AMERICAN UNIVERSITY OF BEIRUT

mIR452 FUNCTION IN BREAST CANCER

by
ASMA TOUFIC ZAHREDDINE

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AN ABSTRACT OF THE THESIS OF

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Background: Breast cancer is the most frequently diagnosed life-threatening cancer in women. Young Lebanese women have a higher prevalence of breast cancer as compared to their counterparts in western countries. Various hereditary and environmental factors contribute to the initiation and the progression of the disease. microRNAs (miRNAs) have been shown to play a critical role in the development and the progression of breast cancer and other types of cancer. miRNAs, a class of endogenous small noncoding RNAs that are approximately 18-22 nucleotides in length, function as oncogenes and tumor suppressor genes and are essential for the post transcriptional regulation of mRNA gene expression. Recent data in our lab has shown that miR-452 is significantly downregulated in tumor tissues taken from young Lebanese breast cancer patients as compared to normal adjacent tissues upon performing miRNA microarray.

Aim: Our project aims at studying the role of miR-452 in breast cancer through investigating its potential target genes *(WWP1, CDKN1B, HECTD1, SOX7 and BMI1)* that were derived based on PubMed search, experimentally validated and predicted in silico tools.

Methods: The expression of miR-452 in breast cancer cell lines was measured by Reverse Transcription Quantitative Real Time PCR (RT-qPCR). The level of relative expression of target genes in MCF-cells was validated by RT-qPCR. Modulation of miR-452 expression in MCF-7 cells was done by transfecting miR-452 mimics. MTT assay, Trypan Blue Dye Exclusion Assay and Invasion Assay were used to check for the effect of overexpression of miR-452 on proliferation, viability and invasion in transfected cells.

Results: miR-452 was significantly downregulated in all tested breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-453, BT-474). A strongly significant downregulation of miR-452 was seen in MCF-7 cells which are hormone positive and have similar profile to the young Lebanese breast cancer patients. Overexpression of miR-452 decreased the mRNA levels of target genes. No effect on proliferation and viability of MCF-7 cells was seen upon transfection with miR-452 mimics. However, the invasive capacity of MCF-7 decreased post transfection with miR-452 mimics at the selected time points.

Conclusion: Our study confirms that miR-452 is significantly downregulated and potentially plays a role in breast cancer through regulating the expression of mRNA of selected target genes *WWP1, CDKN1B, HECTD1, SOX7 and BMI,* involved in many cellular processes. Further studies are needed to validate direct interaction between miR-452 and its target genes. Finally, miR-452 can be considered as a promising biomarker that needs further validation and can help in the early screening and detection of this heterogeneous disease.

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ABBREVIATIONS

ASR: Age-Standardized Rate
AJCC: American Joint Committee on Cancer
BMI: B cell-specific Moloney Murine Leukemia Virus Integration site 1
BRCA1&2: Breast Cancer Antigen 1&2 genes
cDNA: Complementary DNA
CEA: Carcinoembryonic Antigen
CK5/6: Cytokeratin 5/6
CDKN1B: Cyclin Dependent Kinase Inhibitor 1B
DCIS: Ductal Carcinoma In Situ
DGCR8: DiGeorge Syndrome Critical
DMEM: Dulbecco's modified Eagle's medium
dNTP: Deoxyribonucleotide triphosphate
ECM: Extracellular Matrix
EGFR: Epidermal Growth Factor
ER: Estrogen Receptor
FBS: Fetal Bovine Serum
FC: Fold Change
FFPE: Formalin Fixed Paraffin Embedded
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HER2: Human Epidermal Growth Factor
HECTD1: HECT Domain E3 Ubiquitin Protein Ligase 1
IARC: International Agency for Research on Cancer
IDC: Invasive Ductal Carcinoma
IHC: Immunohistochemistry
ILC: Invasive Lobular Carcinoma
IGF: Insulin Like Growth Factor
LCIS: Lobular Carcinoma In Situ
M: Mimics
miRNA: microRNA
MKI67: K-i67 gene
MRI: Magnetic Resonance Imaging
mRNA: messenger RNA
MCF-7: Acronym of Michigan Cancer Foundation-7
MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NC: Negative Control Duplex
NRT: No Reverse Transcription Control
NTC: No Template Control
PBS: Phosphate-Buffered Saline
PR: Progesterone Receptor
PI: Propidium Iodide
pre-miRNA: Precursor miRNA
pri-miRNA: Primary miRNA
RPMI: Roswell Park Memorial Institute medium
RT-qPCR: Reverse Transcription Quantitative Real Time Polymerase Chain Reaction
RNU6B: U6 Small Nuclear 6 gene

SEM: Standard Error of Mean
SOX7: SRY-box7
T: Tumor
Ta: Annealing Temperature
TDLU: Terminal Ductal-Lobular Units
TNBC: Triple Negative Breast Cancer
TRI Reagent: Trizol Reagent
UNG: Uracil N-glycosylase
WWP1: WW Domain Containing E3 Ubiquitin Protein Ligase 1
YBC: Young Breast Cancer

CHAPTER I
INTRODUCTION

A. Cancer Overview

Cancer is a leading cause of death worldwide claiming 8 million lives annually (around 15% of all deaths) [1]. Based on estimates from the International Agency for Research on Cancer (IARC) in 2012, there were 14.1 million new cancer cases and 8.2 million cancer deaths reported worldwide. These numbers are expected to grow to 21.7 million new cancer cases and 13 million cancer deaths by 2030 [2]. Cancer is a group of diseases that result from abnormal proliferation of any of the different kinds of cells in the body, so there are more than a hundred types of cancer, which has specific behavior and respond differently to suggested treatment. A tumor can be benign or malignant. Only malignant tumors are referred to as cancer. Cancer can be divided into three main groups (carcinomas, sarcomas, leukemias or lymphomas). Carcinomas constitute 90% of human cancers and are malignancies of the epithelial cells. Leukemias account for approximately 8 % of human cancers and are malignancies of blood forming cells and cells of the immune system. Sarcomas constitute around 2 % of human cancers and are solid tumors of connective tissues such as fibrous tissue, muscle, bone, and cartilage. Cancer is further classified based on the tissue origin and the type of cells involved (i.e. Breast carcinoma). The development of cancer is a multistep process in which cells gradually become malignant through a progressive series of alterations involving mutation, progressively increasing capacity for proliferation, survival, invasion, and metastasis. Malignant cells secrete proteases and

collagenases to digest extracellular matrix components facilitating the invasion of adjacent normal tissues and to penetrate through basal laminae to invade underlying connective tissue. Moreover, cancer cells secrete growth factors to promote angiogenesis (formation of new blood vessels) to support the growth of the tumor and enhance metastasis to different body sites [3]. We are interested in studying breast carcinoma in specific at its molecular level.

B. Breast Carcinoma:

1. Prevalence of Breast Cancer:

a. Worldwide:

Breast cancer is the second most common cancer worldwide after lung cancer, the fifth most common cause of cancer death, and the leading cause of cancer death in women with an estimated 1.7 million new cases and 521,900 deaths in 2012 [4] (Figure 1). The incidence rates of breast cancer are increasing leading to a global burden. The incidence rate of breast cancer is about 120 in 100,000 women in the United States with a 5 year survival rate dropping from ~99% for Stage I patients, to ~27% for Stage IV disease, and thus necessitates early detection [5]. Yap et al mentioned that breast cancer is an increasing health problem in East Asia where the incidence of breast cancer has been rising dramatically over the past few decades. The incidence of breast cancer in younger age groups in several Asian countries has even surpassed that in the United States highlighting an urgent need for prevention and treatment [6]. The peak age of Asian BC is much younger than that in western countries such as the United States, as approximately half of the Asian breast cancer

patients were pre-menopausal whereas only 15−30% of Western breast cancers were pre-menopausal [7, 8]. Breast cancers arising in younger breast patients (YBC) are known to be more aggressive with increased risk of relapse and mortality [9]. Higher proportions of triple negative/basal-like and HER2 subtypes but lower proportions of ER+/Luminal A subtypes were reported in young breast cancer cases than in older breast cancers and usually go with poor prognosis [10]. This sheds the importance on early detection and cutting off this drastic increase of breast cancer incidence.

