined using itrack4U. The results showed that the miR-498–transduced cells covered a significantly greater average distance than the control miR–transduced cells (Figure 3.2.7D). Taken together with the results of *PTEN* suppression by miR-

498 in Figure 3.2.4, these results suggest that miR-498 enhances the migration of TNBC cells by suppressing *PTEN*.

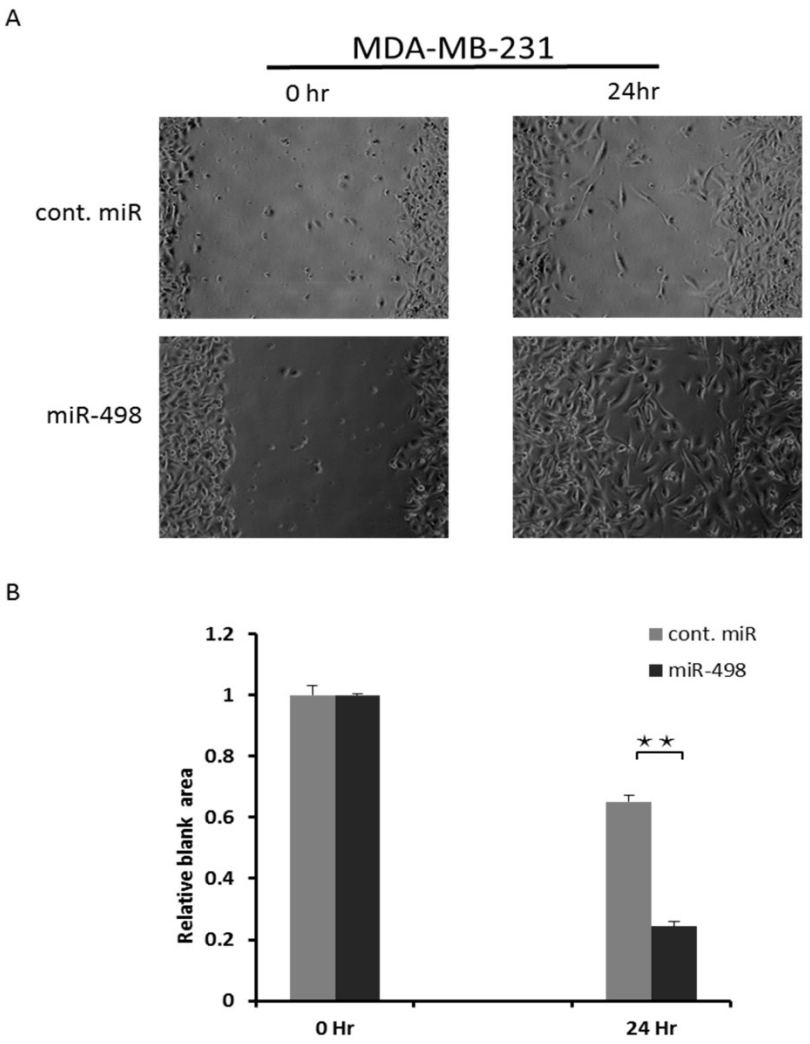


Figure 3.2.7 A & B miR-498 enhanced cell migration.

The miR-498 enhanced cell migration in MDA-MB-231cell. Scratch healing assay was performed to determine the effect of miR-498 on cell migration. (A) The effect of miR-498 on MDA-MB-231 migration in scratch healing assay. (B) The blank wound area of miR-498-transduced cells and of control miR-transduced cells after 24 hours of scratching. The wound images were acquired by phase-contrast microscopy. The wound area was calculated by ImageJ with MRI wound healing tool and statistical analysis of the relative wound area was showed. ⋆⋆ $P<0.01$ (n=3), error bar indicated the SD and the result was the mean value of three independent experiments.

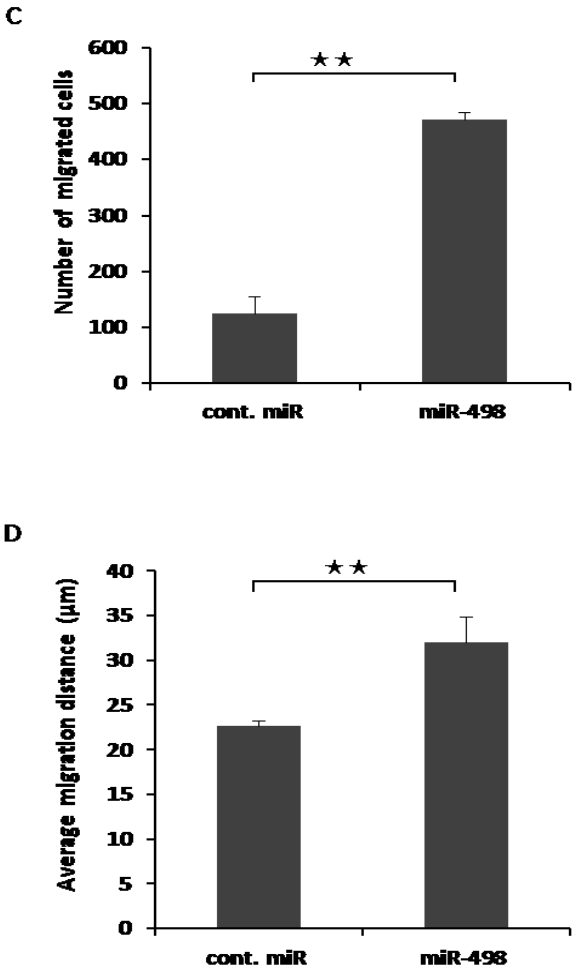


Figure 3.2.7 C & D miR-498 enhanced cell migration.

(C) The effect of miR-498 on amount of migrated MDA-MB-231 cells compared to control miR. (D) The effect of miR-498 on moving distance of MDA-MB-231 cells significantly. ⋆⋆ $P<0.01$ (n=3), error bar indicated the SD and the result was the mean value of three independent experiments.

3.2.7 Oncogenic effects of miR-498 dependent on PTEN suppression and p-AKT activation

As miR-498 promoted the proliferation, cell cycle progression, and migration of TNBC cells, the oncogenic effects of miR-498 were examined. To confirm that the oncogenic effects of miR-498 are dependent on *PTEN* suppression, the *PTEN* ORF was used to restore the PTEN protein levels inhibited by miR-498 and to abrogate the oncogenic phenotypes of miR-498. If the oncogenic phenotypes resulted from miR-498 were abrogated or alleviated by restored PTEN protein, the relation between the phenotypes of miR-498 and PTEN suppression would be confirmed. In the following experiments, the effect of miR-498 on cell viability and proliferation was examined by MTT assay, and the effect of miR-498 on cell migration was examined by scratch healing assay.

To determine the effect of the *PTEN* ORF on PTEN suppression and p-AKT activation, the PTEN wt TNBC cell line MDA-MB-231 and the non-tumorigenic breast epithelial cell line MCF-10A were transduced with miR-498 or the *PTEN* ORF, and then the transduced cells were lysed for immunoblotting. In MDA-MB-231 cells, PTEN protein levels were was decreased by the overexpression of miR-498 but were restored by the introduction of the *PTEN* ORF (Figure 3.2.8A). miR-498 overexpression increased the levels of p-AKT (Ser473) but p-AKT levels recovered to the original levels when PTEN protein levels were restored by introducing the *PTEN* ORF (Figure 3.2.8A). A similar result was found in the MCF-10A cells containing wt PTEN after the *PTEN* ORF had been introduced (Figure 3.2.8B). These data indicate that at protein level, PTEN suppression and p-AKT activation are both abrogated by the *PTEN* ORF.

Simultaneously, to determine the effect of the *PTEN* ORF on the enhanced cell proliferation and viability by miR-498, the cell viability of MDA-MB-231 and MCF-10A cells was examined by MTT assay. The MTT assay was used to directly reflect the cell viability and to indirectly reflect the cell proliferation. The MTT assay showed that the miR-498–transduced MDA-MB-231 cells had significantly enhanced cell viability (75% more) compared to the control miR–transduced MDA-MB-231 cells, which confirmed again that miR-498 has an oncogenic effect on cell viability and cell proliferation. However, the cell viability enhanced by miR-498 was significantly reduced by the *PTEN* ORF, which indicates that the restored PTEN abrogated the enhanced cell viability induced by miR-498 (Figure 3.2.9A). Similar results were observed in the MCF-10A cells, where miR-498 enhanced cell viability but the *PTEN* ORF abrogated this oncogenic phenotype (Figure 3.2.9B). Taken together with the immunoblotting results (Figure 3.2.8) and the MTT assay results (Figure 3.2.9), miR-498 induces the oncogenic phenotype of cell viability and proliferation but introduction of the *PTEN* ORF abrogates it, which suggests that the oncogenic effect of miR-498 on cell viability and proliferation is dependent on *PTEN* suppression and p-AKT activation.

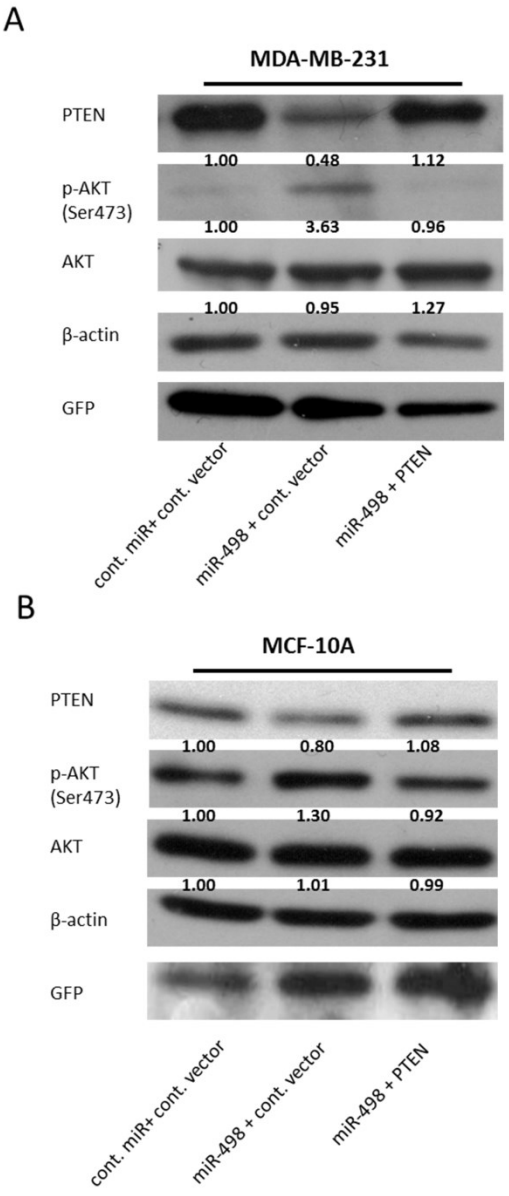

A
MDA-MB-231
PTEN
1.00
0.48
1.12
p-AKT
(Ser473)
1.00
3.63
0.96
AKT
1.00
0.95
1.27
β-actin
GFP
cont. miR+ cont. vector
miR-498 + cont. vector
miR-498 + PTEN
B
MCF-10A
PTEN
1.00
0.80
1.08
p-AKT
(Ser473)
1.00
1.30
0.92
AKT
1.00
1.01
0.99
β-actin
GFP
cont. miR+ cont. vector
miR-498 + cont. vector
miR-498 + PTEN

Figure 3.2.8 Suppression of PTEN by miR-498 was restored by PTEN ORF.

Suppression of PTEN by miR-498 was abrogated by introducing PTEN ORF in MDA-MB-231 and MCF-10A. (A) The protein level of PTEN by miR-498 and by PTEN ORF in MDA-MB-231 cells. (B) The protein level of PTEN by miR-498 and by PTEN ORF in MCF-10A cells. MDA-MB-231 and MCF-10A were transduced with miR-498 or PTEN ORF and transduced cells were lysed for immunoblotting. The protein level of PTEN, p-AKT (Ser473) and AKT were detected by endogenous antibodies and GFP was used as a marker for retroviral transduction efficiency.

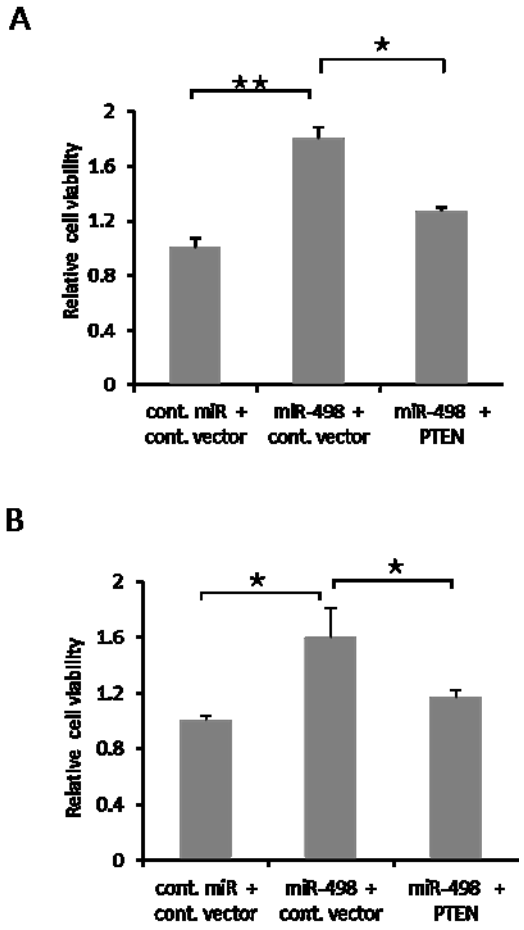


Figure 3.2.9 Restored PTEN inhibited the oncogenic effect of miR-498 on cell viability and proliferation.

Restored PTEN inhibited the oncogenic effect of miR-498 on cell viability and proliferation in MDA-MB-231 and MCF-10A. (A) The cell viability of MDA-MB-231 by miR-498 and by PTEN ORF. Cell viability was examined by MTT assay. (B) The cell viability of MCF-10A by miR-498 and by PTEN ORF. Error bars indicated the SD, ⋆ $P<0.05$, ⋆⋆ $P<0.01$ (n=3) and the result was the mean value of three independent experiments.

In addition, the effect of the *PTEN* ORF on the miR-498–promoted cell migration was investigated via scratch healing assay in both the MDA-MB-231 and MCF-10A cell lines. Considering the prior results in Figure 3.2.8 and Figure 3.2.9, it was expected that the miR-498–promoted cell migration would be abrogated by the *PTEN* ORF. Generally, the results of the scratch healing assay followed this expectation. At 24 hours and 48 hours, the blank wound area of miR-498–transduced MDA-MB-231 cells was significantly less than that of control miR–transduced MDA-MB-231 cells (figure 3.2.10A), which indicates that miR-498 promoted the migration of MDA-MB-231 cells. However, once the *PTEN* ORF was introduced into the miR-498–transduced cells, the blank wound area of the PTEN-introduced cells increased significantly compared with that of the miR-498–transduced cells and recovered to similar levels to the control cells, indicating that the cell migration promoted by miR-498 was inhibited by PTEN ORF in MDA-MB-231 cells (Figure 3.2.10A). Moreover, compared with MDA-MB-231 cells, the MCF-10A cells showed similar results, in that miR-498 promoted cell migration but the *PTEN* ORF abrogated this oncogenic phenotype (Figure 3.2.10B). Generally, miR-498 increased the speed of cell migration in the TNBC cells and normal epithelial breast cells, but the introduction of the *PTEN* ORF decreased it. Taken together with the immunoblotting results (Figure 3.2.8) and scratch healing assay results (Figure 3.2.10), miR-498 induced the oncogenic phenotype in cell migration, but it was abrogated when the *PTEN* ORF was introduced, which suggests that the oncogenic effect of miR-498 on cell migration is dependent on *PTEN* suppression and p-AKT activation.