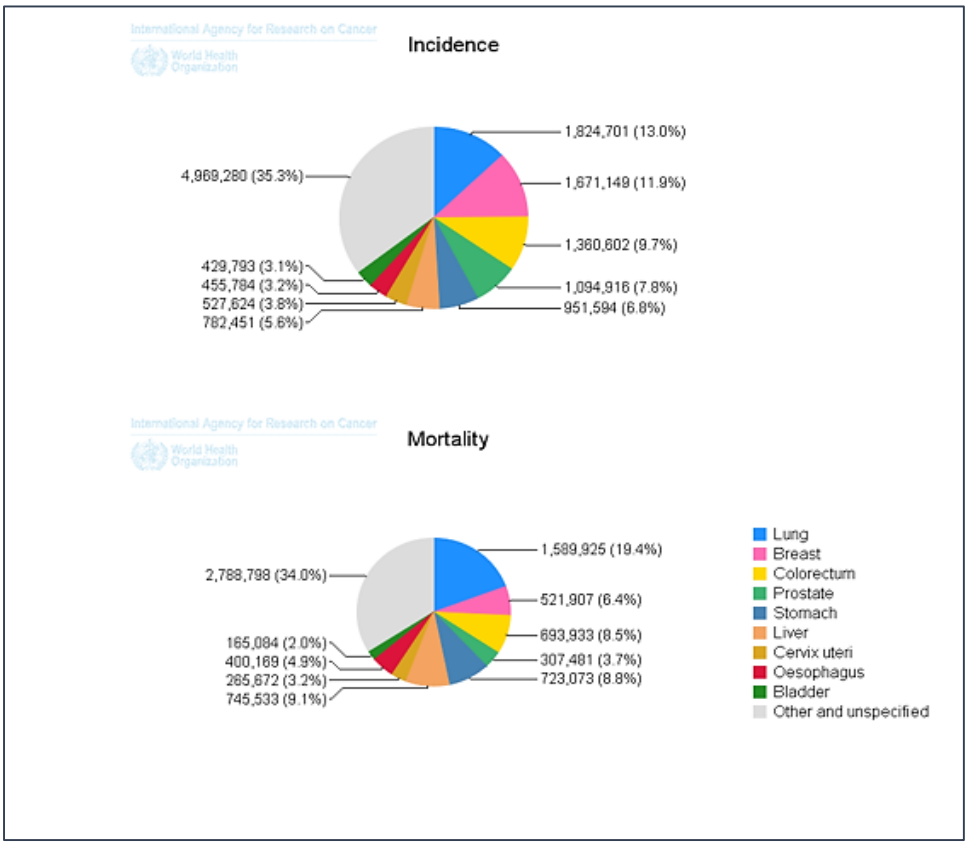

Figure 1 Breast Cancer Incidence and Mortality Worldwide in 2012 (GLOBOCAN 2012). Adapted from [4].

b. Lebanon:

Breast cancer is the leading cause of cancer death among females in Lebanon. Breast cancer accounted for an average of 37.6% of all new female cancer cases in Lebanon during the period of 2004-2010. Moreover, five-year age-specific rates among Lebanese women between 35 and 49 years were among the highest observed worldwide in 2008 [11]. Age Standardized Rate (ASR) for breast cancer remained lower than that observed in developed countries but was substantially higher than in other developing countries of the region or the non-Jewish population. This increase is attributed, in part, to the adoption of screening programs and awareness campaigns that introduce people to risk factors and early signs of breast cancer [12, 13]. Cancer screening methods such as mammography improved cancer detection rates. [14, 15]. However, mammography is not recommended for women less than 40. Almost 50% of the breast cancer patients in Lebanon are below the age of 50 years among which 22% of the cases fall in the age category below 40 compared to around 6% in Western Population[14, 15](Table 1).

Age group	US (%)	Lebanon (%)
<50	25	50
<40	6	22

Table 1 Estimated New Female Breast Cancer Cases by Age in US versus Lebanese Population.

Lebanese women who are diagnosed at an age <35 years presented a more aggressive disease accompanied by a poor survival rate [16]. Based on a cohort of 250 patients with breast cancer and who are at high risk of carrying inherited gene mutations (young age and/or positive family history of breast and/or ovarian cancer), it was reported that the prevalence of *BRCA1* and *BRCA2* (Breast Cancer Types 1 and 2) deleterious mutations contributed to 5.6% [17]. Since there is a rise in the incidence of breast cancer among young Lebanese patients with a slight contribution of *BRCA1* and *BRCA2* mutations, we are interested in studying other biological factors behind breast cancer.

2. *Breast Anatomy and Tumor Initiation*

The knowledge of mammary gland development and mammary stem cell biology has significantly contributed to the understanding of breast cancer. A mammary gland is a complex secretory organ composed of a number of different cell types: epithelial cells, adipocytes, vascular endothelial cells, fibroblasts and immune cells. The mammary epithelium is comprised of two main cell types: luminal (that forms ducts and secretory alveoli and contains populations of cells defined by their hormone receptor status) and the basal (that consists of myoepithelial cells, which generate the outer layer of the gland, and a small population of stem cells).

Breast development involves three main stages: embryonic, pubertal, and reproductive [18]. During embryogenesis, the breast buds, in which networks of tubules are formed, are generated from the ectoderm [19]. These rudimentary tubules will eventually become the matured lactiferous ducts which connect the lobules and the

clusters of alveoli to the nipples [20]. Until puberty, the tubule networks of the breast buds remain rudimentary and quiescent [21]. Puberty initiates branching morphogenesis that requires growth hormone and estrogen, as well as Insulin Growth Factor 1 (IGF1), to create a ductal tree that fills the fat pad. The lateral branches give rise to the basic functional unit of the breast called terminal ductal-lobular units (TDLU) that constitutes of terminal ducts and lobules with numerous secretory ductules, called acini [22]. Each acinus is made up of a luminal epithelial cell layer and covered with an outer layer of myoepithelial cells. This layer of myoepithelial cells is also responsible for creating and maintaining the basement membrane which is a specialized structure composed of collagen IV, laminin and proteoglycans. The basement membrane is surrounded by a stromal extracellular matrix, comprised predominantly of collagen I [23]. Progesterone and prolactin work together upon pregnancy to generate milk producing alveolar cells. During lactation, alveolar luminal epithelial cells secrete milk upon the contraction of myoepithelial cells surrounding them. Lack of demand for milk at weaning initiates the involution process in which the gland is remodeled back to its pre-pregnancy state [18]. The mammary gland is characterized by cellular plasticity, a factor that increases its susceptibility to carcinogenesis. In situ carcinoma involves the formation of tumor within the lobule and/or ducts. It spreads within these interconnected structures and can progress to invasive breast cancer if left untreated. Epithelial Mesenchymal Transition (EMT) precedes lymphovascular invasion and metastasis in which the cells lose their epithelial characteristics and acquire mesenchymal characteristics such as motility and invasiveness. Cancer cells can detach from their primary site, degrade and remodel the ExtraCellular Matrix (ECM), enter the systemic circulation, establish

contacts with the endothelium, adhere to the vascular walls and finally transmigrate across the endothelial layers as single cells or clusters leading to a metastatic tumor that can disseminate to other parts of the body[24] [25-28] . The severity of breast cancer disease raises our concern to emphasize on risk factors and early detection methods.

3. Breast Carcinoma Risk Factors and Prevention

a. Modifiable:

The adoption of a westernized lifestyle in recent generations has been suggested as a major cause of breast cancer. In addition, the higher exposure to environmental pollutants with estrogenic effects among East Asian women may have also contributed to the increasing incidence [29]. Among the lifestyle factors that contribute to breast cancer development, physical inactivity, obesity, excessive alcohol use and smoking stand out as modifiable risk factors, which if avoided, could help in the prevention and management of breast cancer [30, 31]. Physical activity appears to reduce the risk of breast cancer through a number of mechanisms, including body fat reduction, which in turn reduces estrogen and insulin concentrations that have mitogenic effects on mammary cells [32, 33]. A recent study by Nickel et al. illustrated that the factors secreted by adipocytes have a significant impact on the molecular biology of breast cancer cells. Co-culture of triple-negative MDA-MB-231 cells with adipocytes led to the increase of the migratory capacity of this cell line and to the induction of pro-inflammatory genes mainly the genes of the

NF-κB signaling pathway [34]. Other modifiable risk factors include radiation exposure and the use of hormone replacement therapy[35].

b. Non Modifiable:

In addition to the modifiable risk factors mentioned earlier, non-modifiable risk factors include age at menarche and menopause, gender in which females are more prone to have breast cancer than males, genetic factors (5-7%), family history of breast cancer, history of previous breast cancer and proliferative breast disease [36]. Menopausal women can have high levels of estrogen rendering them more prone to breast cancer[37]. Moreover, a study conducted by Nindrea et al. showed that subjects with a family history of first-degree relatives including sisters, mothers or children with breast cancer had a higher risk than those who do not have any family history[36]. Breast cancer is the result of an accumulation of a large number of individual genetic mutations that collectively change the elements of the complex internal signaling system of a cell [38]. The most common hereditary mutations encountered involve *BRCA1* and *BRCA2* genes. *BRCA*1/2 mutations occur more commonly in women with early onset or a suggestive family history [39]. It was estimated that a mutation of *BRCA1* raises the risk of breast cancer to 51% and 85% by the age 50 and 70 years respectively[40].

4. Methods for Breast Cancer Detection and Prevention

Several early detection measurements of breast cancer have been taken in Lebanon and other countries. The most common used screening test is mammography. It is less effective in younger women who have a genetic predisposition to breast cancer

and have denser breasts. Magnetic resonance imaging (MRI) has been introduced for familial breast cancer screening programs in recent years and was intended to improve outcomes in these women. There appears that *BRCA2* carriers benefit from screening with MRI [41]. Moreover, cancer antigen 15-3 (CA 15-3) and carcinoembryonic antigen (CEA) act as prognostic biomarkers that may be found in the blood of people with breast cancer [42].

As for the preventive measurements taken in Lebanon, it has been recommended that a woman has to undergo a breast self-examination (BSE) every month starting at age 20, and a clinical breast examination (CBE) performed by a physician every three years starting age 20 and every year at age 40. An annual CBE and mammography starting age of 40 are also recommended once per year in Lebanon [12, 13].

5. Breast Cancer Classification

Most of the breast malignancies are adenocarcinomas and contribute to more than 95% of breast cancer cases [43]. Breast cancer is a heterogeneous tumor that possesses several subtypes characterized by different biological behaviors, immunopathological and molecular characteristics. Breast cancer can be broadly categorized into two types: in situ carcinoma and invasive (infiltrating) carcinoma. Breast carcinoma in situ is further sub-classified as either ductal or lobular based on the tumor origin and on the level of expression of E-cadherin. E-cadherin is a transmembrane glycoprotein that is involved in epithelial cell-to-cell adhesion and in the regulation of morphogenesis[44-46]. It can act as a phenotypic biomarker in which its absence of expression is more commonly encountered in lobular type of tumors[47].

a. In situ carcinoma:

About one fourth of all newly identified cases of breast carcinoma are diagnosed as breast ductal carcinoma in situ (DCIS) [48]. DCIS is an intraductal neoplastic proliferation of epithelial cells that is separated from the breast stroma by an intact layer of basement membrane and epithelial cells. DCIS is a non-obligate precursor of invasive breast cancer, and it is estimated that up to 40% of these lesions progress to invasive disease if left untreated [49]. The rapid increase in the incidence of DCIS is due to the introduction of mammography screening as the majority of DCIS lesions are detected upon biopsy of mammographic microcalcifications [50]. The vast majority of patients with DCIS are still subjected to surgical treatment followed by radiation and/or prophylactic systemic therapies [49].

The second type of in situ carcinoma is Lobular Carcinoma in Situ (LCIS). LCIS is a lesion with low malignant potential that represents abnormal tissue growth in the lobules (milk secreting tissues of the breast). LCIS is not considered precancerous as DCIS. It can develop with time into invasive type of cancer. The incidence of invasive breast cancer from diagnosis of LCIS at 10 years is 7%. In 2008, lobular carcinoma constituted 11% of all invasive breast cancer diagnosis [51]. E-cadherin staining can help differentiate between LCIS and DCIS in which LCIS cells show lack of E-cadherin that lead to their growth in a diffuse pattern [47]. As for the receptor status, estrogen and progesterone receptors are present and HER2/ neu overexpression is absent in most cases of LCIS [52].

b. Invasive (Infiltrating) Carcinoma (Figure 1a):

Invasive ductal carcinoma (IDC) and Invasive Lobular Carcinoma (ILC) are the two main histological subtypes of breast cancer (Figure 2a). IDC is the most common histologic type of invasive breast cancer contributing to 40-75% of the cases[53]. IDC is classified into many histological subtypes according to a wide range of criteria, including cell type (as in apocrine carcinoma), amount, type and location of secretion (as in mucinous carcinoma), architectural features (as in papillary, tubular, and micropapillary carcinoma) and immunohistochemically profile (as in neuroendocrine carcinoma) [54] [55]. However, ILC accounts for 10–15% of all breast cancers and is characterized by small and round tumor cells growing in stroma [56]. ILC is more prevalent at an older age and in an advanced cancer stage[57, 58]. It is characterized by certain clinical and histological features such as growth, difficulty in detection, and frequent late recurrences due to the lower rate of protein translation and metabolism and the lower detection rate in mammography and PET scanning. ILC has been shown to have a higher activity of almost all types of immune cells based on cell type-specific signatures compared to IDC [59]. E-cadherin loss is the best known genetic hallmark of ILC in which it is present in 90% of ILC cases[47].

Immunohistochemistry (IHC) markers including Estrogen Receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are classically used for breast tumor subtyping and can indicate the therapy outcome [60] (Figure 2b). 70-80% of all breast cancers are positive for ER or PR. However, HER2 protein overexpression is seen in approximately 15–20% of the patients only. Triple negative breast cancer (TNBC), defined by a lack of expression of both ER and PR as

well as HER2 receptors constitute the remaining 10–15% of breast cancers and is associated with a worse prognosis since it does not respond to hormonal therapy and cannot be treated by drug that targets HER2 protein [61].

A class of inhibitors targeting the enzyme poly ADP ribose polymerase (PARP) has shown greater efficacies in treating tumors deficient in homologous recombination repair pathways, such as those harboring *BRCA1* or *BRCA2* mutations. Taken together with the observation of increased *BRCA1/BRCA2* mutation frequencies in younger BCs, these findings raised the possibility that PARP inhibitors could be more effective in treating younger Asian BCs particularly within the TNBC subtype [10].

The molecular profile of a tumor helps in taking therapeutic decisions and establishing more precise prognosis of oncology patients [62]. "Molecular Classification" terminology was proposed by Perou and Sorlie for the first time in 2002 after which a comprehensive study was conducted and showed the differences in mRNA gene expression. This molecular classification was divided into five subtypes associated with different treatment: (a) Luminal A (low proliferative subtype) (Figure 2c): ER/PR positive and Her2 negative, (b) Luminal B (high proliferative subtype): ER and/or PR positive and Her2 positive, (c) Her2neu subgroup – ER,PR negative and Her2 positive, (d) Basal like – ER, PR and Her2 negative, cytokeratin (CK) 5/6 positive and/or Epidermal Growth Factor Receptor (EGFR) positive, and (e) normal like that resembles normal tissue and goes with good prognosis [63, 64]. The basal type of BC does not respond to hormonal and trastuzumab-based treatment [62]. Luminal B cancers

generally grow slightly faster than luminal A cancers and their prognosis is slightly worse [65].

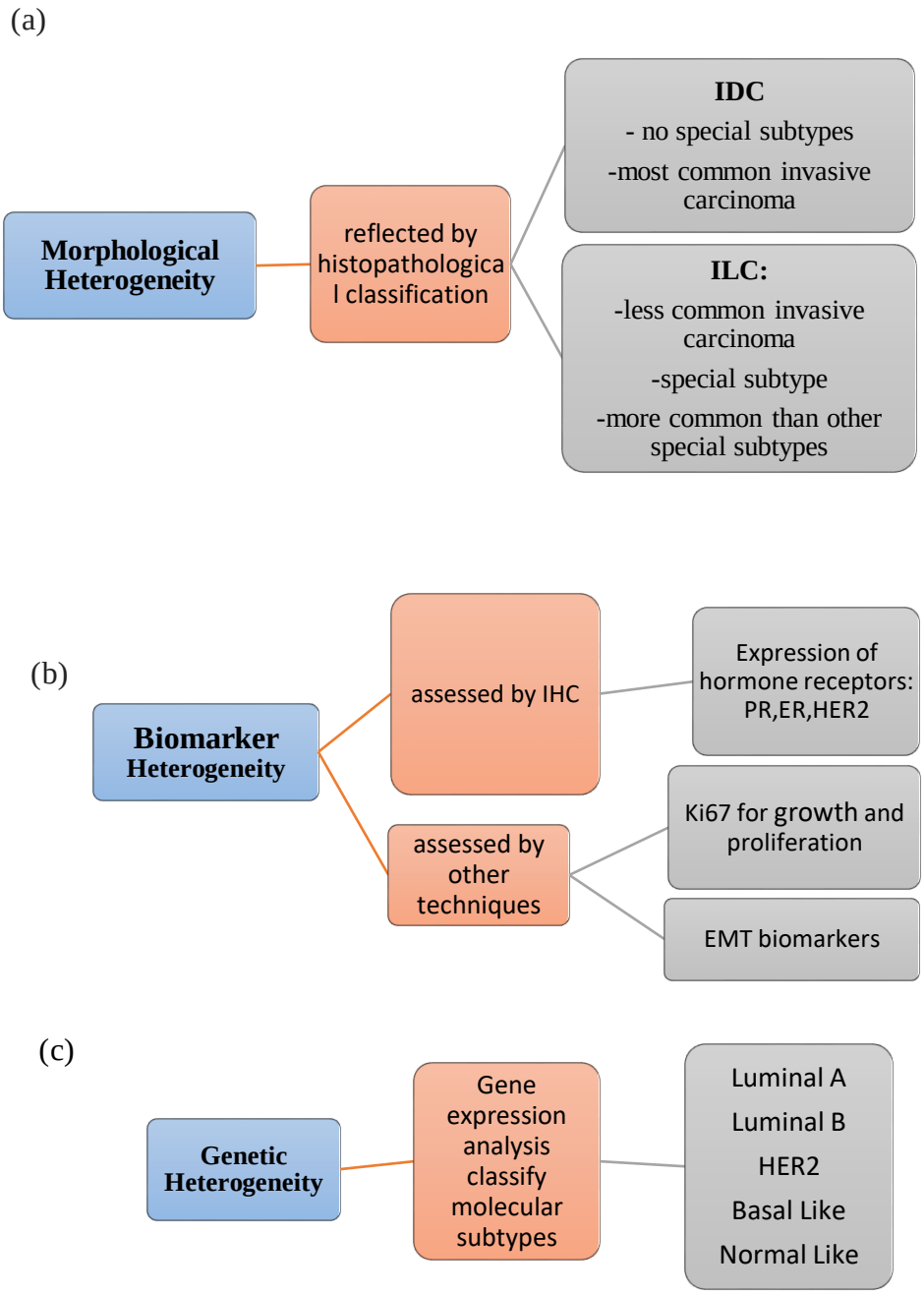

Figure 2. Intertumor Heterogeneity of Breast Cancer Divided into Morphological (a), histological (b) and Genetic Factors (c).