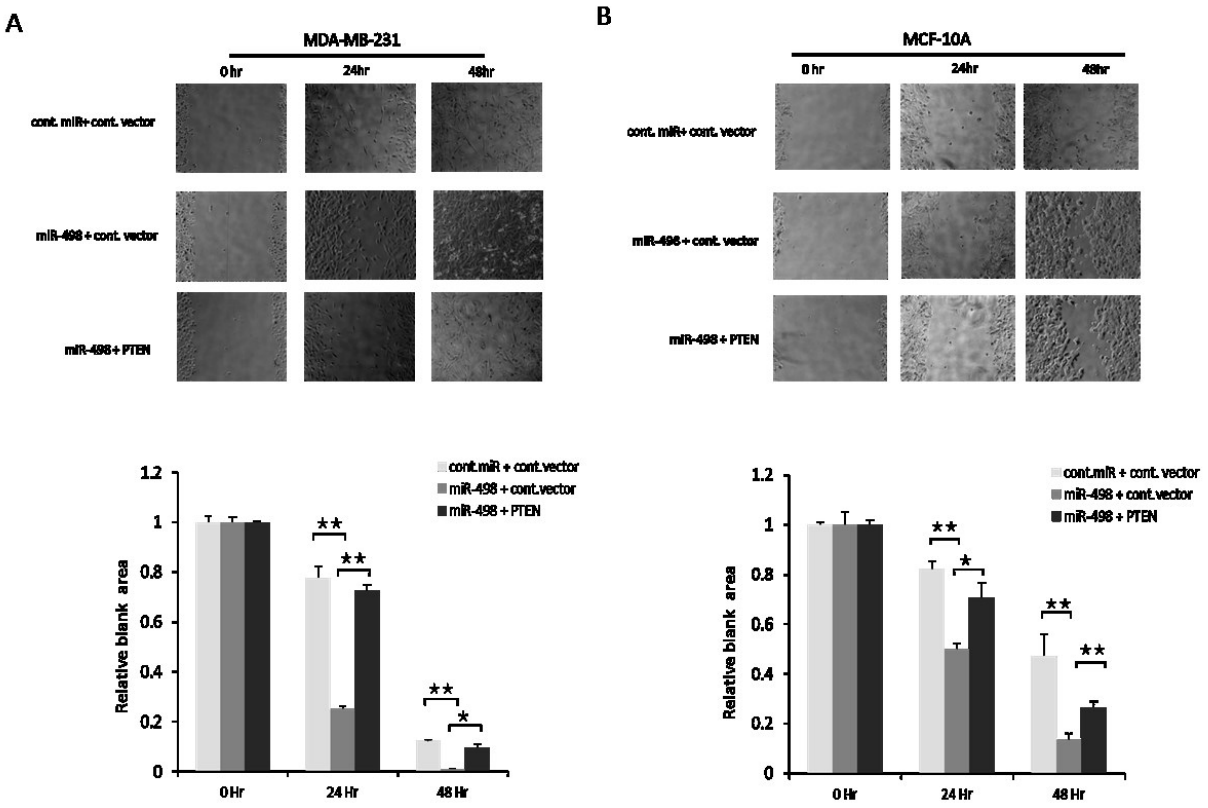


Figure 3.2.10 Restored PTEN inhibited the oncogenic effect of miR-498 on collective cell migration.

Restored PTEN inhibited the oncogenic effect of miR-498 on collective cell migration in MDA-MB-231 and MCF-10A. (A) The cell migration by miR-498 and by PTEN ORF in MDA-MB-231. The speed of wound healing was significantly accelerated by miR-498 but was decreased by introducing PTEN ORF in MDA-MB-231 cells. (B) The cell migration by miR-498 and by PTEN ORF in MCF-10A. Error bars indicated the SD, ⋆ $P<0.05$, ⋆⋆ $P<0.01$ (n=3) and the result was the mean value of three independent experiments.

3.2.8 Oncogenic effect of miR-498 comparable with the effect of PTEN siRNA

To evaluate the oncogenic effect of miR-498, PTEN siRNA was used for comparison with miR-498; the oncogenic effect of miR-498 or PTEN siRNA on cell proliferation and cell migration were screened by the MTT and scratch healing assays.

First, to compare the effect of miR-498 with that of PTEN siRNA on PTEN protein levels and p-AKT activation, the PTEN wt TNBC cell line MDA-MB-231 and the non-tumorigenic breast epithelial cell line MCF-10A were transduced with miR-498 or PTEN siRNA, and the transduced cells were lysed for immunoblotting. In the MDA-MB-231 cells, the protein level of PTEN was decreased by miR-498 and it was comparable with the PTEN levels in PTEN siRNA–transduced cells (Figure 3.2.11A). The p-AKT levels were increased by miR-498 or PTEN siRNA as compared with that in the control miR–transduced cells. Similar results were observed in the MCF-10A cells; either miR-498 or PTEN siRNA led to PTEN suppression and p-AKT activation (Figure 3.2.11B). These results indicate that miR-498 has a comparable effect with PTEN siRNA in suppressing PTEN protein.

Simultaneously, to compare the effect of miR-498 with that of PTEN siRNA on cell proliferation and viability, MDA-MB-231 and MCF-10A cell proliferation and viability were examined by the MTT assay. As miR-498 had a similar effect to PTEN siRNA on PTEN suppression and p-AKT activation, the effect of miR-498 on cell proliferation and viability was expected to rival the effect of PTEN siRNA. The MTT assay showed that the cell viability of miR-498–transduced MDA-MB-231 cells was significantly enhanced by 80% due to miR-498 overexpression as compared to the cell viability of the control miR–transduced cells (Figure 3.2.12A). As expected, similar to the miR-498–transduced cells, the cell viability of PTEN

siRNA–transduced cells was also enhanced by about 100% (Figure 3.2.12A). Similar results were observed in the MCF-10A cells; either miR-498 or PTEN siRNA led to enhanced cell viability (Figure 3.2.12B). Taken together with the results in Figure 3.2.11 and Figure 3.2.12, these data suggest that the oncogenic effect of miR-498 on cell proliferation and viability is comparable with that of PTEN siRNA, both of which result in PTEN suppression.

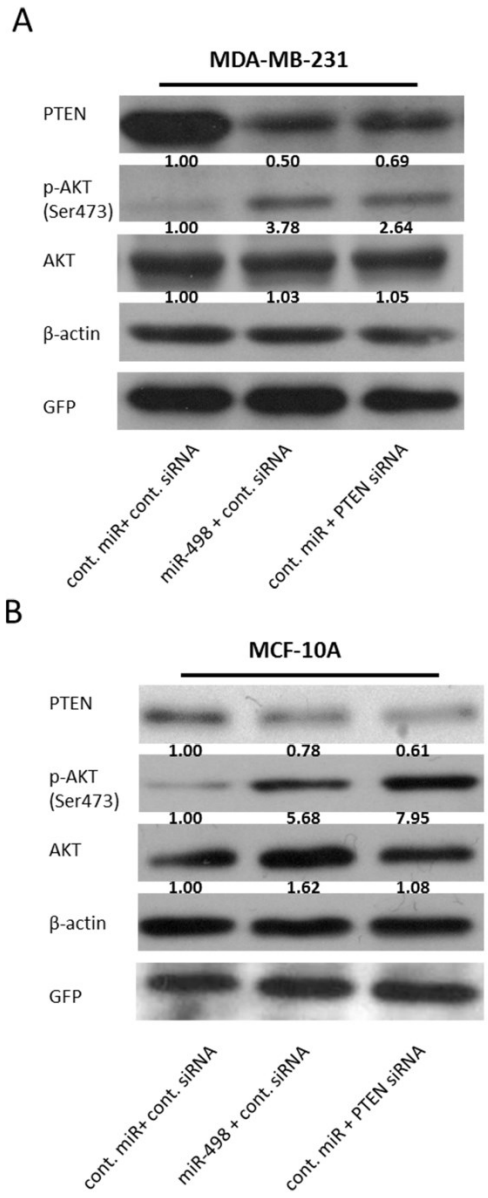


Figure 3.2.11 Suppression of PTEN protein by miR-498 compared with by PTEN knockdown by siRNA.

Suppression of PTEN protein by miR-498 was compared with by PTEN siRNA in MDA-MB-231 and MCF-10A. (A) The protein level of PTEN by miR-498 and by PTEN siRNA in MDA-MB-231 cells. (B) The protein level of PTEN by miR-498 and by PTEN siRNA in MCF-10A cells. The protein level of PTEN, p-AKT (Ser473) and AKT were detected by endogenous antibodies and normalized to the β-actin. GFP was used as a marker for retroviral transduction efficiency.

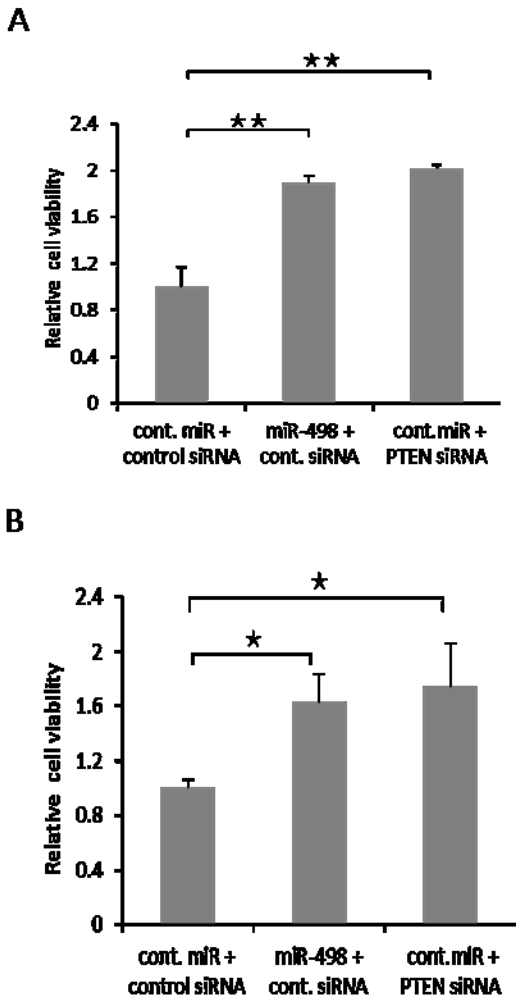


Figure 3.2.12 Oncogenic effect of miR-498 on cell proliferation comparable with PTEN siRNA.

The oncogenic effect of miR-498 on cell proliferation was comparable with PTEN siRNA in MDA-MB-231 and MCF-10A. (A) The cell viability of MDA-MB-231by miR-498 and by PTEN siRNA. Cell viability was examined by MTT assay. (B) The cell viability of MCF-10A by miR-498 and by PTEN siRNA. Error bars indicated the SD, ⋆ $P<0.05$, ⋆⋆ $P<0.01$ (n=3) and the result was the mean value of three independent experiments.

Furthermore, to compare the effect of miR-498 with that of PTEN siRNA on cell migration, the scratch healing assay was performed in both MDA-MB-231 and MCF-10A cells. As miR-498 had a similar effect to PTEN siRNA on PTEN suppression and p-AKT activation, the effect of miR-498 on cell migration was expected to rival the effect of PTEN siRNA. At 24 hours, the blank wound area of miR-498–transduced cells was significantly less than the blank wound area of the control miR–transduced cells (Figure 3.2.13A), which indicated that miR-498 promoted cell migration. As expected, the blank wound area of PTEN siRNA–transduced cells was significantly decreased and was similar with that of the miR-498–transduced cells, which indicated that miR-498 is comparable to PTEN siRNA in promoting MDA-MB-231 cell migration (Figure 3.2.13A). Moreover, compared with MDA-MB-231 cells, the MCF-10A cells showed similar results; either miR-498 or PTEN siRNA led to enhanced cell migration (Figure 3.2.13B). Generally, miR-498 accelerated cell migration, which was comparable with PTEN siRNA in the TNBC cell line MDA-MB-231 and the normal epithelial breast cell line MCF-10A. These results further prove that miR-498 has similar functions to PTEN siRNA in suppressing PTEN and promoting cell migration.

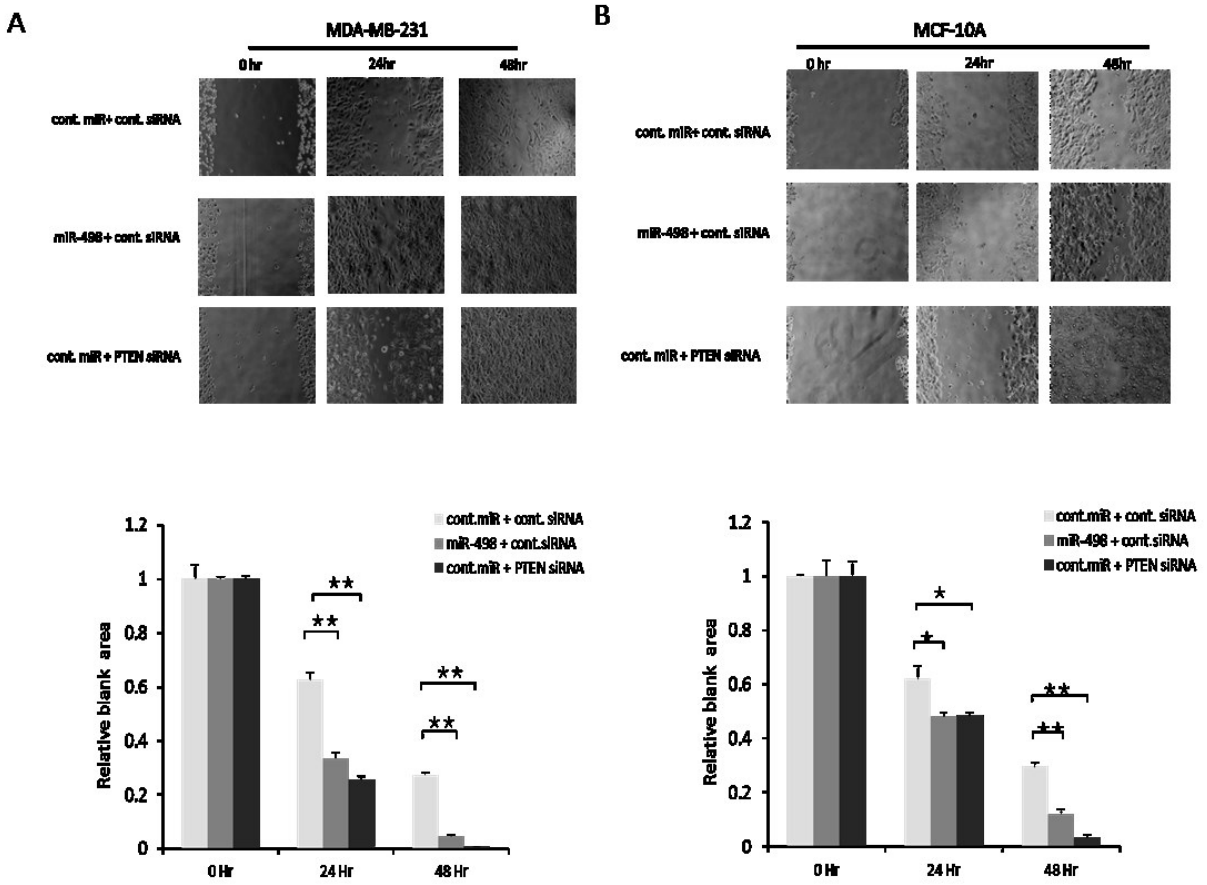


Figure 3.2.13 Oncogenic effect of miR-498 on cell migration comparable with PTEN siRNA.