6. Breast Cancer Detection and Prognosis

The treatment of breast cancer usually require surgical excision (lumpectomy or quadrantectomy), radiation to the tumor and the implementation of appropriate adjuvant therapy (chemotherapy and/or targeted therapy)[66]. Immunohistochemistry (IHC) markers, together with clinicopathological variables such as tumor size, tumor grade, axillary lymph node involvement, hormone receptor status, HER2/ neu status and surgical margins, have been used for prognosis, prediction and treatment selection [67, 68].

a. Breast Cancer Stage

The intertumor heterogeneity of breast cancer is illustrated by the clinical staging. The most often staging system used for breast cancer is the American Joint Committee on Cancer (AJCC) TNM system[69] (Table 2).

Breast Cancer Clinical Stage	
(T): Tumor Size	T0: no primary tumor T1: 2cm or < 2cm T2: >2cm but <5 cm T3: >5 cm T4: tumor growing into chest wall or skin.
(N): Nodal Involvement	**N0:** no spread to nearby lymph nodes **N1:** Cancer has spread to 1 to 3 axillary lymph node(s) and/or internal mammary lymph nodes. **N2:** Cancer has spread to 4 to 9 lymph nodes or enlarged the internal mammary lymph nodes. **N3:** extensive lymph node involvement
(M): Metastasis	M0: no metastasis M1: distant metastasis

Table 2. Breast Cancer Stages Based on American Joint Committee on Cancer (AJCC) TNM system

a. Hormone Receptor Status

The expression of hormone receptors by tumor cells can help in the decision of therapy taken. Hormone receptor negative breast cancers ER-/PR- do not respond to hormonal therapy; however, hormone receptor positive breast cancer cells respond well to hormonal therapy in which hormonal drugs can lower the level of estrogen or can stop estrogen from acting on breast cancer cells. Moreover, hormone receptor-positive cancers tend to grow more slowly, have better outcome but can sometimes come back many years after treatment as compared to those that are hormone receptor-negative [69].

b. HER2/ neu Status:

HER2 is an oncogene which is involved in proliferative and anti-apoptosis signals and is the major driver of tumor development in breast cancer[70]. Breast cancer cells with higher than normal levels of HER2 receptors are called HER2-positive cells. Invasive breast cancers should be tested for HER2 as HER2 cells grow fast. Breast cancers that are HER2 positive tend to respond to drugs that target HER2 such as Herceptin [69].

c. Ki67 staining:

The immunohistochemistry of Ki67 (also known as MKI6) which is a nuclear protein that is associated with proliferation, is also performed as a prognostic method to differentiate luminal A from luminal B subtypes. Luminal A subtypes result in low Ki67 scoring while luminal B subtypes result in high Ki67 scoring [71].

In addition to the prognostic factors mentioned earlier and in order to predict the risk of recurrences, certain multigene prognostic tests are performed to check for late

recurrences in ER positive breast cancers[72]. First-generation prognostic signatures (Oncotype DX, MammaPrint, Genomic Grade Index) are substantially more accurate to predict recurrence within the first 5 years than in later years. However, the usage of the latter has become a limitation with the availability of effective extended adjuvant endocrine therapies. Newer tests (Prosigna, EndoPredict, Breast Cancer Index) appear to have a better prognostic value for late recurrences while also remaining predictive of early relapse[73] [72].

C. microRNA:

miRNAs are a class of endogenous small noncoding RNA molecules (18-22 nucleotides in length) that control post transcriptional gene expression in several cellular processes[74]. miRNA can act as a biomarker and provide a potential tool for the diagnosis and prognosis of human cancers [75].

1. miRNA Biogenesis

miRNA biogenesis in the human cell is a multistep complex process. It begins in the nucleus where miRNA genes are transcribed by RNA polymerase II to form large capped and polyadenylated primary miRNA transcripts (pri-miRNAs). RNase III enzyme Drosha, coupled with its binding partner DGCR8 cleaves pri-miRNAs into 70–90 nucleotide precursors (pre-miRNA) which consist of an imperfect stem-loop hairpin structure. Then, Exportin-5 transports pre-miRNAs from the nucleus into the cytoplasm in a Ran-GTP-dependent process. Dicer and its binding partner, the transactivator RNA-binding protein TRBP cleave the hairpin precursors into a small dsRNA duplex that

contains both the mature miRNA strand and its complementary strand. Only the single stranded mature miRNA is preferentially incorporated into a miRNA-associated RNA-induced silencing complex (miRISC) to carry out its function. RISC targets complementary mRNA sequences, exerting its functionality via mRNA cleavage if there is a perfect pairing between the miRNA and the mRNA target or translational repression if there is imperfect or partial pairing between the miRNA and the mRNA target (Figure 3) [76, 77].

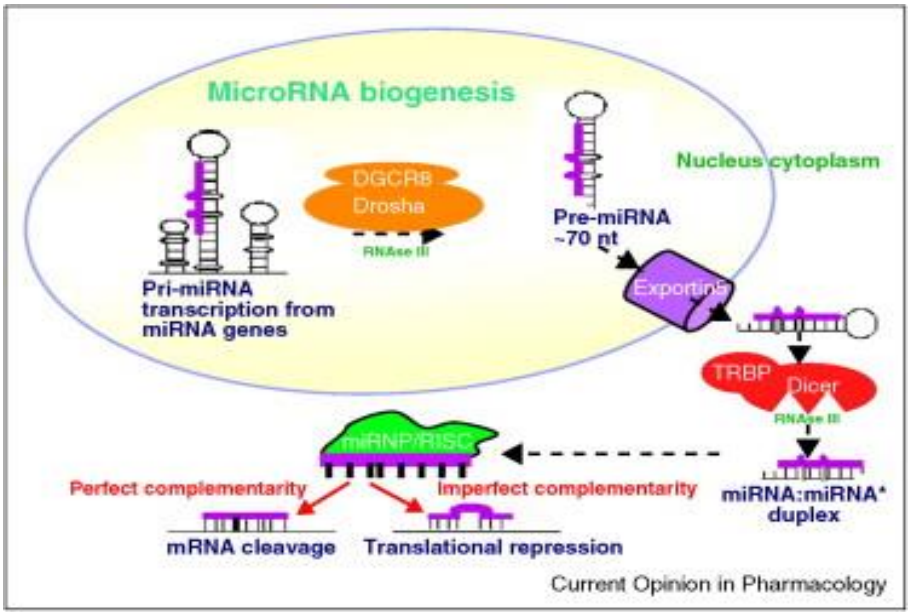

Figure 3. Regulatory Pathway of miRNA .Adapted from [78].

2. miRNA Function and Regulation

More than 2000 distinct human miRNAs capable of targeting thousands of genes have been identified [79]. miRNAs are small noncoding RNAs that binds to the

3′ untranslated region (UTR) of mRNA and can effectively reduce their translation. miRNAs have been shown to play a role in normal biological processes. For example, miR-124a and miR-9 have been shown to have an effect on neural lineage differentiation while miR-223 governs granulocytic differentiation[80, 81].Moreover, miR-1 and miR-133a are involved in skeletal muscle development[82]. On the other hand, miRNAs regulate oncogene and tumor suppressor gene expression and studies have shown that miRNA gene loss or mutation play a significant role in tumorigenesis.

3. miRNA and Cancer:

miRNAs are dysregulated in cancer and involved in the whole process of tumorigenesis[83]. The first correlation between miRNA and cancer was determined in Chronic Lymphocytic Leukemia (CLL) in which the homozygous deletion of mir-15a and mir-16-1 led to overexpression of the targeted mRNAs resulting in tumor proliferation and invasion. Then, it has been shown that miRNA is correlated with other types of cancer[84]. miR-143 and miR-145 were shown to be downregulated in lung cancer while miR-17 and miR-92 were upregulated in B-cell lymphoma, lung cancer and T-cell Acute Lymphoblastic Leukemia[85-88]. Moreover, It has been shown that dysregulation of specific miRNAs leads to drug resistance in different cancers and that the modulation of these miRNAs using miRNA mimics or inhibitors can normalize the gene regulatory network and signaling pathways and revert resistance [73].

4. miRNA as a Potential Biomarker? Why?

miRNAs are present in clinical samples such as formalin-fixed paraffin-embedded tissues, serum, plasma, urine, and saliva. The strong correlation between miRNA expression and patient prognosis implies that miRNAs may ultimately prove to be valuable cancer diagnostic analytes [75]. Moreover, miRNAs have unique characteristics in which their stability, tissue specificity, ease of detection and manipulation will aid in determining individualized cancer treatment [78]. As mentioned earlier, there is a significant percentage of young breast cancer patients in Lebanon upon which their mammography results are not accurate and they are presented with an aggressive state of the disease. This emphasizes the importance of studying the biological mechanism that lies behind breast cancer and the discovery of new biomarkers for early detection of that heterogeneous disease.

5. miRNA and breast cancer:

The dysregulation of miRNAs in breast cancer was first detected in 2005 by Iorio et al [89]. microRNAs have been shown to modulate tumor suppressor and oncogenic pathways; hence, playing a significant role at different stages of breast cancer and acting as regulators of cell cycle, progression, apoptosis, angiogenesis, epithelial to mesenchymal transition, invasion and metastasis [90]. Several studies have shown the role of miRNAs in understanding breast cancer which further describes how miRNA profiling can play a better role than mRNA in molecular subtyping of breast cancer.

miR-221 and miR-222 were shown to play an oncogenic role in breast cancer [91] . miR-10b promoted motility and proliferation by increasing FUT8 and activating AKT in breast cancer cells[92]. miR-148b was found to be a potential biomarker for young breast cancer patients and miR-155 was shown as a significant biomarker for menopausal state, PR and HER2 status while miR-10 b was correlated with ER and PR status among Lebanese patients[93]. It has been shown that miR-183-5p exerts oncomiRs effects in breast cancer, and may have broad impacts on the field of using antimiRs as anti-cancer drugs for breast cancer [94]. Moreover, miR-126 regulated breast cancer cell invasion by targeting ADAM9 [95]. miR-452 has been shown to play an important role in cancer initiation and progression. Its function is illustrated briefly in the following paragraph and its contribution to breast cancer is of our main interest. miR-452 has been shown in our laboratory to be significantly downregulated among young Lebanese women.

6. miR-452

miR-452 has been studied in several types of cancer. The expression of miR-452 in malignant tumors is controversial [96]. miR-452 is downregulated in several types of cancer such as non-small cell lung cancer, prostate cancer(PCa), breast cancer and glioma [97-99]. For example, miR-452 was significantly downregulated in PCa tissues in which transfection with mature miR-452 inhibited the migration and invasion of PCa cells[100]. Moreover, miR-452 is downregulated in pancreatic cancer in which its overexpression suppressed migration and invasion in pancreatic cancer cells. However,

this miRNA was highly expressed in esophageal cancer, urothelial carcinoma and hepatocellular carcinoma[101-103] .

miR-452 target genes are involved in the 'regulation of transcription', 'nucleoplasm', 'protein binding' and 'cell cycle' pathways [104]. The significant function of miR-452 mentioned in the latter accompanied by the results that was achieved in our laboratory, in which miR-452 is downregulated in young Lebanese breast cancer patients, stimulated our interest in studying the molecular function of miR-452 in breast cancer which can also render it as a promising biomarker in the future.

D. Aim of the Study:

miR-452 has been proven recently in our laboratory to be significantly downregulated in young Lebanese women as compared to normal adjacent tissues upon performing miRNA microarray. Lebanese breast cancer patient under age 40 are not recommended to undergo mammography examination; hence, another detection method is needed, as well as studying the biological factors of early breast cancer initiation and progression. Many miRNAs were found to be dysregulated in Lebanese breast cancer patients. For this study, we chose to focus on miR-452. We will test the functional role of miR-452 by investigating its potential targets that have been chosen based on their role in breast cancer. Functional assays such as cell cycle, cell proliferation, viability and invasion assays will be performed on transfected MCF-7 cells with miR-452 mimics. The effect of overexpression of miR-452 on the selected target genes will be tested and validated using reverse transcription quantitative

polymerase chain reaction. In this way, the function of miR-452 will be determined by emphasizing how molecular factors can contribute to the heterogeneity of breast cancer and how miR-452 can act as a promising biomarker and therapeutic agent.

II. MATERIALS AND METHODS

A. Cell culture

Breast cancer cell lines (MCF-7, MDA-MB-453, MDA-MB-231, BT-474 and AU565) were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with 10% fetal bovine serum (FBS) (Sigma Aldrich, St Louis, MO), 1mM sodium pyruvate (Sigma Aldrich, St Louis, MO), 100U/ml penicillin/ streptomycin (Lonza, Belgium) and Kanamycin (0.1 mg/ml) (Amresco, USA). Non-neoplastic MCF-10A human mammary epithelial cells were cultured and maintained in DMEM/F12 (Sigma Aldrich, St Louis, MO) supplemented with 5% Horse Serum, 1 µl /500 ml EGF(20ng/ml), 25 µl Hydrocortisone (0.5 ng/ml), 5 µl cholera toxin (100 ng/ml), 50 µl insulin (10 ng/ml) and 500 µl Penicillin/ Streptomycin (100U/ml). Cells were maintained at 37 °C with 5% CO2.

B. Total RNA Extraction for cDNA Synthesis

Pellets of breast cancer cells grown in culture were used for RNA extraction. The media was removed and cells were washed with 5 ml 1xPhosphate Buffered Saline (PBS). 5 ml 1x PBS was added to scrape the cells. Another 5 ml 1xPBS was added to

further scrape. Then centrifugation was done at 900 rpm for 5 minutes. Pellet was washed with 1x PBS and stored at -80ºC for RNA extraction.

Total RNA was extracted from the cells using the TRI Reagent (Sigma Aldrich) according to the instructions provided by the manufacturer. 1ml of TRI Reagent was added to the cells and cells were incubated for 5 minutes for complete dissociation of the nucleoprotein complex. Then 200 µl of chloroform was added per 1ml of TRI Reagent to each sample, mixed for 15 seconds and cells were incubated for 2 minutes. Cells were spun at 12000xg for 15 minutes at 4°C in which the phases were separated into: a lower red phenol chloroform phase, an interphase and a colorless aqueous phase. The tube was angled at 45°C and the aqueous solution containing RNA was retrieved by being transferred into a new tube. The remaining phases were discarded. 500 µl of 100% isopropanol was added, vortexed and followed by incubation at room temperature for 10 minutes and cells were spun at 12000 xg for 15 minutes to collect the RNA pellet. The supernatant was removed. Two washes with 75% ethanol in DEPC treated water were further done in which each was followed by centrifugation at 7500 xg for 5 minutes at 4ºC. Supernatant was removed and tubes were kept open for 5 minutes to get rid of ethanol residues. Then 40 µl of RNase-free water was added to the pellet. Tubes were incubated at 55 °C on a heat block for 15 minutes. The optic density 260nm of each sample was read using Denovix (DS11 Spectrophotometer). Blank was set using DEPC water. Each sample of volume 2 µl was added to the Denovix stage and gave a ratio 260nm/280nm between 1.8 and 2.1 and met the criteria needed. Samples were then stored at -80°C and were processed later for cDNA synthesis.

C. cDNA using Multiplex PCR to be Used in RT-qPCR for miRNA Expression

Specific dilutions were performed so that a total of 10 ng is reversely transcribed. TaqMan® microRNA Reverse Transcription Kit was used according to the manufacturer's guidelines (Applied Biosystems). Multiplex cDNA master mix was prepared on ice. 2 µl DEPC treated water, 0.1 µl 100mM deoxynucleotide triphosphates (dNTP),1 µl 10x Reverse Transciption Buffer (RT Buffer), 0.13 µl RNase inhibitor, 0.67 µl MultiScribe Reverse Transcriptase RT enzyme, 1 µl of each 5x primers (RNU6B as an endogenous control, miR-126-3p and miR-452-5p) were used per each reaction. 6.9 µl of the master mix was added to each reaction tube on ice in addition to 3.1 µl of 10 ng RNA. No template control (NTC) containing no RNA sample was used as a negative control for the sample. Reaction tubes were well mixed and loaded in the BioRad T100 Thermal Cycler (Germany). The protocol included the following steps:

- 30 minutes incubation at 16°C
- 30 minutes incubation at 42°C
- 5 minutes incubation at 85°C
- Infinite hold at 4°C.