The oncogenic effect of miR-498 on cell migration was comparable with that of PTEN siRNA in MDA-MB-231 and MCF-10A. (A) The cell migration by miR-498 and by PTEN siRNA in MDA-MB-231 cells. The images of wound scratches at 0 hr, 24 hr and 48 hr were showed in the upper panel as indicated. The wound area was calculated by ImageJ with MRI wound healing tool and statistical analysis of the relative wound area was showed in the lower panel. (B) The cell migration by miR-498 and by PTEN siRNA in MCF-10A cells. Error bars indicated the SD, ⋆ $P<0.05$, ⋆⋆ $P<0.01$ (n=3) and the result was the mean value of three independent experiments.

In summary, in this section, I investigated the role of miR-498 in TNBC cells. First, the expression levels of PTEN and miR-498 in TNBC cell lines were examined, and there was an inverse relationship between PTEN expression and miR-498 in the cells. Second, miR-498 led to *PTEN* suppression in TNBC cells that had lower expression of miR-498. Third, the oncogenic effects of miR-498 on cell viability and proliferation, cell cycle progression, and cell migration were demonstrated by clonogenic assay, MTT assay, flow cytometry, and scratch wound healing assay, respectively. Furthermore, the *PTEN* ORF was introduced to restore PTEN in TNBC cells and non-tumorigenic breast cells. The restored PTEN abrogated the oncogenic phenotypes induced by miR-498, which confirmed that the oncogenic effects of miR-498 are dependent on *PTEN* suppression. Finally, PTEN siRNA was introduced to TNBC cells and non-tumorigenic breast cells for comparison with miR-498. The oncogenic effects of miR-498 on cell migration, viability, and proliferation were comparable with that of PTEN siRNA. Based on the above results, the regulation of PTEN by miR-498 was illustrated in TNBC cells, and the oncogenic role of miR-498 is dependent on PTEN suppression.

3.3 miR-498 decoy acts as a negative regulator of miR-498

The miRNAs are potential targets to be directly used in cancer therapy. The anti-miRNA oligonucleotides or miRNA mimics can be delivered to tumor cells directly or indirectly via the circulation system, in which aberrant miRNA expression will be rescued and its oncogenic effect will be diminished. Prior studies have demonstrated the plausibility of anti-miRNA oligonucleotides or miRNA mimics in cancer cells or animal models. For instance, the oncogenic miR-21was overexpressed in breast cancer cells and tissues, anti-miR-21oligonucleotides was used to reduce miR-21 expression and inhibit tumor growth in xenograft mice (Yan LX, 2011); due to its downregulated expression in multiple cancers, miR-145 mimic was introduced to suppress its oncogenic targets in breast cancer cells or xenograft mice (Tang L, 2016; Inamoto T, 2015). Acting as a negative regulator of microRNA, microRNA decoy is an oligonucleotide that contained the complementary sequence of specific mature microRNA and can attract its complementary microRNA to bind on it (Wang B, 2017). Previously, I have demonstrated that miR-498 can suppress tumor suppressor PTEN through binding to PTEN mRNA and show the oncogenic effects of miR-498 on cell proliferation, cell cycle progression and cell migration in TNBC cells. Therefore, to develop a miR-498 decoy for inhibiting miR-498 and protecting PTEN from miR-498 binding is possible to abrogate the oncogenic effects of miR-498.

In this section, I designed a specific miR-498 decoy that contained a luciferase gene and six tandem repeats of a complementary sequence of mature miR-498. I examined the capability of miR-498 decoy to attract the binding of miR-498 by luciferase assay. The reduction of miR-498 and the restoration of PTEN by miR-498 decoy were determined in the endogenous miR-498-overexpressing cells. Furthermore, the function of a miR-498 decoy on inhibiting the

oncogenic effects of miR-498 was investigated by clonogenic assay and wound healing assay in TNBC cells. Based on this work, the miR-498 decoy was determined to be a negative regulator of miR-498 to inhibit the miR-498 expression and oncogenic effects.

3.3.1 Construction of miR-498 decoy

Since miR-498 was overexpressed in TNBC cells and played an oncogenic role in cell proliferation, cell cycle progression and cell migration through suppressing PTEN, the effective way of reducing its expression was necessary to be investigated. The specific microRNA decoy was an efficient tool to inhibit its microRNA by reducing the microRNA expression level in vitro (Ebert MS, 2007); the specific microRNA decoy was used as a negative regulator to suppress its target microRNA and as a positive regulator to rescue microRNA-target genes in cancer studies (Wang B, 2017; Nishi H, 2011). The structure of the miR-498 decoy that I constructed in this project was seen in figure 3.3.1A; six repeats of complementary sequence of miR-498 were inserted into a luciferase reporter following the luciferase gene. The transcription product of miR-498 decoy contained a luciferase ORF sequence and six repeats of complementary sequence of miR-498. The free miR-498 in the cytoplasm bound to the complementary sequence of the miR-498 decoy and the concentration of free miR-498 in the cytoplasm was reduced.

To determine that miR-498 exclusively bound to miR-498 decoy and suppressed the translation of miR-498 decoy, the luciferase assay was performed. The miR-498 decoy with miR-498 or control miR or negative control miR-1-1 was co-transfected into HEK293 cells and after 48 hours the luciferase activity of each transfection was measured. It was expected that the miR-498 exclusively bound to the six repeats of miR-498 complementary sequence on miR-498

decoy but neither control miR nor the negative control miR-1-1. Luciferase assay results showed that only miR-498 reduced the luciferase activity significantly, which suggested that miR-498 successfully bound to the transcript product of miR-498 decoy (Figure 3.3.1 B). This indicated that miR-498 decoy was specific to miR-498 and was capable of attracting miR-498 binding. Therefore, this miR-498 specific decoy acted as an inhibitory tool for negative regulating miR-498 expression.

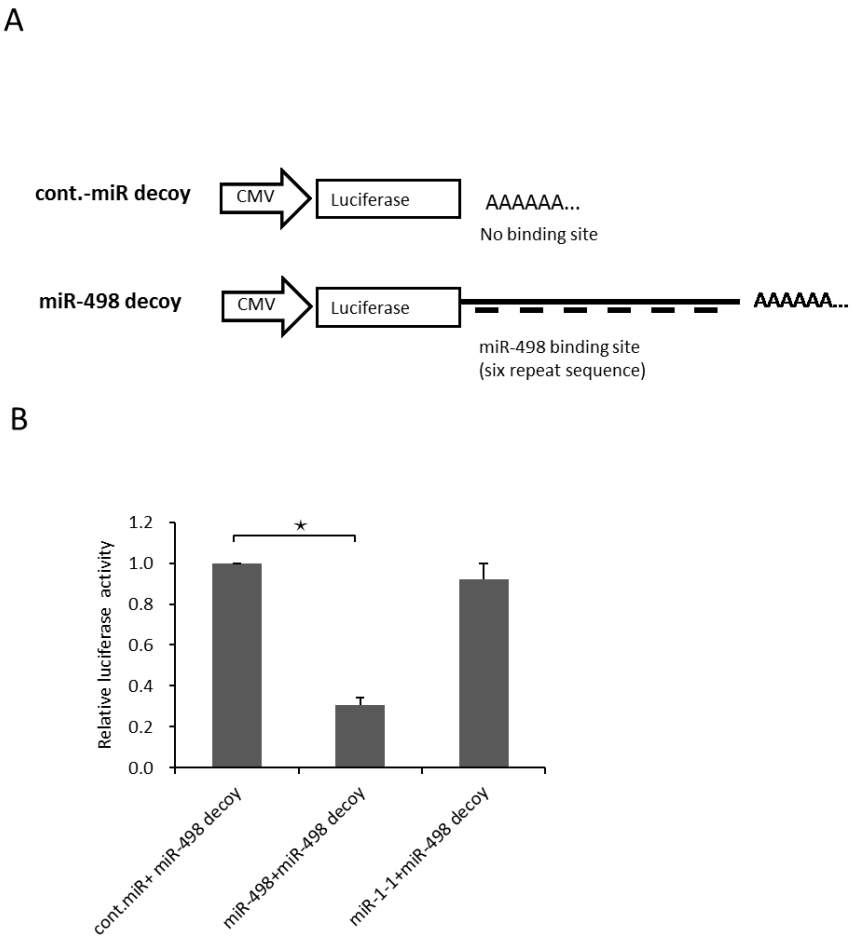


Figure 3.3.1 Construction of miR-498 decoy that was specific to miR-498.

The miR-498 decoy was constructed and its specificity to miR-498 was confirmed. (A) The structure of the miR-498 decoy. (B) The binding of miR-498 on its decoy. The miR-498 or control miR or negative control miR-1-1 was co-transfected with miR-498 decoy into HEK293 cells and after 48 hours the luciferase activity of each transfection was measured. Error bars indicated the SD, ⋆$p<0.05$ (n=3) and the result was the mean value of three independent experiments.

3.3.2 miR-498 decoy suppressed miR-498 overexpression and restored PTEN

Though the miR-498 decoy was constructed and its specificity to miR-498 was tested by luciferase assay, the effects of the miR-498 decoy on miR-498 overexpression and the protein level of PTEN were still needed to be examined. Theoretically, miR-498 decoy resulted in the reduction of free miR-498 in the cytoplasm and the restoration of PTEN protein. Thus, once miR-498 decoy was introduced to miR-498-overexpressing TNBC cells, the high level of miR-498 was suppressed and the protein level of PTEN was restored.

To observe an obvious effect of the miR-498 decoy on inhibiting miR-498 overexpression and restoring PTEN, the stable miR-498-overexpressing Hs578T was generated. TNBC Hs578T cells were transfected with miR-498 and the stable miR-498-overexpressing Hs578T cells were prepared by Hygromycin-resistant selection. And the stable Hs578T cells were examined by immunoblotting for PTEN suppression and by qRT-PCR for miR-498 overexpression. In figure 3.3.2A, compared with stable control miR-expressing cells, PTEN protein level was significantly decreased by miR-498 in the stable miR-498-overexpressing Hs578T cells. Specifically, PTEN protein in miR-498-stable cells was reduced by 67% compared with the control miR-overexpressing cells (Figure 3.3.2B). Moreover, to confirm the overexpression of miR-498, the miR-498 expression in the stable miR-498-overexpressing cells was detected by qRT-PCR using TaqMan probe. Compared to the stable control miR-expressing cells, the miR-498 expression level in stable miR-498-overexpressing cells was increased by 120-fold (Figure 3.3.2C). Based on these results, the stable miR-498-overexpressing Hs578T cells were established.

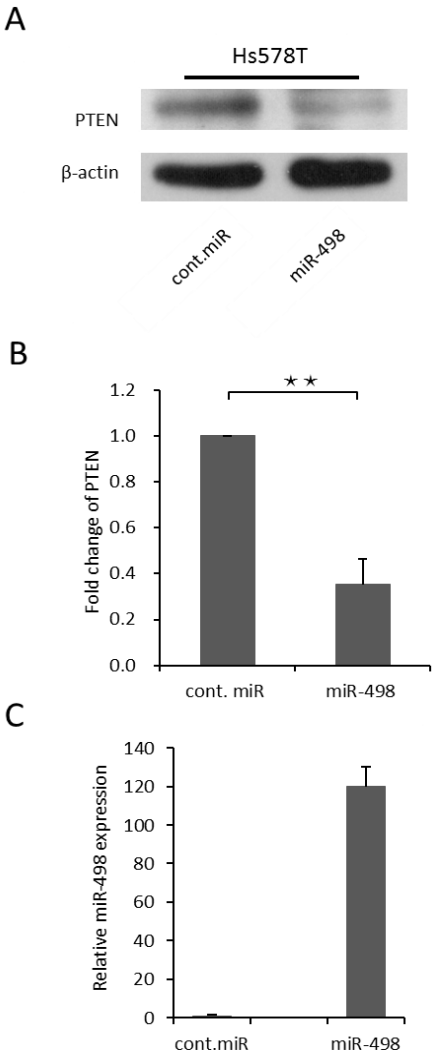


Figure 3.3.2 MiR-498-overexpressing Hs578T cells established.

MiR-498-overexpressing Hs578T cells were established and the endogenous PTEN was suppressed by miR-498. (A) The PTEN protein level of miR-498-overexpressing cells. (B) Fold change of PTEN protein in miR-498-overexpressing Hs578T cells was analyzed comparing with control miR-overexpressing cells. PTEN protein in miR-498-stable cells was reduced by 67% compared with cont. miR-stable cells. (C) The overexpression of miR-498 in miR-498-overexpressing cells. Compared to the stable control miR-expressing cells, the level of miR-498 in stable miR-498-expressing cells was increased by 120-fold. Error bars indicated the SD, ⋆⋆ $p<0.01$ (n=3) and the result was the mean value of three independent experiments.

Next, I examined the effect of the miR-498 decoy on inhibiting miR-498 overexpression and restoring PTEN protein. The miR-498 decoy was transduced to the stable miR-498-overexpressing Hs578T cells and the levels of PTEN protein and miR-498 were examined by immunoblotting and qRT-PCR. As I expected, miR-498 decoy restored the protein level of PTEN in miR-498-overexpressing Hs578T cells, compared to the control decoy (Figure 3.3.3A). Specifically, PTEN protein in miR-498 decoy transduced cells was increased over 4-fold compared with PTEN protein in control miR decoy transduced cells (Figure 3.3.3B). Moreover, to determine that the cellular concentration of miR-498 was reduced by the miR-498 decoy, the expression level of miR-498 was examined by qRT-PCR using TaqMan probe. Results showed that compared to control miR decoy, miR-498 decoy obviously reduced the miR-498 expression in stable miR-498-expressing cells and the remaining miR-498 was only 7% of its original expression (Figure 3.3.3C). Taken together these data, it indicated that miR-498 decoy was specific to miR-498 binding and was capable of reducing miR-498 expression and restoring PTEN.

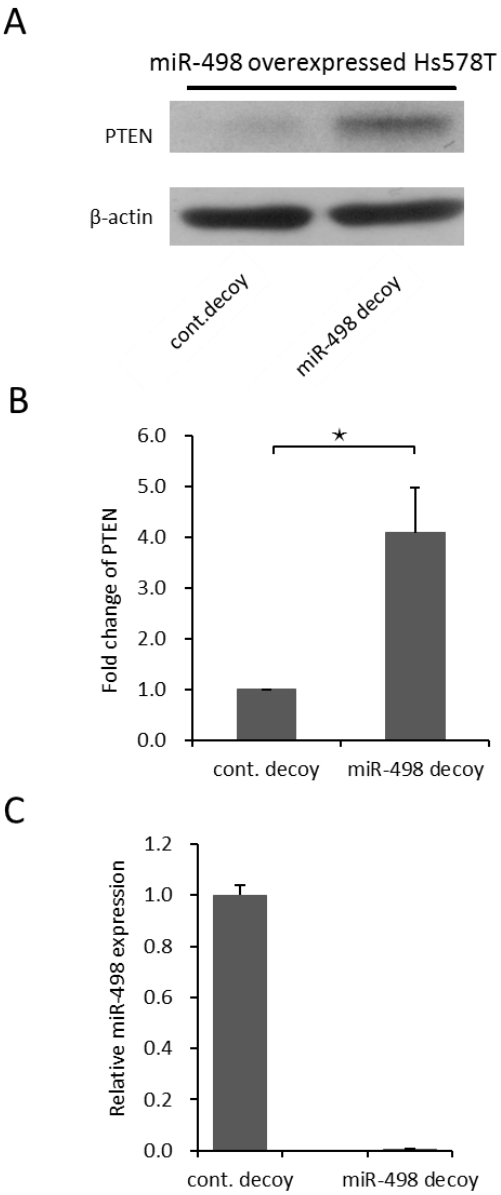


Figure 3.3.3 MiR-498 decoy reduced miR-498 expression and restored PTEN protein level.