Samples were then diluted in DEPC treated water and were placed at -20°C for long term storage.

D. miRNA Expression in Cultured Breast Cancer Cell Lines by Reverse Transcription Quantitative Real Time PCR (RT-qPCR)

TaqMan® microRNA Assays and 2X TaqMan® Universal Master Mix with no Amperase Uracil N-glycosylase (UNG) (Applied Biosystems) were used to check for the relative expression of specific miRNAs (miR-126-3p and miR-452-5p) and RNU6B in the breast cancer cell lines used.

Briefly, Master mix was prepared using 5 µl 2x Universal Master Mix, 0.5 µl of the corresponding 20 x miRNA probe and 2µl of DEPC treated water. 7.5 µl of the master mix and 2.5 µl of the cDNA were added per each reaction well of a BioRad 96 well skirted plate or the 384 well skirted plate. NTC was also included in the plate. The plate was centrifuged briefly at 3000 rpm for 1 min and then loaded in the BioRad qPCR machine by running the following steps:

-10 minutes hold at 95 °C

- 40 cycles of a 15 seconds denaturing step at 95 °C and 60 seconds of annealing and extension at 60 °C.

Fold change was used to check for the relative quantitative expression of the miR-452 and miR-126 in the cultured breast cancer cell lines compared to MCF-10A cell lines using RNU6B as an endogenous control. RNU6B was used to normalize the experimental Ct values. The mean of the duplicate wells of each sample was determined. ΔCt was derived by subtracting Ct value of RNU6B from miR-452 and miR-126 for each sample.

The formula for ΔCt= Ct (miR-452-5p or miR-126-3p) – Ct *(*RNU6B*)*

Then ΔΔCt was derived by subtracting the ΔCt of normal cells used (MCF-10A) from ΔCt of breast cancer cell lines used (BT-474, MCF-7, MDA-MB-231, AU-565 and MDA-MB-453). The latter is summarized in the following formula:

ΔΔCt= ΔCt (breast cancer cell line)- ΔCt (normal cell line used).

Then the derived ΔΔCt was used to determine the fold change via using the following formula:

Fold change = **2^-ΔΔCt**

E. In Silico Predicted and Experimentally Validated Target Tools

Predicted Target Database (TargetScan 6.2) and experimentally validated TarBase (Diana Tools) were used in order to find the targets of miR-452-5p. Further PubMed search was done to check for target genes based on their relation with miRNA 452- 5p in breast cancer and in other types of cancer. The following target genes were selected: HECT Domain E3 Ubiquitin Protein Ligase 1 *(HECTD1),* SRY-box7 *(SOX7),* B cell-specific Moloney Murine Leukemia Virus Integration site 1 *(BMI1),* WW Domain Containing E3 Ubiquitin Protein Ligase 1 *(WWP1)* and Cyclin Dependent Kinase Inhibitor 1B *(CDKN1B).*

Primer bank was used by accessing the following website https://pga.mgh.harvard.edu/primerbank/.

The sequences of primers SOX7 and BMI1 were derived from cited articles Guo and Li et al respectively. The specificity, amplicon size and melting temperature were checked by inserting the exact sequence of the forward and reverse primers using Primer Blast (PubMed).

Target gene	Primer name	Sequence	Amplicon Size	Concentration (nM)	Melting Temperature Tm °C based on Primer blast	Ta °C	Reference
BMI1	*BMI*-1-Pf	CGTGTATTGTTCGTTACCTGGA	82	350	58.42 58.69	60	Li et al, 2017
	BMI-1-Rf	TTCAGTAGTGGTCTGGTCTTGT					
WWP1	*WWP1*-Pf	TGCTTCACCAAGGTCTGATACT	108	200	59.09 59.64	60	Primer Bank
	WWP1-Rf	GCTGTTCCGAACCAGTTCTTTT					
CDKN1B	*CDKN1B*-Pf	ATCACAAACCCCTAGAGGGCA	77	150	60.84 58.98	60	Primer Bank
	CDKN1B-Rf	GGGTCTGTAGTAGAACTCGGG					
SOX7	*SOX7*-Pf	GCCAAGGACGAGAGGAAAC	168	350	58.16 59.67	63.30	Guo et al, 2008
	SOX7-Rf	GTTGGGGTAGTCCTGCATGT					
HECTD1	*HECTD1*-Pf	TGTGCTTACCTTCATTCGTGAC	83	350	58.94 58.71	60	Primer Bank
	HECTD1-Rf	AGAGTCTTGATACCACAGCCATA					
GAPDH	GAPDH-Pf	ACAACTTTGGTATCGTGGAAGG	101	500	58.59 59.79	60	Primer Bank
	GAPDH-Rf	GCCATCACGCCACAGTTTC					

Table 3. Sequence, Concentration and Annealing Temperature (Ta) of primers for miR-452 -5p target genes and GAPDH (reference control) (TIB, MOLBIOL)

F. Transfection of MCF-7 Breast Cancer Cell Line:

700,000 MCF-7 breast cancer cells were seeded per well in a 6 well plate (one plate for each of the three time points 24, 48 and 72 hours) and 3000 cells per well were seeded in a 96 well plate (one plate for each of the three time points 24, 48 and 72 hours). Seeded cells were incubated overnight at 37°C with 5% CO2. The confluency that has to be 60-70% was checked on the second day. Culturing media was removed and replaced with antibiotic free media 2 hours before transfection. The transfection was done using Lipofectamine RNAiMAX Reagent (Invitrogen, USA) and Opti-

MEM® Medium (Invitrogen, USA) based on the manufacturer's instructions. A duplicate was done per each of the three conditions: MCF-7 cells transfected with miR-452 mimics (M), MCF-7 cells transfected with Negative Control duplex (NC) (GenePharma, Shanghai) and untransfected MCF-7 cells. Lipofectamine and 30 pmol of M and NC were diluted with Opti-MEM, incubated for 15 minutes and added to MCF-7 cells (250 µl/well in 6 well plates). Lipofectamine and 5 pmol of M and NC were diluted with Opti-MEM, incubated for 15 minutes and transfected to MCF-7 cells grown in 96 well plates (10 µl/well). Cells grown and transfected in 96 well plates were used for MTT assay at the time points allocated. Cells grown in 6 well plates were then harvested after 24 hours, 48 hours and 72 hours. Wells were washed with 1ml 1x PBS and then 500 µl of trypsin was added. The plate was incubated at 37^{o}C and detachment of cells was checked and followed by resuspension of cells with 1 ml DMEM. Trypan blue dye exclusion assay was done on the trypsinized cells. Then a pellet was derived upon centrifugation at 900 rpm for 5 minutes and kept at -80°C for RNA extraction and RT-qPCR. More than one plate of seeded MCF-7 cells for 24 hours and 48 hours were transfected to be used in invasion assay and Propidium Iodide staining procedure for cell cycle analysis. 24 and 48 hours time points were chosen for further functional tests.

G. MTT Assay

Cells grown in 96 well plate (at a seeding concentration of 3000 cells/ml) and transfected with Lipofectamine RNAiMAX as mentioned earlier were used for MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay. 10 µl of MTT dye/well was added 24, 48 and 72 hours post transfection. The plate was incubated at

37°C with 5% CO2 for 4 hours after which 100 µl of MTT stop solution was added. Absorbance was measured at 595 nm using TriStar2 S LB 942 (Multimode reader) from Berthold Technologies.

H. Trypan Blue Dye Exclusion Assay:

DMEM media was removed 24 and 48 hours post transfection from MCF-7 breast cancer cells grown in 6 well plates. The supernatant was centrifuged and the pellets were resuspended with 50 µL 1x PBS. 10 µL of the mixed pellet was added to 10 µL trypan blue and then 10 µL of this mixture was loaded into a hemocytometer chamber. The supernatant was used to calculate the percentage of dead cells per each condition. As for the viable cells, cells were trypsnized using 500 µL of 1x Trypsin with EDTA and then resuspended with 1 ml DMEM and counted by loading 10 µL of (10 µL cells+10 µL trypan) mixture into the chamber.

I. Cell Cycle Analysis:

MCF-7 transfected cells were harvested into 1.5 ml tubes and centrifuged at 1500 rpm for 5 minutes at room temperature. Cells were washed once with 1ml 1xPBS and resuspended with ice cold 1x PBS. Centrifugation was further performed to get the pellet. Pellet was resuspended with 1ml 1xPBS, and transferred to a 15 ml tube after which 4 ml of 100% ethanol was added for fixation. Fixed cells were kept at -20°C. The next day, ethanol was removed by spinning down at 200xg then cells were washed with PBS. Cells were treated with 100 µL Ribonuclease/pellet (working concentration 0.2 mg/ml) and incubated for 40 minutes at 37°C to ensure that only DNA is stained.