MiR-498 decoy reduced the endogenous miR-498 expression level and restored PTEN protein level. (A & B) The effect of miR-498 decoy on PTEN protein in miR-498-overexpressing Hs578T cells. (C) The effect of miR-498 decoy on the endogenous miR-498 expression in miR-498-overexpressing Hs578T cells. Error bars indicated the SD, ⋆$p<0.05$, (n=3) and the result was the mean value of three independent experiments.

3.3.3 Oncogenic effects of miR-498 were inhibited by the miR-498 decoy

Since the function of inhibiting miR-498 overexpression by miR-498 decoy was confirmed by my previous results, it was expected that miR-498 decoy alleviated the oncogenic effects of miR-498. Thus, the effects of the miR-498 decoy on the enhanced cell proliferation and cell migration by miR-498 were examined in two TNBC cell lines (PTEN wild type MDA-MB-231 and PTEN null BT-549). Firstly, to examine the protein level change induced by miR-498 or miR-498 decoy, miR-498 or miR-498 decoy was transduced into MDA-MB-231 cells. The expected result was that miR-498 suppressed PTEN and increased p-AKT in MDA-MB-231 cells but miR-498 decoy alleviated or abrogated the oncogenic effect of miR-498. In figure 3.3.4A, compared to the control miR and control decoy (lane 1, from left to right), miR-498 suppressed endogenous PTEN protein in MDA-MB-231 cells (lane 2) but miR-498 decoy restored PTEN protein in the presence of miR-498 (lane 4). As expected, at the level of p-AKT, miR-498 increased p-AKT greatly in MDA-MB-231 but miR-498 decoy alleviated the enhanced p-AKT by miR-498 (Figure 3.3.4A). These results indicated that miR-498 decoy acted as a competitive target of miR-498, which avoided PTEN mRNA from the binding of miR-498 and then protected the translation of PTEN mRNA. Because BT-549 cell had no wild type PTEN, it was used as negative control cells to examine whether the function of miR-498 decoy was dependent on PTEN. In PTEN null BT-549 cell, since the endogenous PTEN was not detected, p-AKT was not obviously affected by miR-498 or miR-498 decoy, which suggested that the enhanced p-AKT by miR-498 and the alleviated p-AKT by miR-498 in MDA-MB-231 were dependent on PTEN suppression by miR-498 (Figure 3.3.4B). Therefore, these results confirmed that miR-498 decoy was an effective tool to inhibit the PTEN suppression induced by miR-498 and to restore the PTEN protein level.

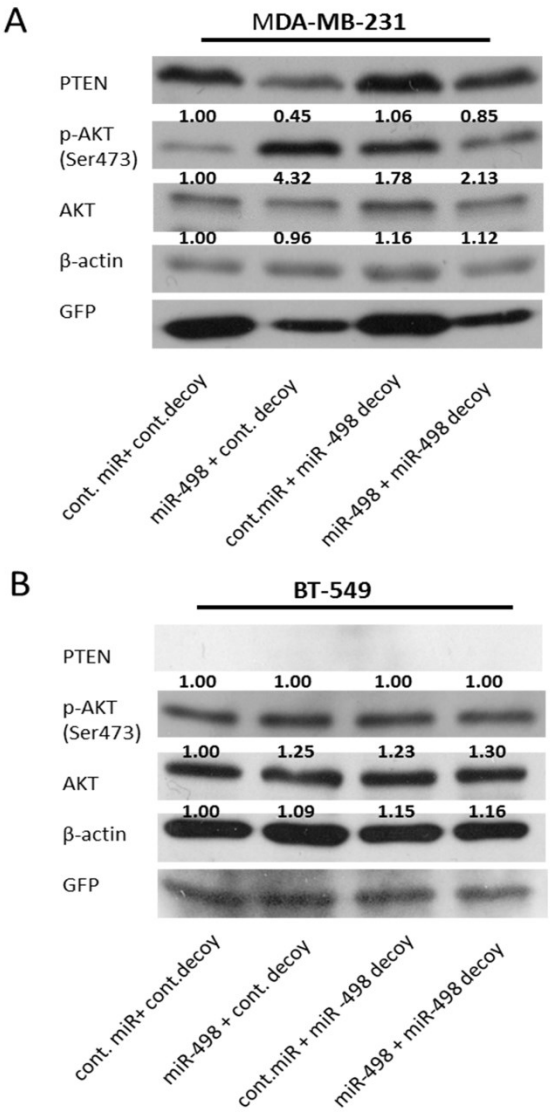


Figure 3.3.4 miR-498 decoy restored PTEN by inhibiting miR-498.

MiR-498 decoy restored PTEN by inhibiting miR-498 in PTEN-Wt MDA-MB-231 cells. (A) miR-498 decoy and PTEN or p-AKT protein level in MDA-MB-231cell. (B) miR-498 decoy and PTEN or p-AKT protein level in BT-549 cells. The endogenous PTEN was not detected and p-AKT was not obviously affected by miR-498 or miR-498 decoy, which suggested that the enhanced p-AKT by miR-498 and the alleviated p-AKT by miR-498 decoy in MDA-MB-231 were dependent on PTEN suppression by miR-498.

Next，to determine whether miR-498 decoy alleviated the oncogenic effect of miR-498 on cell proliferation, a clonogenic assay was performed. Since the miR-498-induced PTEN suppression was alleviated by miR-498 decoy (Figure 3.3.4), it was expected that the oncogenic effect of miR-498 on cell proliferation was alleviated. As results shown in figure 3.3.5A, miR-498 significantly increased the ratio of cell colony by 53% in MDA-MB-231 compared to the control miR (upper panel); however, miR-498 decoy significantly reduced the ratio of cell colony in MDA-MB-231, compared with miR-498 (lower panel). These results supported the expectation that miR-498 promoted cell proliferation and this oncogenic effect was alleviated by the miR-498 decoy. The clonogenic assay result of MDA-MB-231 cell was statistically analyzed and shown in figure 3.3.5B. In contrast, in the negative control cell line BT-549 where endogenous PTEN did not exist, no significant change in cell colony ratio was found after transducing miR-498 or miR-498 decoy (Figure 3.3.6). These results indicated that the PTEN-dependent oncogenic role of miR-498 on cell proliferation was alleviated by miR-498 decoy in vitro.

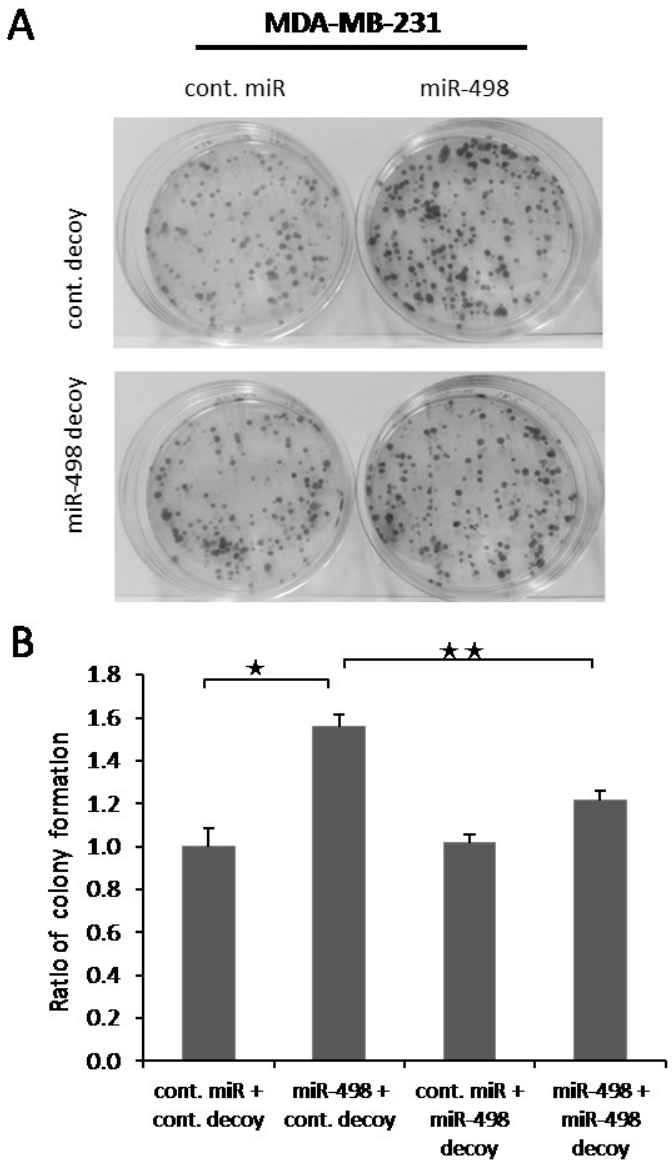


Figure 3.3.5 Oncogenic effect of miR-498 on cell proliferation alleviated by the miR-498 decoy.

Oncogenic effect of miR-498 was alleviated by miR-498 decoy in PTEN-Wt cell MDA-MB-231. (A) The oncogenic effect of miR-498 on cell proliferation by miR-498 decoy in MDA-MB-231. The effect of miR-498 on cell proliferation was examined by clonogenic assay. (B) The clonogenic assay result was statistically analyzed. ⋆$p<0.05$, ⋆⋆ $p<0.01$ (n=3) and the result was the mean value of three independent experiments.

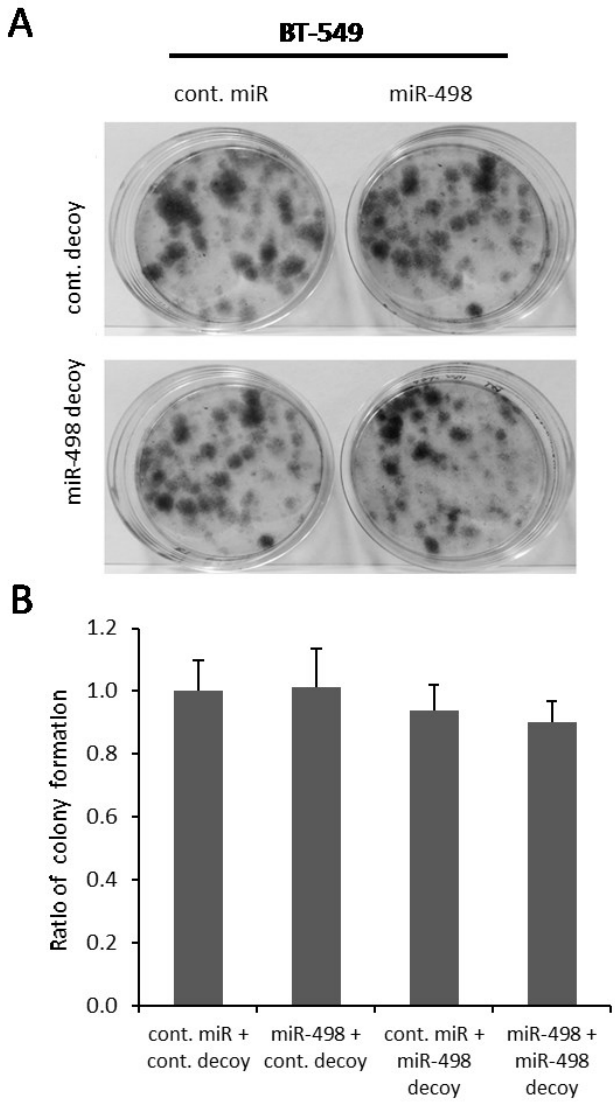


Figure 3.3.6 The miR-498 decoy had no significant effect on cell proliferation in PTEN-null cell line BT-549.

The miR-498 decoy had no significant effect on cell proliferation in PTEN-null cell line BT-549. (A) The effect of miR-498 decoy on cell proliferation of BT-549 cells. (B) The clonogenic assay result was statistically analyzed. The miR-498 decoy had no significant effect on cell proliferation in PTEN-null BT-549. The effect of miR-498 on cell proliferation was examined by clonogenic assay. The result was the mean value of three independent experiments.

To determine whether miR-498 decoy abrogated or alleviated the oncogenic effect of miR-498 on cell migration, I used scratch healing assay to visualize the cell migration of TNBC cells. Previous data indicated that miR-498 decoy inhibited miR-498 overexpression in TNBC cells and restored PTEN protein. It was expected that the oncogenic phenotype that miR-498 promoted cell migration through suppressing PTEN was abrogated or alleviated by the miR-498 decoy. As I expected, in PTEN wild type MDA-MB-231 cells, the results of wound healing assay indicated that at the time point of 24-hour, the blank area of miR-498 transduced cells (the second row) was significantly reduced comparing with that of control miR transduced cells (the first row); but the blank wound area of miR-498 and miR-498 decoy transduced cells (the fourth row) was restored to the level of control miR transduced cells by miR-498 decoy (Figure 3.3.7). These results showed that the cell migration rate of miR-498 transduced cells was significantly faster than that of control miR transduced cells; however, the miR-498 decoy inhibits the acceleration of cell migration which was resulted from miR-498 overexpression. The statistical analysis of the relative blank wound area was shown in the lower panel of figure 3.3.7. These data confirmed that miR-498 decoy inhibited the oncogenic effect of miR-498 on cell migration, which was also consistent with the immunoblotting results that PTEN protein was suppressed by miR-498 and restored by the miR-498 decoy.

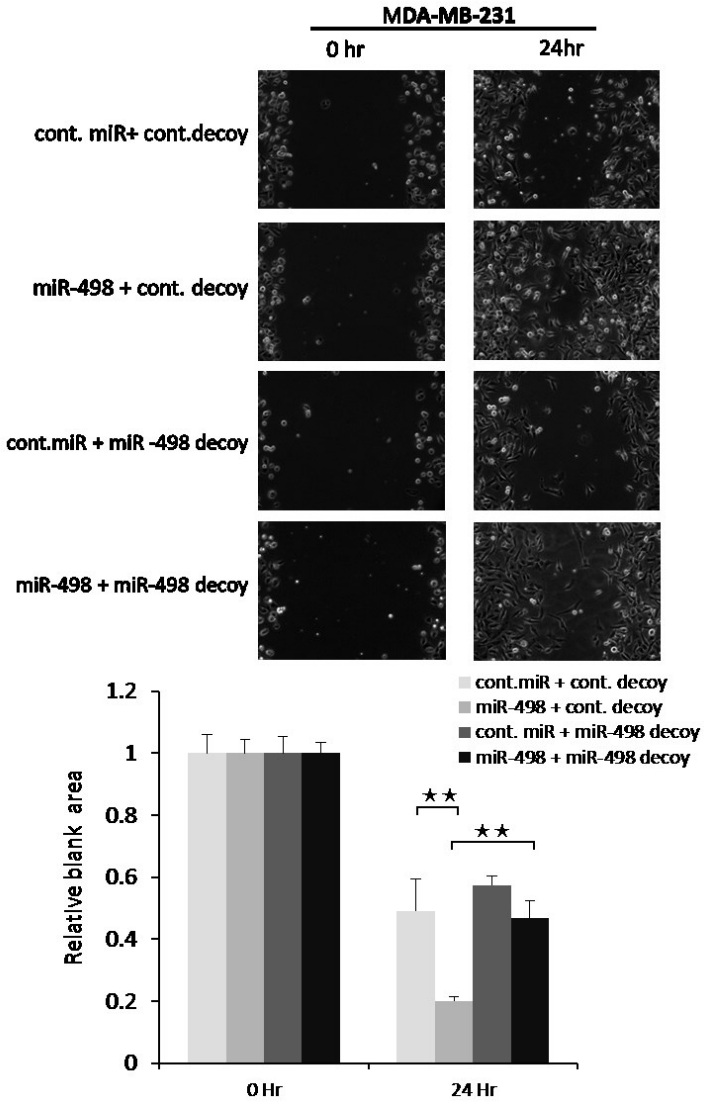


Figure 3.3.7 Oncogenic effect of miR-498 on cell migration was inhibited by the miR-498 decoy.

The oncogenic effect of miR-498 decoy on cell migration in PTEN-Wt cell MDA-MB-231. The statistical analysis of the relative blank wound area was shown in the lower panel of figure 3.3.7. The result was the mean value of three independent experiments. ★★P<0.01 (n=3).

In contrast, in the negative control cell line BT-549 where endogenous PTEN did not exist, no significant change in blank wound area was found after transducing miR-498 or miR-498 decoy. Specifically, at time point of 24 hour, there were no significant difference in blank wound area between the cells transduced with miR-498 and the cells transduced with control miR, which suggested that the cells transduced with miR-498 (the second row) did not exhibit a faster rate of migration, compared to the cells transduced with control miR (the first row) (Figure 3.3.8). Furthermore, in the presence of miR-498 overexpression, there was also no significant difference in blank wound area between the cells transduced control miR decoy (the second row) and the cells transduced miR-498 decoy (the fourth row), which suggested that the cells transduced with control miR decoy (the second row) did not exhibit a faster rate of migration, compared to the cells transduced with miR-498 decoy (the fourth row). Taken together, these results in the negative control cell line indicated that the miR-498 decoy had no effect on cell migration in PTEN-null cell line BT-549, which was also consistent with the immunoblotting data that p-AKT was not changed (Figure 3.3.4).

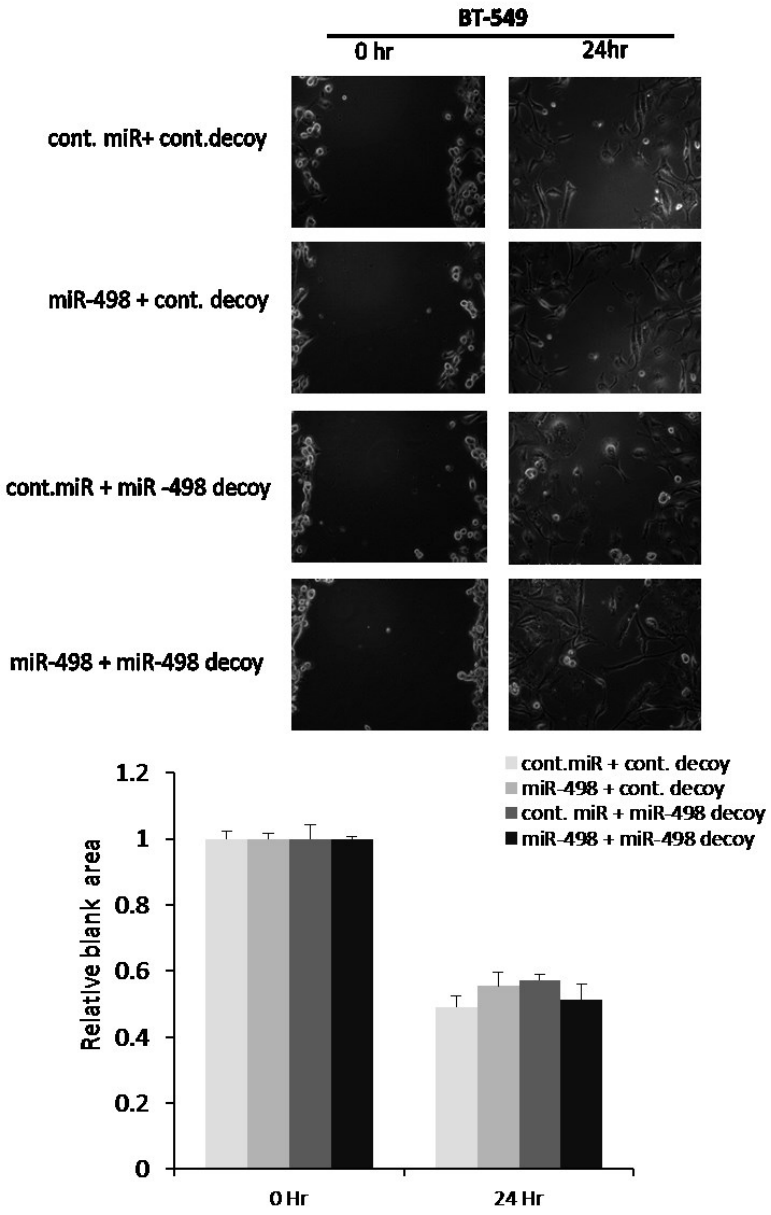


Figure 3.3.8 The miR-498 decoy had no significant effect on cell migration in PTEN-null cell line BT-549.

The miR-498 decoy had no significant effect on cell migration in PTEN-null cell line BT-549.. The statistical analysis of the blank wound area was shown in the lower panel of Figure 3.3.8. The result was the mean value of three independent experiments.

Summarily, in this section, I designed the miR-498 decoy, which was demonstrated to act as a negative regulator of miR-498 for inhibiting miR-498 and protecting PTEN from miR-498 binding. First, I confirmed the capability of the miR-498 decoy on attracting the binding of miR-498 by luciferase assay. The miR-498 decoy led to the reduction of miR-498 and the restoration of PTEN in the endogenous miR-498-overexpressing cell. Furthermore, miR-498 enhanced cell migration and cell proliferation by suppressing PTEN and activating p-AKT in PTEN wild type MDA-MB-231cells, whereas miR-498 decoy inhibited these oncogenic effects of miR-498 through protecting PTEN from miR-498. However, in negative control cell line PTEN-null BT-549 cells, since endogenous PTEN and the PTEN-dependent oncogenic phenotypes of miR-498 did not exist, miR-498 decoy had no effect on cell proliferation and migration. These results also further confirmed that the promoted cell migration and cell proliferation by miR-498 were dependent on PTEN suppression.

Chapter 4 Discussion and Conclusion

4.1 Discussion

4.1.1 Interpretation of results

The aim of this study was to identify oncogenic microRNA(s) that negatively regulate tumor suppressor PTEN in breast cancer.

In section 3.1 of chapter 3, to identify novel PTEN-targeting microRNAs, I examined 19 predicted PTEN-targeting microRNAs through immunoblotting and luciferase reporter assay. Eventually, I found that miR-498 was a novel PTEN-targeting microRNA and a potential oncomiR in breast cancer. As a tumor suppressor, PTEN was reported to be absent or expressed at a low level in multiple cancers including prostate cancer, gastric cancer, colorectal cancer, non–small cell lung cancer and breast cancer (Whang YE, 1998; Kang YH, 2002; Goel A; 2004; Soria JC, 2002; Garcia JM, 2004). PTEN inactivity is associated with large tumor sizes, considerable lymph node metastasis and an aggressive triple-negative phenotype in breast cancer (BCa) (Li S, 2017). PTEN expression is much lower in BCa tissue (57.5%, 84/117) than in the normal tissue (100%, 10/10); however, PTEN mutation rates are approximately 5% in BCa, which indicates that other mechanisms negatively regulate PTEN expression (Rhei E, 1997; Zhang HY, 2013).MicroRNA is a small non-coding RNA that has been shown to regulate PTEN. Previous studies had reported some PTEN-targeting microRNAs in various cancers: miR-21 was a PTEN-targeting microRNA and acted as oncogenic miR in hepatocellular cancer (Meng F, 2007); the oncogenic miR-4534 was determined to negatively regulate PTEN in prostate cancer (Nip H, 2016); miR-221 and miR-222 were overexpressed in hepatocellular carcinoma and non-small cell lung cancer (NSCLC) and they promoted tumorigenesis by targeting PTEN (Garofalo M, 2009). Since the studies of PTEN regulation by microRNAs in breast cancer (BCa) are still

limited, the identification of novel miRNAs that result in the PTEN inactivation is of great importance to improving the breast cancer treatment, which makes it necessary to determine PTEN-targeting microRNAs in BCa.

To screen for PTEN-targeting microRNAs, nineteen predicted PTEN-targeting microRNAs were examined by immunoblotting in the breast cancer cell line MCF-7 and in the lung cancer cell line A549 as a control. Immunoblotting results indicated that miR-425, miR-454, miR-301b, miR-498 and miR-374b reduced endogenous PTEN protein over 50% in breast cancer cell line MCF-7; miR-22, miR-425, miR-29a, miR-374b, miR-454, miR-301b, miR-498 and miR-29c negatively regulated the protein level of PTEN in lung cancer cell line A549 (Figure 3.1.3). Among nineteen miRNAs, five predicted PTEN-targeting miRNAs exhibited a negative effect on PTEN protein in MCF-7 breast cancer cells and eight PTEN-targeting miRNAs did in A549 lung cancer cells. This suggested that the prediction for PTEN-target microRNAs by online databases was redundant but not highly accurate. Moreover, the predicted binding sites of microRNAs on PTEN mRNA by online databases were also redundant but not highly accurate, compared with the actual binding sites determined by experimental results. For example, there were three distinct binding sites of miR-498 on PTEN mRNA predicted by database microRNA.org; the binding site A, site B and site C located in 26-48bp, 833-855bp, 1242-1269bp of PTEN mRNA 3'UTR, respectively. All of them had a mirSVR score lower than -0.05, which meant that miR-498 would be highly probable to bind to these sites. However, these predicted binding sites of miR-498 were examined by luciferase reporter assay and only the binding site B was confirmed by experimental results. Thus, the predicted results for PTEN-targeting microRNAs by online databases only gave a preliminary but not accurate list of PTEN-targeting microRNAs and showed some possible microRNA binding sites, which was far away

from the solid determination of a PTEN-targeting microRNA. These results indicated that the experimental examination by immunoblotting and luciferase assay in my study was required for determining PTEN-targeting miRNAs.

Furthermore, the tissue-specific or cell-type-specific difference in down-regulating PTEN protein level by microRNAs was observed in this study. I found that miR-29a and miR-29c obviously reduced the endogenous PTEN in A549 cells (over 50%), but they did not exhibit the effect on down-regulating 50% of PTEN in MCF-7 cells. This phenomenon is possibly due to the different expression files of target mRNAs between these two cell lines. In addition to PTEN mRNA, there were other mRNA targets of miR-29a or miR-29c and they competitively interacted with miR-29a or miR-29c, which were capable of influencing the PTEN suppression by these two microRNAs. For instance, the mRNA of transcription factor KLF4 that was identified as the target of miR-29a was overexpressed in breast cancer cell line T47D (Cittelly DM, 2013). It is possible that in breast cells the transfected miR-29a mimics bind to the abundant KLF4 mRNA instead of PTEN mRNA, which results in the less binding to the PTEN mRNA and subsequently alleviated PTEN suppression. Furthermore, this phenomenon was also possible to be related to the high native expression level of miR-29a and miR-29c in breast cancer and the low expression in non-small cell lung cancer (NSCLC). It was reported that miR-29a was highly expressed in breast cancer tissues and miR-29c was highly expressed in luminal A breast cancer (Rostas JW 3rd, 2014; Pei YF, 2013; Myhre S, 2016). Whereas prior studies had demonstrated that miR-29a was down-regulated in NSCLC cell A549 and NSCLC tissues and acted as a tumor suppressor (Li Y, 2017; Kamikawaji K, 2016); miR-29c was downregulated in 77% of NSCLC patients and negatively regulated PI3K-AKT pathway (Wang H, 2013; Sun DM, 2018).

Considering miR-29a/29c low expression in NSCLC but high expression in breast cancer, the transfection of miR-29a/29c induced a dramatic decrease of PTEN protein in A549 cells but not in MCF-7 cells. Except for miR-29a and miR-29c, the PTEN protein levels in both MCF-7 and A549 were decreased by six predicted miRNAs, whereas the expression levels of these miRNAs were also distinct between different cancers. For instance, miR-454 was found to be down-regulated in 80% osteosarcomas tissues (24/30) and four osteosarcomas cell lines and it functioned as a tumor-suppressing miRNA (Niu G, 2015); whereas in hepatocellular carcinoma (HCC), miR-454 was highly expressed in 85% of cancer tissues (34/40) and four HCC cell lines and it promoted cell proliferation and invasion as an oncomiR (Yu L, 2015). These results on the miRNA expression indicated that the expression level of a specific miRNA was distinct between different cancer tissues, which also explained the observation that the same miRNA exhibited distinct effects on PTEN suppression in a different tissue context.

To determine specific microRNAs targeting and regulating PTEN, the evidence that PTEN protein was down-regulated by microRNA transfection was not sufficient. Instead of immunoblotting, luciferase assay was capable of showing the direct evidence for the binding of microRNAs on PTEN mRNA. Therefore, the four predicted PTEN-targeting microRNAs that passed the immunoblotting screening were tested by luciferase assay (Figure 3.1.4). The luciferase results showed that miR-498 and miR-374b obviously reduced the luciferase activity among four predicted microRNAs, which further confirmed that miR-498 and miR-374b were capable of binding to PTEN mRNA 3' UTR and reducing the translation of PTEN (Figure 3.1.4).

In order to determine the oncogenic role of microRNAs and further investigate the biological function in a specific type of cancer, it was necessary to comprehensively analyze the microRNA expression in a specific tissue context. I examined prior publications about the

expression levels of miR-498 and miR-374b in multiple cancers. Previous studies found that oncogenic miR-498 was overexpressed in retinoblastoma and triple negative breast cancer (TNBC) tissues and cell lines where it promoted cell proliferation (Matamala N, 2016; Yang L, 2018). Regarding of the immunoblotting result that PTEN was suppressed by miR-498, it was rational to speculate that there might be a causal relationship between PTEN suppression by miR-498 and the oncogenic role of miR-498 in breast cancer. The results from the Oncomine and Kaplan-Meier database also supported this speculation on PTEN and miR-498. The Oncomine results showed that PTEN expression in breast cancer tissues was significantly lower than in normal breast tissues (Figure 3.1.9). The Kaplan-Meier survival curves indicated that miR-498 expression was negatively correlated with the overall survival of patients with BCa (Figure 3.1.11). However, miR-374b was down-regulated in multiple cancers including colon and T-cell lymphoblastic lymphoma (T-LBL), and its high expression was associated with a favorable outcome of TNBC patients (Qian D, 2015; Qu R, 2018; Liu Y, 2015). These studies suggested that miR-374b probably acted as tumor-suppressing microRNA in cancers, which did not support my hypothesis that microRNA acted as an oncomiR to target PTEN mRNA and down-regulate PTEN protein level in breast cancer. Therefore, taken together with my experimental results and the bioinformatics analysis of microRNA expression in specific tissues, it was the first time that miR-498 was identified as a PTEN-targeting microRNA, and miR-498 was chosen to further investigate its oncogenic effects on PTEN suppression in TNBC.