Centrifugation at 400xg was further done to retrieve the pellet. 200 µL of 180 µL PBS + 20 µL propidium iodide staining (working concentration = 1 mg/ml) was added per each sample and samples were incubated for 40 minutes at 37°C. Pellet was washed and suspended with 500 µL 1x PBS.Cells were analyzed using a Guava easyCyte Flow Cytometry machine (Merck Millipore). (Yellow-Lin) fluorescence intensity and side scatter were measured. DNA peaks of G0/G1 and G2/M phases were adjusted upon loading the control (NC). 10,000 events were collected. The percentage of MCF-7 cells previously transfected with mimic 452 (M) and Negative Control (NC) at 24 hours and 48 hours, were quantitated in each cell cycle phase. This experiment needs further optimization. For that reason, results were not reported.

J. Invasion Assay:

The insert of pore diameter 8 µm (BD Falcon) was coated in a 24 well plate with 80 µL (4 µL matrigel and 76 µL incomplete DMEM media) in the center and the plate was agitated to make sure that the matrigel covers the bottom of the insert. Media was then removed and the matrigel was washed with 500 µL antibiotic and additive free DMEM medium. 50,000 MCF-7 cells untransfected and transfected with mimic 452 (M) and Negative Control duplex (NC) for 24 hours or 48 hours as mentioned earlier were seeded on the top of the matrigel suspended in 250 µL DMEM medium (0% FBS). 500 µL of DMEM medium with 10% FBS was added under each insert. Cells were incubated overnight at 37°C with 5% CO2 and are left to invade the matrigel for 24 hours. Confluency of the cells that has to be 70% was checked on the second day. Media was removed from above and below the insert. Then, fixation with ice cold

methanol (500 µL above and 250 µL below the insert) was performed for 20 minutes at room temperature. Methanol was removed followed by removing matrigel and the cells towards the inside of the insert using a cotton swab dipped in water. The bottom of the insert was stained with 600 µl of 1 µl Hoechst (10mg/ml) in 10 ml 1x PBS at a ratio of 1:10000 Hoechst for 7 minutes. Hoechst was then removed and the bottom of the insert where the invading cells reside was washed with 500 µl 1x PBS. The insert was then cut and mounted on a slide where the back of the insert that is cut has to be oriented upward. One drop of anti-fade was added and the slide can be read on the next day. Zeiss Observer ZI Microscope was used to examine the cut insert that was divided into six zones. Zen Program was used, RL illumination as well as the 488 nm wheel where turned on to check for fluorescence and to snap the live acquired pictures.
10,000 MD-MB-231 cells were also seeded to check for the invasion of these breast cancer cells. The same procedure mentioned earlier was performed.

K. Primer Optimization:

mRNA expression of selected target genes (*BMI1, WWP1, SOX7, HECTD1 and CDKN1B*) in MCF-7 breast cancer cells was checked. cDNA was done on RNA extracted from MCF-7 cells.

a. <u>cDNA synthesis for mRNA expression of target genes in untransfected MCF-7 cells</u>:

1 µg of total extracted RNA were reverse transcribed according to the following protocol. 1 µl of a random hexamer (5ng/µl) (Amersham Bioscience) was added to a total volume of 11 µl total RNA diluted with DEPC Nuclease free water. The tube containing the hexamer and the RNA added was incubated in BioRad T100 Thermal

Cycler (Germany) at 65°C for 10 minutes and then transferred directly on ice where it was kept to chill for 10 minutes.7 µl of the master mix consisting of the reagents mentioned in the below (Table 4) was added to the 12 µl mixture:

5x First Strand Buffer (Invitrogen)	4µl
1 M Dithiothreitol	0.2 µl
10 mM dNTP mix (Promega)	1µl
Nuclease free water	1.8 µl

Table 4: The volume of Each Reagent Added for Master Mix Preparation is mentioned.

1µl of SuperScript™ II RT polymerase (Invitrogen) was added to each reaction to obtain a final volume of 20 µl. Samples were then incubated in the thermal cycler following these steps: 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes followed by a 4°C hold.A tube for no reverse transcription (NRT) was prepared in which it contains the master mix and the mixture of RNA and hexamer but excludes any addition of SuperScript™ II RT polymerase.

b. RT-qPCR optimization for the expression of mRNA of selected target genes in MCF-7 cells:

The following master mix illustrated in (Table 5) was added per each primer concentration. 24 µl of master mix was added with 1 µl cDNA in 96 well skirted plate.

NRT and NTC samples were included. The cycle steps of all primers except SOX7 were as follows:

- 10 minutes at 95°C
- 40 cycles of 15 seconds at 95°C, 60 seconds at annealing temperature (Ta) and 1 min at 60°C as well as a melting curve of 55ºC to 95ºC with 0.5ºC increment.

The annealing temperature was optimized for SOX7 in which a gradient was set and the optimized temperature used was 63.3°C.Using the ΔΔCt equation, the relative expression of the experimental mRNA was determined in MCF-7 breast cancer cells using *GAPDH* as a reference gene control.

Primer (nM)	100	150	200	250	300	350	400	450	500	550	600
SYBR µL	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Pf µL	0.25	0.375	0.5	0.625	0.75	0.875	1	1.125	1.25	1.375	1.5
Pr µL	0.25	0.375	0.5	0.625	0.75	0.875	1	1.125	1.25	1.375	1.5
water µL	11	10.75	10.5	10.25	10	9.75	9.5	9.25	9	8.75	8.5
cDNA µL	1	1	1	1	1	1	1	1	1	1	1
Total µL	25	25	25	25	25	25	25	25	25	25	25

Table 5. The dilutions performed for primer optimization. iTaq™ Universal SYBR® Green Supermix (BioRad) was used.

L. mRNA Expression of Target genes in MCF-7 Transfected Cells by RT-qPCR

cDNA synthesis for mRNA expression of target genes was done on MCF-7 untransfected cells and MCF-7 transfected cells with miR-452 mimics (M) and Negative Control duplex (NC) as mentioned earlier.

RT-qPCR was performed on cDNA of MCF-7 transfected cells and the selected target genes (*BMI1, CDKN1B, WWP1, SOX7 and HECTD1*). A master mix including 12.5 µl

iTaq™ Universal SYBR® Green Supermix (BioRad) and a specific volume of forward and reverse primers as well as DEPC Nuclease free water depending on the primer concentration was prepared (Table 5). NTC and NRT samples were included. The cycle steps of all primers except SOX7 included the following steps:

- 10 minutes at 95°C
- 40 cycles of 15 seconds at 95°C, 60 seconds at annealing temperature (Ta) and 1 min at 60°C as well as a melting curve of 55ºC to 95ºC with 0.5ºC increment.

  The cycle SOX7 followed different conditions where the optimized temperature used was 63.3°C.

Using the ΔΔCt equation, the relative expression of the experimental mRNA was determined in MCF-7 transfected breast cancer cells (miR-452 mimics transfected cells compared to NC transfected cells) using *GAPDH* as a reference gene control.

M. Statistical Analysis:

Statistical analyses were performed using GraphPad Prism 6. One sample t-test, Mann -Whitney test and two-way ANOVA were used and specified accordingly for each test in the figure legend. A p-value <0.05 was considered statistically significant.

III. RESULTS

A. miR-452 Expression in Breast Cancer Cell Lines:

In order to assess the level of miR-452 in breast cancer cells, RT-qPCR was used to check for the relative expression of miR-452 in breast cancer cell lines with different hormonal receptors profile (MCF-7, MDA-MB-231, MDA-MB-453, BT-474

and AU-565) compared to the normal non-tumorigenic epithelial cell line MCF-10A using RNU6B as an endogenous control (Table 6). Figure 4 shows that miR-452 was significantly downregulated in all tested breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-453, BT-474) except for AU-565 which showed slight decrease when cultured in RPMI. Importantly, no effect in the trend of miR-452 dysregulation in AU-565 and BT-474 cell lines when these cells were cultured with RPMI (Figure 4a) or DMEM (Figure 4b). A strongly significant downregulation of miR-452 was seen in MCF-7 cells which are hormone receptor positive. This stimulated our interest to investigate the role of miR-452 in MCF-7 cells.

cell line	Organism and Age	Origin	Primary Tumor	Receptors
MCF-7	Human, 69 years old	mammary gland, breast, derived from metastatic site; pleural effusion	luminal type, invasive ductal carcinoma	ER+ PR+ HER2-
MDA-MB-231	Human, 51 years old	mammary gland, breast, derived from metastatic site; pleural effusion	basal type adenocarcinoma	ER- PR- HER2-
MDA-MB-453	Human , 48 years old	mammary gland, breast, derived from metastatic site; pericardial effusion	invasive apocrine breast carcinoma	androgen receptor + ER- PR- HER2 -
BT-474	human, 60 years old	mammary gland/ breast duct	invasive ductal carcinoma of breast	ER+ PR+ HER2+
AU-565	human 43 years old with radiation steroids cytoxan and 5-	mammary gland, breast, derived from metastatic site; pleural	Adenocarcinoma	HER2 + ER- PR-