In section 3.2 of chapter 3, the relation of miR-498 and PTEN expression was clarified in TNBC cell lines and the oncogenic effects of miR-498 in TNBC were investigated. The TNBC is the most lethal BCa subtype and the traditional BCa therapies like the hormone replacement therapy are not effective to TNBC patients (Perou CM, Nature, 2000; Sørlie T, PNAS, 2001).

Therefore, the study to investigate new therapeutic targets or methods to repress TNBC is very urgent and necessary. Previous studies indicated that miR-498 was overexpressed in TNBC tissue samples (19/31) (Matamala N, 2016); the results of Kaplan Meier analysis revealed that the expression levels of miR-498 in breast cancer patients were negatively related with their overall survival time (Lánczky A, 2016). My prior results also demonstrated that miR-498 was a PTEN-targeting microRNA and a potential oncomiR in breast cancer. Thus, I intended to determine the oncogenic role of miR-498 in TNBC cells by suppressing PTEN.

At first, the relation between miR-498 expression and PTEN expression in TNBC cell lines was examined by qRT-PCR and immunoblotting. The qRT-PCR results indicated that compared to normal epithelial breast cell line MCF-10A, two TNBC cell lines had a higher level of miR-498 expression. This was consistent with the results that miR-498 expression in TNBC tissues was significantly higher than that in normal breast tissues from a prior study on miR-498 (Matamala N, 2016); whereas, the PTEN expression of four TNBC cell lines in both protein level and mRNA level was lower than that of MCF-10A. These results indicated that there was possibly a reverse relation between miR-498 and PTEN in TNBC. Moreover, the expression of miR-498 was up-regulated in TNBC tissues and PTEN inactivity was associated with the aggressive triple-negative phenotype (Li S, 2017). However, since the tissue samples of TNBC patients in these studies were not adequate for large-scale statistical analysis, there was no strong evidence to confirm the correlation between PTEN inactivation and miR-498 overexpression in TNBC patients. Therefore, my results revealed a possible negative relation between miR-498 expression and PTEN expression in TNBC cell lines, though it still needed to test PTEN and miR-498 expression in a larger sample pool of TNBC tissues and paired normal breast tissues.

Next, the effect of miR-498 overexpression on endogenous PTEN was examined in TNBC cells lines MDA-MB-231 and MDA-MB-157. These two cell lines contained relatively low miR-498 expression and wild-type PTEN protein. The efficiency of transfection to these two cell lines by the calcium phosphate method was very low. However, prior studies indicated that retrovirus transduction could be used for microRNA overexpression and showed high efficiency in MDA-MB-231 cells (Qin H, 2002; Yin Q, 2008; Croset M, 2018). Therefore, I used viral transduction to overexpress miR-498 in MDA-MB231 cells to investigate the effects of miR-498. The overexpression of miR-498 by viral transduction in two TNBC cell lines led to the downregulation of PTEN, in which approximately 50% of PTEN protein was reduced. Simultaneously, miR-498 expression was measured by qPCR, and the relative miR-498 expression of miR-498-tranduced cells was increased by over 100-fold compared to control-miR-transduced cells. This phenomenon may possibly be explained by the following aspects. First, the protein components of miRISC (microRNA-induced silencing complex) are present in insufficient amounts to fully utilize the over 100-fold miR-498 overexpression for miR-498-induced PTEN suppression. Mature miR-498, together with the miRISC protein components AGO and GW182, targets to PTEN mRNA and leads to PTEN suppression. AGO is the key component to guide miRNA to the binding site on its target mRNA (Bartel DP, 2009), which means that AGO is a rate-determining component for miRNA-induced translation suppression. Thus, though miR-498 is increased by over 100-fold, miR-498 is over abundant for cooperation with the amount of AGO protein, which might result in the observation that only 50% of PTEN protein was reduced by miR-498 overexpression. Also my results (Figure 3.1.8) indicated that 7-fold miR-498 overexpression by calcium phosphate transfection was capable of resulting in a 40% reduction of endogenous PTEN in MCF-7 cells. Despite different cell lines, there was no

dramatic difference in PTEN downregulation at 7-fold or 100-fold overexpression of miR-498, which also supports my speculation that the amount of miRISC components (e.g.AGO) were insufficient for utilizing 100-fold miR-498 overexpression.

Second, other targets of miR-498 may dilute the activity of miR-498 on PTEN suppression. As stated in the introduction, the mRNA of PTEN pseudogene PTENP1 is a homolog of PTEN mRNA and acts as a competing endogenous RNA for PTEN-targeting miRNAs (Thomson DW, 2016). The PTENP1 mRNA can compete with PTEN mRNA to attract the binding of miR-498, which means that partial miRISC containing miR-498 and AGO will bind to PTENP1 mRNA but not PTEN mRNA. In addition to PTENP1 mRNA, circular RNA circFADS2 was found to interact with miR-498 and inhibit miR-498 in lung cancer (Zhao F, 2018). Thus, these potential targets of miR-498 may explain the observed reduction in PTEN expression.

Third, the inhibitory effect of miR-498 on PTEN protein is limited to its functional binding sites. The number of functional binding sites is related to the inhibitory effects on protein translation. Although there are multiple predicted binding sites of miR-498 on PTEN mRNA 3'UTR, only one binding site of miR-498 was experimentally confirmed as a functional one for regulating PTEN translation, which limits the inhibitory function of miR-498 on the translation of PTEN. Fourth, the native PTEN expression level in different tissue or cell contexts might also be related to this phenomenon. In tissues or cells where relatively high PTEN expression exhibited a crucial role in maintaining tissue/cell-specific features, the PTEN protein reduction by miR-498 overexpression might trigger the PTEN compensation mechanism to alleviate PTEN reduction, e.g., p53-mediated PTEN transcription activation (Stambolic V, 2001). Taken together,

these four aspects may explain how only 50% of PTEN protein was reduced by viral-transduced miR-498 overexpression in TNBC cells.

Subsequently, the oncogenic effects of miR-498 on cell proliferation and migration were demonstrated in TNBC cells. These oncogenic effects were dependent on the suppression of PTEN by miR-498. My results originally showed that miR-498 overexpression led to PTEN suppression and AKT activation in TNBC cell lines (Figure 3.2.3 & 3.2.4). And recent studies indicated that the reduced PTEN expression was commonly found in BCa tissues and was positively associated with tumor size, lymph node metastasis and an aggressive triple-negative phenotype (Li S, 2017); the activated AKT promoted cell proliferation and cell cycle progression and enhanced cell migration (Manning BD, 2017). Therefore, it was rational that miR-498 played an oncogenic role on cell proliferation, cell cycle progression and migration, and I intended to investigate the oncogenic effects of miR-498 through PTEN suppression in TNBC cells.

Firstly, the oncogenic effect of miR-498 on cell proliferation was demonstrated by clonogenic assay. It was well established that PTEN was a negative regulator of cell proliferation and cell survival in multiple cells and the clonogenic assay was widely used to evaluate cell reproduction and survival (Groszer M, 2001; Stambolic V, 1998; Franken NA, 2006). My clonogenic assay results originally showed that miR-498 significantly increased the colony formation ratio in PTEN-Wt MDA-MB-231 cells but not PTEN-Null BT-549 cells, which demonstrated that miR-498 had the oncogenic effect on the proliferation and viability of TNBC cells with wild-type PTEN (Figure 3.2.5). These results told us that as a PTEN-targeting miRNA, miR-498 promoted cell proliferation in TNBC cells, which was consistent with other PTEN-

targeting microRNAs, such as miR-214 in gastric cancer cells and miR-153 in prostate cancer cells (Yang TS, 2013; Wu Z, 2013).

Secondly, the oncogenic effect of miR-498 on cell cycle progression was demonstrated by flow cytometry. Prior studies had identified the role of PTEN on inhibiting cell cycle progressing at G1 checkpoint and this inhibitory role of PTEN was mainly dependent on its phosphatase activity through inhibiting the activation of PI3K-AKT pathway (Li DM, 1998; Furnari FB, 1998; Weng LP, 2001). My flow cytometry result originally showed that miR-498 increased the proportion of cells in S phase and reduced the proportion of cells in G1 phase, which suggested that miR-498 promoted the transition from G1 phase to S phase and promoted cell cycle progression (Figure 3.2.6). This oncogenic effect of miR-498 on cell cycle progression was well explained by my prior results that miR-498 suppressed PTEN and activated AKT in TNBC cells, which eventually promoted cell proliferation. Similarly, my results demonstrated that miR-498 promoted cell cycle progression in TNBC cells, which was consistent with other PTEN-targeting microRNAs found in gastric cancer, cervical cancer and prostate cancer (Zhang LY, 2012; Yang TS, 2013; Wu Z, 2013).

Thirdly, the oncogenic effect of miR-498 on cell migration was demonstrated by wound healing assay. The cancer metastasis is one of the most challenging problems in cancer treatment and the active migration of cancer cells is a prerequisite for cancer metastasis (Entschladen F, 2004). It was reported that the expression of miR-498 was significantly higher in metastatic medullary thyroid carcinoma than in primary medullary thyroid carcinoma, which indicated that miR-498 was possibly associated with cancer cell migration (Santarpia L, 2013). However, in TNBC, the role of miR-498 on cell migration was not clear. To evaluate the effect of miR-498 on cell migration, three parameters were analyzed in my wound healing assay, the relative cell-

free wound area, the average migration distance and the number of migrated cells. The relative cell-free wound area of miR-498 transduced cells was significantly less than control miR transduced cells at the time point of 24-hour after scratching, which indicated that miR-498 could enhance the migration capacity of TNBC cell (Figure 3.2.7 A&B). Next, to further determine that the motility of individual cell was enhanced by miR-498, the average migration distance of each cell and the number of migrated cells were analyzed. The results showed that miR-498 significantly increased the number of migrated cells and also significantly enhanced the average migration distance (Figure 3.2.7 C&D). Taken together, these results in my study demonstrated for the first time that miR-498 had an oncogenic effect on promoting the migration capability of TNBC cells, which was similar with other PTEN-targeting microRNAs found in different types of cancers (Zhang LY, 2012; Kong G, 2011; Zhang JG, 2010).

Although the oncogenic effects of miR-498 on cell proliferation and cell migration were determined, it was still not determined that whether these effects of miR-498 resulted from the suppression of PTEN or from other protein targets of miR-498. Since other protein targets of miR-498 were identified and some of them also functioned as a tumor suppressor in different cancers (e.g. FOXO3), it was necessary to identify that whether these oncogenic effects of miR-498 on cell proliferation and cell migration were dependent on inhibiting PTEN by miR-498. Thus, in order to clarify this question, PTEN ORF was introduced into the miR-498-transduced cells and the phenotypes on cell proliferation and migration were observed. As expected, PTEN ORF restored PTEN protein level in miR-498 transduced cells and restored PTEN significantly inhibited cell proliferation and cell migration which was accelerated by miR-498, which confirmed the hypothesis that miR-498 suppressed PTEN and promoted cell proliferation and migration (Figure 3.2.8& 3.2.9 & 3.2.10). And also PTEN siRNA was used as a positive control

to compare with miR-498 on PTEN suppression and oncogenic phenotypes. The oncogenic phenotypes induced by PTEN siRNA were similar to the ones induced by miR-498 (Figure 3.2.11& 3.2.12 & 3.2.13). Therefore, these results further demonstrated that the oncogenic effects of miR-498 were largely dependent on PTEN suppression, which clarified the oncogenic mechanism of miR-498 in TNBC cells and expanded understanding of the miR-498 function.

In section 3.3 of chapter 3, the miR-498 decoy was determined to be a negative regulator of miR-498 and to inhibit the miR-498 expression and the oncogenic effects in TNBC cells. Due to its poor prognosis and the lack of effective therapies, TNBC is the most lethal subtype of BCa (Dent R, 2007); the median survival of metastatic TNBC patients is only 13 months (Kassam F, 2009). Therefore, it is urgent and necessary to find effective targets to prevent the progression of TNBC. A prior study demonstrated that the tumor suppressor PTEN played an important role in the development of TNBC (Li S, 2017); Matamala et al reported miR-498 overexpression in TNBC tissues according to microarray analyses (Matamala N, 2016). My prior results showed that miR-498 was overexpressed in TNBC cell lines and led to PTEN suppression. Hence, these results suggested that miR-498 was a potential target for TNBC patients and it was plausible to build an effective tool to inhibit miR-498 overexpression and rescue PTEN in TNBC. In section 3.3, a miR-498 decoy was successfully constructed and demonstrated to specifically reduce the endogenous level of miR-498 (Figure 3.3.1 & 3.3.3). Furthermore, I confirmed that the miR-498-induced PTEN suppression and AKT activation was abrogated by the miR-498-specific decoy in TNBC cells and the oncogenic effects of miR-498 were also abrogated through rescuing PTEN (Figure 3.3.4 & 3.3.5 & 3.3.7). These data originally demonstrated that the miR-498-specific decoy was an efficient tool to inhibit miR-498 and its oncogenic effects in TNBC cells.

Considering the effectiveness of the specific microRNA decoy on inhibiting its oncogenic microRNA, therapeutic microRNA decoys or anti-miR oligonucleotides are capable of being used in pre-clinical or clinical trials of cancers. For instance, therapeutic anti-miRNA oligonucleotides anti-miR-221 and anti-miR-10b are still in development by Regulus Therapeutics for hepatocellular carcinoma treatment and glioblastoma treatment, respectively (Nguyen DD, 2017). Taken together, in this section, my results demonstrated that miR-498 was potential to be used as a potential treatment target and miR-498-specific decoy was possible to be developed as a therapeutic method for TNBC patients, which provided new information for the development of TNBC therapies.

4.1.2 Limitation of this study

Prior to the clinical trial of miR-498 decoy in other types of cancer, there are still some issues to be addressed. Firstly, the role that miR-498 plays in cancers appears to be complicated. The expression level of miR-498 varies in different cancer tissues or different stages of cancer. MiR-498 was expressed at low levels in non–small cell lung cancer and ovarian cancer (Cong J, 2015; Wang M, 2015); while the overexpression of miR-498 was observed in TNBC and retinoblastoma (Matamala N, 2016; Yang L, 2018). In addition, the expression of miR-498 was significantly higher in metastatic medullary thyroid carcinoma than in primary medullary thyroid carcinoma (Santarpia L, 2013). These results indicated the diversity of miR-498 expression profiles in different tissues or different stages of the tumor.

In this study, I found that high expression of miR-498 was significantly associated with short overall survival of breast cancer patients and promoted cell proliferation and migration,

which suggested that miR-498 was an oncogenic player in breast cancer. However, in terms of different subtypes of breast cancer, the relation between miR-498 expression and overall clinical survival appeared more complicated and required further investigation. Generally, the high expression of miR-498 indicated a short overall survival in breast cancer patients (Figure 3.1.11); however, it seems that the survival prediction role of miR-498 was diverse in different subtypes. In the TCGA database, for ER-positive and PR-positive breast cancer patients(n=393), miR-498 high expression was significantly associated with short overall survival(HR=4.11, P=9.9E-7), which suggested it worked as a death-predicting marker. However, for TNBC patients(n=97), miR-498 high expression was not associated with short overall survival (HR=0.2, P=0.002) (Supplement Figure 1). This indicated that predicting overall survival in breast cancer by assessing miR-498 expression level was not fully determined. The clinical role of miR-498 as an overall survival marker is distinct among different breast cancer subtypes. This phenomenon is possible due to the diversity of patient samples and the variability among individual patients. The genetic and environmental background of individual patients could have a more significant impact on the overall survival than just miR-498 overexpression. For instance, miR-498-induced PTEN suppression might not be the dominant factor to determine the overall survival in some breast cancer patients highly expressing wild-type p53. Furthermore, it was possible that when miR-498 simultaneously downregulated PTEN, it bound to other oncogenic targets and inhibited their expression, which contributed to the longer overall survival of patients and opposed the miR-498 oncogenic effect by PTEN suppression. This could lead to the positive relationship between high expression of miR-498 and better survival. In addition, considering only 97 TNBC patients in TCGA database, the smaller sample size of TNBC patients might also affect the

survival outcome of miR-498. Therefore, to clarify the role of miR-498 as a survival prediction marker, further investigation is needed in more breast cancer patient samples.

Next, I used qPCR to screen the expression of miR-498 in four TNBC cell lines and the results showed miR-498 expression in TNBC cells was higher than in non-tumorigenic MCF-10A cells. The miR-498 expression in Hs578T cells was significantly increased 7-fold compared to that in the MCF-10A cells. Meanwhile, the expression of PTEN was inhibited in these TNBC cell lines on the protein level. In particular, the PTEN protein in Hs578T cells was 57% less than in MCF-10A cells. These results indicated that miR-498 expression was negatively associated with the PTEN protein in Hs578T cells. Similarly, the same trend was observed in MDA-MB-231 cells. However, since there were only three PTEN-wt TNBC cell lines that are available in lab cell stocks, the small sample size limited analysis for statistical correlation between PTEN expression and miR-498 expression. Therefore, the negative correlation between miR-498 and PTEN expression could not be determined in this study. My results implied that there was possibly a negative relationship on PTEN expression and miR-498 expression, and provided a clue that miR-498 might be involved the inhibition of PTEN in TNBC cells. Moreover, there was evidence of miR-498 overexpression in TNBC tissues. A previous study reported high expression of miR-498 in TNBC tissues compared with in normal tissues (Matamala N, 2016). However, the PTEN expression in each of TNBC patients was not screened simultaneously, so the analysis to identify whether there was a statistical correlation between miR-498 and PTEN expression was not ideal. If possible, future statistical analysis between miR-498 and PTEN expression should be done simultaneously in samples of TNBC patients in order to directly demonstrate whether a negative correlation exists between miR-498 and PTEN in TNBC.

I then examined the effect of miR-498 overexpression on PTEN inhibition in TNBC cells by using retroviral transduction to overexpress miR-498 in two TNBC cell lines (MDA-MB-231 and MDA-MB-157) that expressed relatively low levels of miR-498. Immunoblotting showed that 50% of PTEN protein was reduced by miR-498 overexpression, while qPCR revealed miR-498 was overexpressed more than 100-fold by retroviral transduction. I speculated that the 100-fold miR-498 overexpression was not necessary to achieve a 50% reduction in PTEN protein. Considering my results in Figure 3.1.8, in MCF-7 cells, a 7-fold overexpression of miR-498 by calcium phosphate transfection led to a 40% decline in PTEN protein that was comparable to the effect of a 100-fold miR-498 overexpression on PTEN protein expression in TNBC cells. A possible reason for this phenomenon is that the availability of miRISC components (e.g.AGO) in TNBC cells was insufficient to fully utilize the 100-fold miR-498 overexpression to reduce PTEN protein expression. To confirm this speculation, TNBC cells would be transduced with a lower concentration of miR-498 viral supernatant to observe whether a similar PTEN reduction was obtained. If miR-498 transduction with a low concentration virus supernatant was capable of inducing a 7-fold overexpression of miR-498 and a reduction in PTEN protein that was similar to the reduction in PTEN protein observed with 100-fold miR-498 overexpression, it would illustrate that considerable PTEN inhibition resulted from as low as 7-fold miR-498 overexpression, which would be consistent with the miR-498 overexpression I screened in TNBC Hs578T cells.

Another issue I have to point out is that the effect of exogenous miR-498 overexpression on PTEN inhibition under experimental conditions is not equal to the effect of endogenous miR-498 overexpression under physiological conditions. In this study, the 100-fold miR-498 overexpression induced by viral transduction in MDA-MB-231 cells was significantly higher

than the endogenous 7-fold miR-498 overexpression in TNBC Hs578T cells, and this led to the promotion of cell proliferation and migration in MDA-MB-231 cells. According to this, it might be possible that introduction of the physiological miR-498 overexpression (7-fold increase) into MDA-MB-231 cells, may not promote cell proliferation and migration to the same extent as miR-498 viral transduction. The dramatic difference between more than 100-fold miR-498 overexpression and 7-fold miR-498 overexpression could result in the gap in their oncogenic effects. This is a reminder to not overestimate the PTEN-dependent oncogenic effects of physiological miR-498 overexpression *in vivo*. Although the 100-fold miR-498 overexpression exhibited significant oncogenic effects on PTEN inhibition *in vitro*, the physiological miR-498 overexpression may have a different outcome in vivo reflecting an example of biological complexity. Therefore, the oncogenic effects of miR-498 overexpression at physiological levels were not determined and need further investigation by experiments such as those that I have mentioned above.

Furthermore, in addition to PTEN, other targets of miR-498 were reported. For instance, telomerase reverse transcriptase that enhanced cell growth in an ER-α -dependent manner was an oncogenic target of miR-498; Vitamin D–induced miR-498 bound to telomerase reverse transcriptase mRNA and suppressed its protein level in ovarian cancer cells where miR-498 functioned as a tumor suppressor (Kasiappan R, 2012). However, in TNBC, which has no ER-α expression, miR-498 was highly expressed in tissues and cell lines and negatively regulated the tumor suppressor PTEN, which contributed to tumorigenesis. Therefore, to some extent, the diversity of miR-498 targets and expression levels explained the conflicting reports of the effects of miR-498 in different cancers.

The mechanisms of miR-498 overexpression in TNBC are still not clear. The miR-498 overexpression was observed in retinoblastoma and triple negative breast cancer (TNBC) tissues and cells where it acted as an oncomiR (Matamala N, 2016; Yang L, 2018). Unfortunately, the mechanisms that induced the overexpression of miR-498 in retinoblastoma and TNBC were not clarified. Generally, some studies revealed the regulatory mechanisms of PTEN-targeting microRNAs in multiple cancers. Kasiappan R et al found that miR-498 overexpression was induced by 1,25-dihydroxy vitamin D3 in human ovarian tumor cell lines (Kasiappan R, 2012). The mechanism of the miR-498 overexpression was that the vitamin D receptor and its co-activators bound to a functional vitamin D response element in the 5'prime regulatory region of the miR-498 genome and then activated the transcription of miR-498. Moreover, it was reported that inflammatory stress activated PTEN-targeting microRNA through NF-kappaB activation; the pro-inflammatory cytokine IL-1β induced NF-kappaB activation in gastric cancer and NF-kappaB bound to the promoter of miR-425 which enhanced its transcription (Ma J, 2014). Therefore, it is possible that inflammatory stress also contributes to the overexpression of miR-498 in TNBC. Moreover, the other non-coding RNAs were also involved in the regulation of miR-498 in cancers. Long non-coding RNA (lncRNA) UFC1 was determined to interact with miR-498 and decreased the free miR-498 in gastric cancer, which inhibited the binding of miR-498 to its target Lin28b (Zhang X, 2018); the circular RNA circFADS2 was also found to interact with miR-498 and inhibit miR-498 in lung cancer (Zhao F, 2018). Although these non-coding RNAs was overexpressed and acted as a negative regulator of miR-498 in gastric and lung cancers, their expression in TNBC was not determined. It is probable that these non-coding RNAs are down-regulated in TNBC which contributes to the overexpression of miR-498 in TNBC.

Since microRNA is just one of the mechanisms that inactivate PTEN, other mechanisms also contribute to the inactivation of PTEN in cancers. Epigenetic dysregulation of PTEN was found to result in the PTEN inactivation in multiple cancers, including hypermethylation of PTEN promoter and posttranslational modification of histone. It was reported that the PTEN promoter was hypermethylated in 48% of sporadic breast cancer cases, 16% of hepatocellular carcinoma cases and 50% of thyroid cancer cases (Garcia JM, 2004; Wang L, 2007; Alvarez-Nunez F, 2006). The transcription factor Evi1 was ectopically expressed in approximately 10% of AML patients, and it recruited polycomb complex to the PTEN locus and repressed PTEN transcription through the methylation of histone H3 at Lys27 (Yoshimi A, 2011); the transcription factor SALL4, associated with Mi-2/Nucleosome Remodeling and Deacetylase (NuRD) complex, bound to the promoter region of PTEN and repressed the transcription of PTEN, which led to PTEN suppression and potentially explained the development of acute myeloid leukemia (Lu JY, 2009). The contribution of these epigenetic mechanisms of regulating PTEN has not been determined in TNBC. Moreover, posttranslational modification, such as ubiquitination, also resulted in the inactivation of PTEN through promoting PTEN degradation. Ubiquitin E3 ligase NEDD4-1 was highly expressed in some types of human cancers and ubiquitination of PTEN mediated by NEDD4-1 accelerated the PTEN degradation and decreased the protein level of PTEN (Wang X, 2007). Additional ubiquitin E3 ligases XIAP and WWP2 were identified for PTEN ubiquitination and the hyperactivation of these E3 ligases resulted in the PTEN degradation (Van Themsche C, 2009; Maddika S, 2011). In addition, competing endogenous RNA (ceRNA) also contributed to the PTEN suppression in cancers. The PTEN pseudogene PTENP1 had three non-coding transcripts which were involved in the regulation of PTEN (Poliseno L, 2010). Among these transcripts, PTENP1 antisense RNA isoform1 bound to

the PTEN promoter and inhibited PTEN transcription by recruiting epigenetic repressor complexes (Johnsson P, 2013). Some protein-coding mRNAs also acted as competing endogenous RNA for PTEN-targeting miRNAs. For instance, zinc finger E-box binding homeobox2 (ZEB2) was capable of interacting with PTEN-targeting miRNAs and the abrogation of ZEB2 expression in melanoma released these miRNAs which resulted in PTEN suppression and tumorigenesis (Karreth FA, 2011). These mechanisms of PTEN suppression have not been confirmed in breast cancer, but they probably exist in breast cancer. Therefore, multiple mechanisms including miRNAs contribute to the suppression of PTEN and it is necessary to comprehensively investigate these mechanisms for PTEN suppression in breast cancer.

The investigation of PTEN in a specific tissue context is necessary before choosing PTEN as a therapeutic cancer target. As a tumor suppressor, low expression of PTEN was frequently observed in some types of cancers; however, for different types of cancer, the low expression of PTEN was associated with distinct clinical outcomes. Low PTEN expression was significantly associated with short overall survival of patients with lung adenocarcinoma (n=720, P=5.9E-6) or with liver cancer (n=370, P=0.31), while low PTEN expression was associated with longer but not significant increased survival of patients with bladder cancer (n=404, P=0.29) (Supplement Figure 2). These results suggested that low PTEN expression was associated with distinct survival outcomes of cancer patients, which provided a hint that PTEN might act in a different role other than as tumor suppressor in a different tissue context. However, for the distinct subtypes of breast cancer, low PTEN expression in patients with luminal A and luminal B subtypes was associated with short overall survival; in patients with HER2+ and basal subtypes with lymph node metastasis, low PTEN expression was significantly associated with short overall survival. These results were consistent with PTEN tumor-suppressing function in

breast cancer. This function was consistent with my finding that down-regulation of PTEN by miR-498 promoted the migration of MDA-MB-231 cell, a TNBC cell line that is basal-like and derived from a metastatic breast cancer. Taking my findings with what has been reported in literature, it was necessary to undertake a comprehensive investigation of PTEN in specific tissue contexts.

Although in this study, I found miR-498 was a death-prediction marker for overall survival of breast cancer, the clinical significance of miR-498 as a prediction marker for overall survival in other cancers is still in debate. The Keplan-Meier plots illustrate that high expression of miR-498 is associated with short overall survival of patients in breast cancer, pancreatic carcinoma and head-neck squamous cell carcinoma, which supports miR-498 acts as a death marker. However, in liver cancer and bladder caner, high expression of miR-498 is associated with long overall survival of patients, which indicates that miR-498 serves as a survival marker. The differences in outcomes for miR-498 as a marker of survival might be due to the specific expression of miR-498 in different tissue contexts and the regulatory function of miR-498 in regulating other putative targets and regulating downstream pathways in different cancers. Therefore, one should be cautious about using miR-498 as a pragmatically therapeutic target in certain types of cancers, such as liver and bladder cancer. More investigation and assessment of miR-498 should be performed because the oncogenic role of miR-498 remains controversial in many cancers.

Therapeutic translation of miRNA or anti-miRNA (antisense miRNA or miRNA decoy) in breast cancer is still under investigation. Although introducing miRNA or anti-miRNA by viral vector was effective to deliver miRNA or anti-miRNA into cancer cells, problems like an immune response to a virus or insertional mutagenesis restricted their clinical use (Chen Y, 2003;

Boulaiz H, 2005). The miR-498 decoy I constructed in this study, it was capable of being transduced into proliferating cancer cells and it highly expressed long term. However, due to the features of the retrovirus, the sequence of miR-498 decoy would be integrated into the genome of host cancer cells, which might induce insertional mutagenesis. This insertional mutation could result in the inactivation of a tumor suppressor gene or the hyperactivation of an oncogenic gene, which in contrast would promote tumor progression or metastasis. For instance, insertion of miR-498 decoy sequence into the ORF of tumor suppressor TP53, would generate a frame-shift mutation, transcription truncated p53 or mutated p53 mRNA and the loss of its tumor suppressor function. To solve this problem, it is necessary to investigate how to precisely control the insertion of miR-498 into the host cell genome. It is possible to avoid the side effects of genome insertion by guiding the miR-498 decoy sequence to locate at a downstream sequence of the miR-498 promoter, so that, transcription of the miR-498 decoy would follow the transcription of miR-498 without interrupting other functional genes.

Moreover, anti-miR-498, an oligonucleotide containing a complementary sequence of mature miR-498, also had the potential to be used in the treatment for TNBC patients with miR-498 overexpression. Generally, anti-miR-498 can be delivered into cancer cells by liposomes to inhibit overexpression of endogenous miR-498. However, there are some challenges to be overcome for clinical use of anti-miR-498 in breast cancer patients. The stability of naked oligonucleotides (miRNAs or anti-miRNA) was relatively low and was easy to be degraded by nucleases, which resulted in fail to reach its target cancer cells (Mitchell PS, 2008). Also, problems of nonspecific delivery to target cells and low uptake by target cells need to be addressed (Muthiah M, 2013). Recently, advances in nanotechnology offered a promising approach to overcome these issues, where nanoparticles were designed for delivering

oligonucleotides into cancer cells (De Jong WH, 2008; Provenzale JM, 2009). Synthetic miRNA or anti-miRNA oligonucleotides were conjugated to nanoparticles or encompassed by nanoparticles, which gave them resistance to degradation by nucleases (Reynolds AR, 2003); modification of the reactive surface of nanoparticles by a biocompatible coating reduced the immune response (Kumar MNVR, 2004). The surface of nanoparticles was also easily modified by different molecules to enhance the specific delivery (Yuba E, 2008). For instance, the nanoparticle surface modified with carboxyl and amino groups was easily conjugated with proteins, antibodies and carbohydrates, which facilitated the interaction of nanoparticles with the specific ligand on target cells and enhanced the specific delivery of miRNA/anti-miRNA (Davies OR, 2008; Yuba E, 2008). Excitingly, recent studies confirmed the anti-tumor effect of this modified nanoparticle in vitro and in vivo. Antisense miR-21 or antisense miR-10b conjugated PLGA-b-PEG polymer NPs elevated the stability of oligonucleotides in serum and the efficiency of cellular uptake; their function of blocking endogenous miR-21 and miR-10b was confirmed in both TNBC cells and mouse xenograft tumors (Devulapally R, 2015). It was also reported that antisense miR-21 conjugated on the surface of nanoparticles enhanced apoptotic their effect in breast cancer MDA-MB-231 cells and SKBR-3 cells (Bhargava-Shah A, 2016). Therefore, the clinical use of miRNA or anti-miRNA in breast cancer is promising, but it requires more investigation before its prevalent use in breast cancer patients.

4.2 Conclusion

My results supported my hypothesis that PTEN was regulated by microRNA. Among nineteen microRNAs predicted to target PTEN, only miR-498 was capable of binding to the site of 833-855bp on PTEN mRNA 3' UTR and reducing PTEN protein level in BCa cell lines. Next, I found that the expression of the tumor suppressor PTEN was lower both at the protein level and at the mRNA level in TNBC cell lines than in the breast epithelial cell. This is the first report of miR-498 overexpression leading to PTEN suppression and activation of survival pathway PI3K-AKT in TNBC cells. Moreover, miR-498 promoted cell proliferation and cell migration in TNBC cells in a PTEN-dependent manner. Suppressing the miR-498 overexpression impaired the oncogenic effects of miR-498 on cell proliferation and cell migration. Finally, when I designed and used a miR-498 decoy, miR-498 was reduced and PTEN protein level was restored. Furthermore, the oncogenic effects of miR-498 on cell proliferation and cell migration were inhibited by miR-498 decoy in TNBC cells (Figure 4.1).

Generally speaking, the hypothesis in this study was well supported by experimental results. miR-498 was overexpressed in TNBC cells and acted as an oncogenic player by negatively regulating the tumor suppressor PTEN. These oncogenic effects of miR-498 were dependent on PTEN suppression. This study expanded the understanding of miR-498 function and provided new information for the development of novel TNBC therapies.

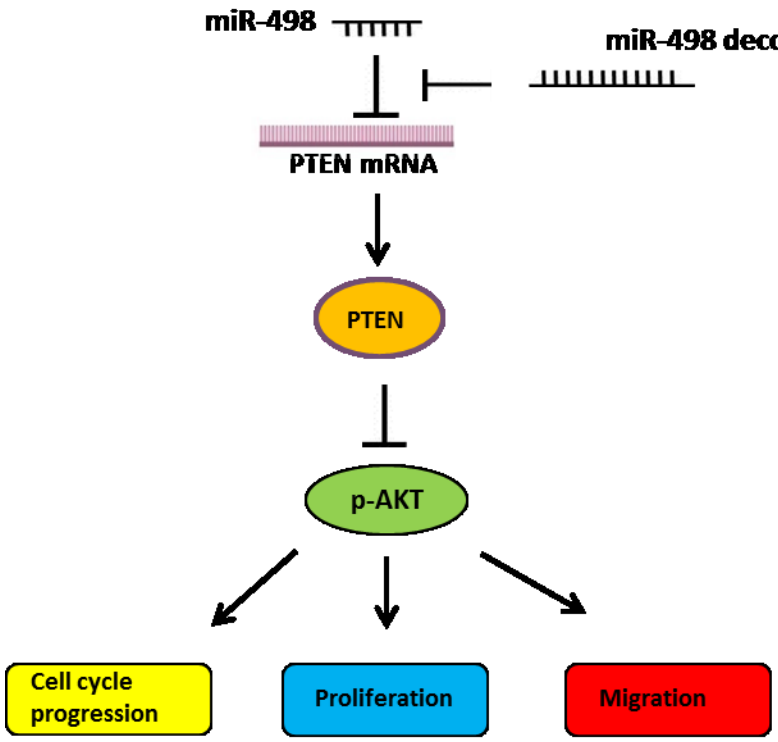


Figure 4.1 A model diagram illustrating that miR-498 suppresses PTEN and activates p-AKT.

The miR-498 binds to the PTEN mRNA and suppresses the tumor suppressor PTEN; suppressed PTEN results in the increment of p-AKT and the activation of AKT pathway; activated AKT promotes the cell proliferation, migration and cell cycle progression in breast cancer cells.

Chapter 5 Future Directions

5.1 PTEN suppression by miR-498 and cell apoptosis

In this study, I screened nineteen predicted PTEN-targeting miRNAs and I determined that miR-498 was overexpressed in TNBC cell lines and suppressed PTEN by directly binding to the 3'UTR of PTEN mRNA. The oncogenic effects of miR-498 on cell proliferation, cell cycle progression and cell migration were also determined. However, there are still some questions on miR-498 that are needed to be addressed.

Results in this study confirmed that miR-498 overexpression led to PTEN suppression and AKT activation which promoted cell proliferation, cell migration and cell cycle progression. However, the effect of miR-498 on apoptosis is still not clear. Previous studies found that phosphorylated AKT acted as a cell survival regulator and inhibited apoptosis (Datta SR, 1999). Specifically, phosphorylated AKT inhibited apoptosis through phosphorylating and sequestrating the pro-apoptotic protein BAD and BIM (del Peso L, 1997; Kelly PN, 2010). AKT also repressed the function of transcription factor FOXO3A by phosphorylation and thereby inhibited the transcription of pro-apoptotic gene Bim, which indirectly prevented apoptosis and promoted cell survival (Zhu S, 2008). The apoptosis that was inhibited by PTEN suppression and AKT activation was reported in multiple cancers, including prostate cancer and colorectal cancer (Persad S, 2000; Luo H, 2013). Since the PTEN suppression by miR-498 was determined in TNBC cell lines, I will examine the effect of miR-498 on apoptosis through suppressing PTEN in TNBC cells in the future investigation. TNBC cell MDA-MB-231will be used as the cell model to observe the effect of miR-498 on apoptosis. The effect of miR-498 on apoptosis will be examined by flow cytometry and immunoblotting. It is expected that miR-498 leads to the reduced expression of pro-apoptotic protein BIM through inhibiting the transcription activity of

FOXO3A and the increased phosphorylated level of BAD through AKT activation in TNBC cells.

5.2 Effect of miR-498 on PTEN protein level in the nucleus and DNA repair

Although the anti-tumor function of PTEN is mainly dependent on its lipid phosphatase activity, PTEN also contributes to its anti-tumor function acting as a transcription factor in cell nucleus (Trotman LC, 2007). In the nucleus, PTEN played an important role in maintaining genomic stability; for instance, double-strand break DNA repair was positively regulated by nuclear PTEN through the transcriptional activation of RAD51, which was a key protein in the double-strand repair (Shen WH, 2007). This function of PTEN did not require its activity of lipid phosphatase. There is convincing evidence to support the conclusion that the absence of PTEN in the nucleus is associated with the aggression of malignant cancers (Trotman LC, 2007; Gimm O, 2000). In my study, I only examined the effects of miR-498 on the suppression of total PTEN and the subsequent AKT activation in TNBC cells. However, whether miR-498 affected the protein level of PTEN in nucleus and DNA repair is still not clear. Therefore, I will investigate the effect of miR-498 on the function of nuclear PTEN. The effect of miR-498 on PTEN in the nucleus and PTEN-dependent DNA damage repair will be assayed in TNBC cells by subcellular fractionation, protein quantification and DNA damage repair analysis.

5.3 Mechanism of miR-498 overexpression in TNBC

Since miR-498 was overexpressed in TNBC cell lines and patient tissues and resulted in the suppression of tumor suppressor PTEN, it was identified as an oncomiR in TNBC (Matamala N, 2016). Unfortunately, the mechanisms that induced the overexpression of miR-498 in TNBC are still not clear. According to prior studies, miR-498 overexpression was observed in human ovarian tumor cell lines; the transcription of miR-498 was activated by the vitamin D receptor (VDR), which bound to the 5'prime regulatory region of the miR-498 genome (Kasiappan R, 2012). Thereby, I will examine whether this mechanism regulating the miR-498 transcription exists in breast cancer cell lines. Furthermore, epigenetic mechanisms will be considered to explain the miR-498 overexpression in TNBC. It was reported that the gene of the oncogenic microRNA let-7a-3 was heavily methylated by DNMT1 and DNMT3B in normal lung tissues but was hypomethylated in some lung adenocarcinomas, which led to the elevated expression of let-7a-3 in lung cancer (Brueckner B, 2007). Thus, the overexpression of miR-498 may be resulted from the hypomethylation of miR-498 genomic DNA. The DNA methylation level at the miR-498 gene will be examined in TNBC cell lines where the miR-498 was overexpressed (MDA-MB-231 and Hs578T) by methylation-specific PCR (MSP). If the unmethylation or hypomethylation of the miR-498 promoter gene is found in TNBC cells, the overexpression of miR-498 will be well explained. Finally, the down-regulation of some ceRNA may also result in the high expression level of miR-498 in TNBC. As mentioned before, circular RNA circFADS2 was found to interact with miR-498 and inhibit miR-498 in lung cancer (Zhao F, 2018). It is also hypothesized that circFADS2 is down-regulated in TNBC and thereby leads to a high level of miR-498. Therefore, if possible, I will examine the expression of circFADS2 by qPCR in TNBC cell lines (MDA-MB-231 and Hs578T) to determine the low expression level of circFADS2.

And then I will use the luciferase assay to examine the interaction between miR-498 and circFADS2 in TNBC cell lines.

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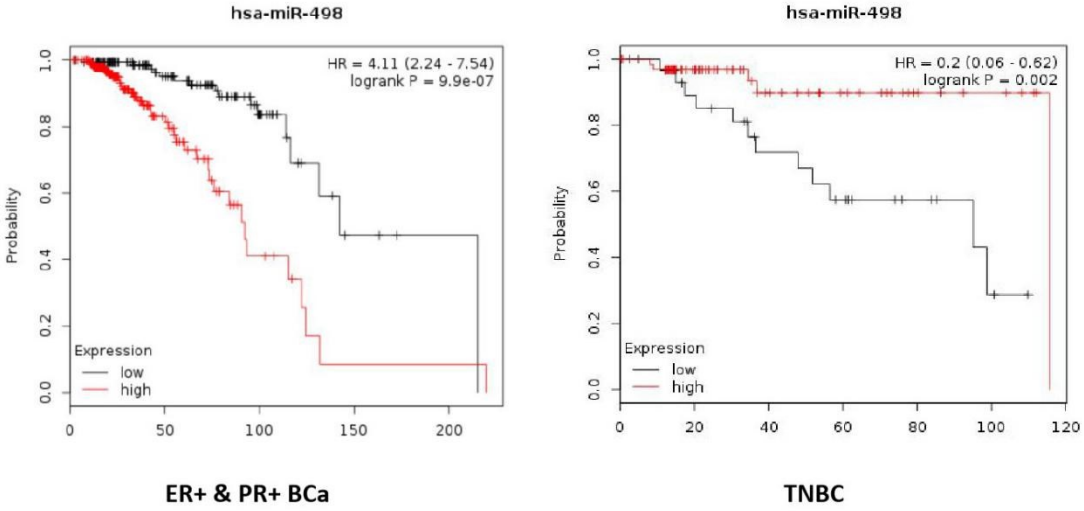
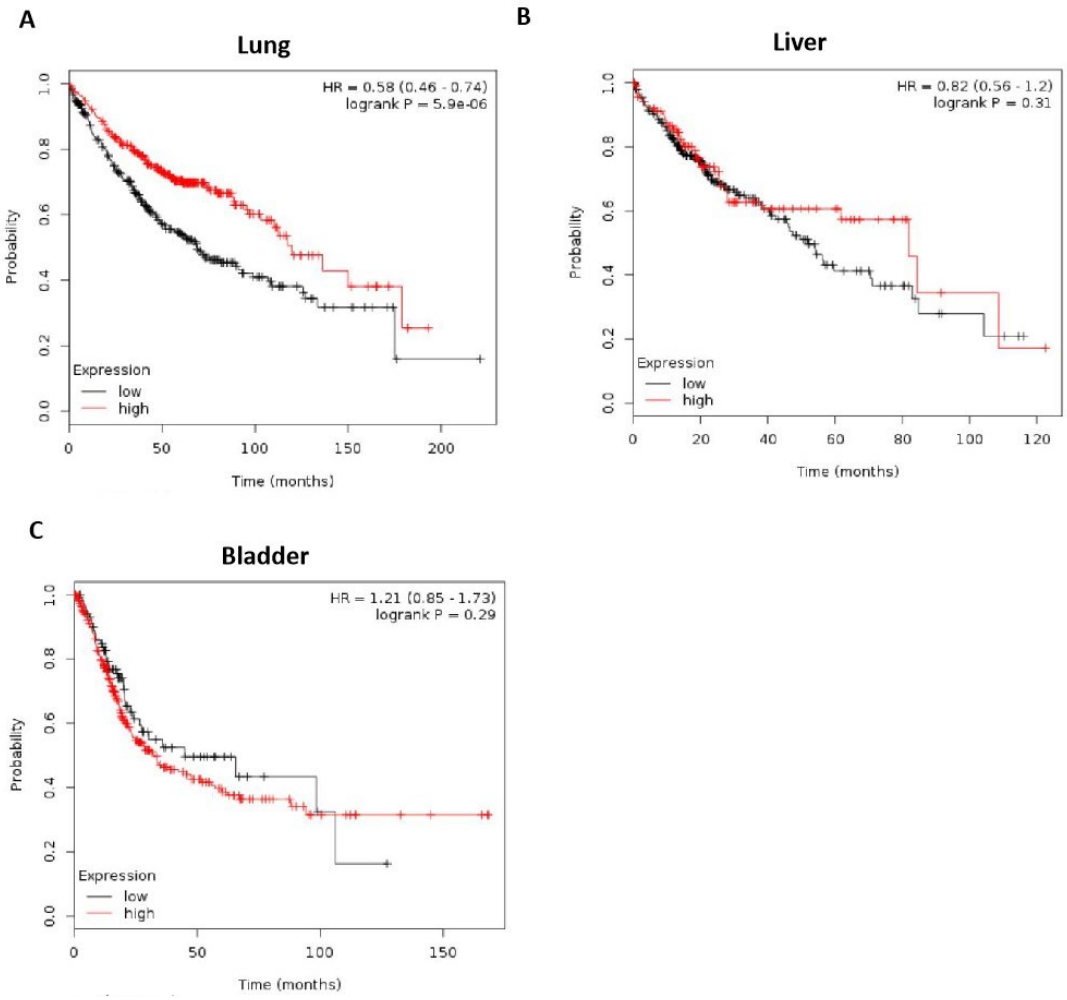
Appendices



Supplement Figure 1 miR-498 was differently associated with overall survival in TCGA database. (Left) In ER+ and PR+ BCa patients, high expression of miR-498 was significantly associated with short overall survival. (Right) In TNBC patients, high expression of miR-498 was significantly associated with long overall survival.



Supplement Figure 2 PTEN expression associated with distinct survival outcomes in different types of cancers. (A) low expression of PTEN in lung cancer patients was significantly associated with short overall survival. (B) low expression of PTEN in liver cancer patients was associated with short overall survival. (C) low expression of PTEN in lung cancer patients was associated with long overall survival.