Table 6: **List of breast cancer cell lines showing their origin, primary tumor and receptor status.**

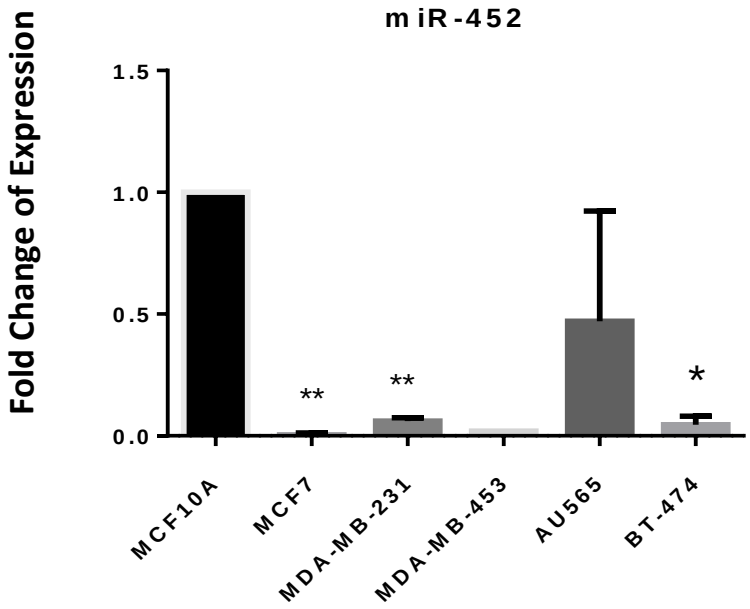

Figure 4a. Relative miR-452 Expression in Breast Cancer Cell Lines Compared to non-tumorigenic epithelial cell line MCF-10A. AU565 and BT-474 were cultured in RPMI media. RNU6B was used as an endogenous control. Replicates were two times per each cell line (n=2). One Sample t-test test was used, * denotes $p < 0.05$, and ** denotes $p < 0.01$

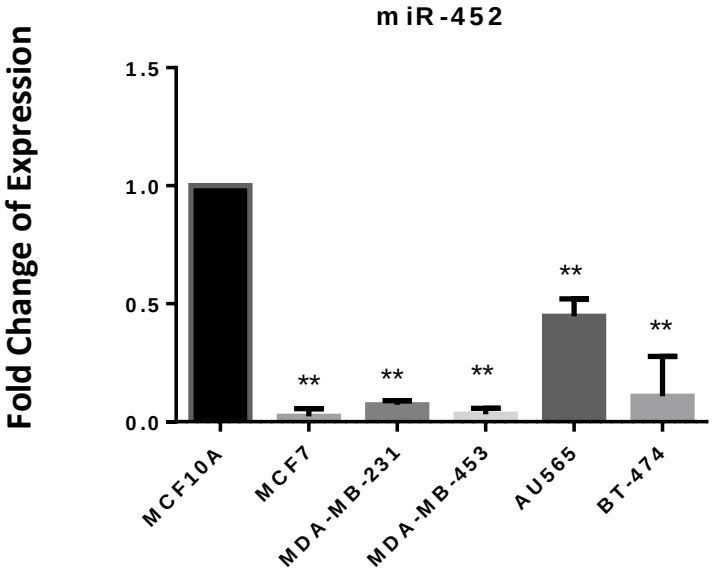

Figure 4b. Relative miR-452 Expression in Breast Cancer Cell Lines Compared to non-tumorigenic epithelial cell line MCF-10A. AU565 and BT-474 were cultured in DMEM media. RNU6B was used as an endogenous control. Replicates were four times per each cell line (n=4).

B. Predicted and Experimentally Validated mRNA Target of miR-452

miR-452 target genes were selected based on PubMed Search, Predicted Target Database (TargetScan 6.2) and experimentally validated TarBase (Diana Tools). The selection criteria was based on the prediction (TargetScan) whether miR-452 can act on this specific gene, whether this specific gene has been experimentally validated to be a direct target of miR-452 (TarBase), whether miR-452 has been shown to act on the mRNA of this target gene in breast cancer and other types of cancer, and whether this target gene has been shown to play a role in breast cancer. The following target genes were selected: HECT Domain E3 Ubiquitin Protein Ligase 1 *(HECTD1),* SRY-box7 *(SOX7),* B cell-specific Moloney Murine Leukemia Virus Integration site 1 *(BMI1),*

WW Domain Containing E3 Ubiquitin Protein Ligase 1 *(WWP1)* and Cyclin Dependent Kinase Inhibitor 1B *(CDKN1B)* (Table 7).

Table 7: **Criteria of Selection of Target Genes of miR-452**

C. miR-452 Overexpression in Breast Cancer Cell Lines Upon Transfection with miR-452 Mimics

To investigate the function of miR-452 in breast cancer, MCF-7 breast cancer cell line was chosen for this study as the endogenous level of miR-452 was significantly downregulated in these cells. To overexpress miR-452 in MCF-7, cells were transfected with miR-452 mimics (M) and Negative control (NC) using RNAiMAx reagent. Transfected cells were harvested at 24, 48 and 72 hours. RT-qPCR was performed to monitor transfection efficiency and validate the level of expression of miR-452 in MCF-7 cells transfected with miR-452 mimics compared to NC. RNU6B was used as an endogenous control. miR-452 was significantly overexpressed upon transfection with miR-452 mimics. miR-452 overexpression was prominent at 24 hours (Figure 5a) ($p<0.01$) and at 48 hours ($p<0.05$) (Figure 5b). The overexpression of miR-452 in transfected MCF-7 cells decreased at 72 hours (Figure 5c). Accordingly, the 24 and 48 hours time point were chosen for further functional tests.

Target gene based on literature	Name	Target Scan	TarBase	miRNA and target	Relation of Breast cancer and target	Role of Target in Literature	Expression of target in cancer and mode of expression of miR-452 in the cancerous cells
BMI1	B cell-specific Moloney Murine Leukemia Virus Integration site 1	YES	NO	YES	The high expression of BMI1 in basal-like breast cancer may be related to the aggressiveness of that subtype.	potential driver oncogene	. Upregulated in NSLC .Downregulated in pancreatic cells
WWP1	WW Domain Containing E3 Ubiquitin Protein Ligase 1	NO	NO	YES	WWP1 increases KLF5 protein degradation that promotes breast cell proliferation, survival and tumorigenesis.	ubiquitin-protein ligase	.Downregulated in prostate cancer cells
SOX7	SRY-box7	YES	NO	YES	SOX7 is downregulated in breast cancer.SOX7 co-regulates Wnt/β-catenin signaling with Axin-2: both expressed at low levels in breast cancer.	transcription factor	.Upregulated in hepatocellular cancer cells
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B	NO	NO	YES	CDKN1B/p27KIP1 is upregulated in ERBB2+ BC cells	prevents the cell from entering the cell cycle	.Upregulated in hepatocellular cancer cells
HECTD1	HECT Domain E3 Ubiquitin Protein Ligase 1	NO	YES	YES	Condensins positively regulate ligand-dependent enhancer activation at least in part by recruiting HECTD1. Condensin complexes exhibit an unexpected, dramatic estrogen-induced recruitment to estrogen receptor α.	controls the protein level of IQGAP1 to regulate the dynamics of adhesive structures	.Upregulated in renal cancerous cells

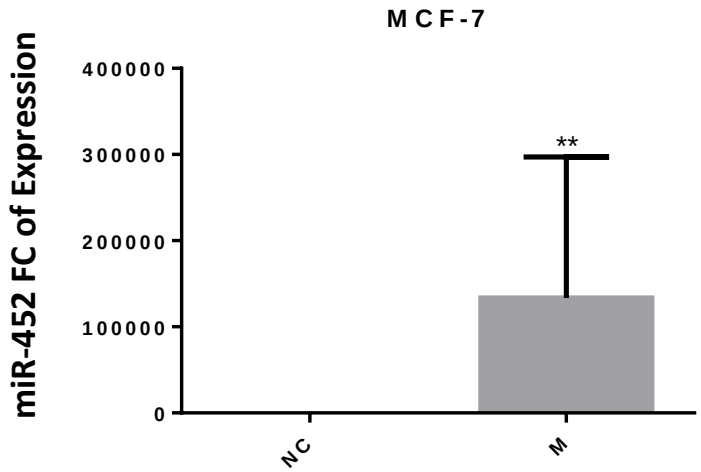

Figure 5a. Transfection efficiency of miR-452 mimics in MCF-7 cells. miR-452 expression was validated by RT-qPCR at 24 hours after transfection with miR-452 mimics and NC. RNU6B was used as an endogenous control. Mann-Whitney test was utilized. Error bars present SEM (n=5) ** denotes $p<0.01$. FC: fold change

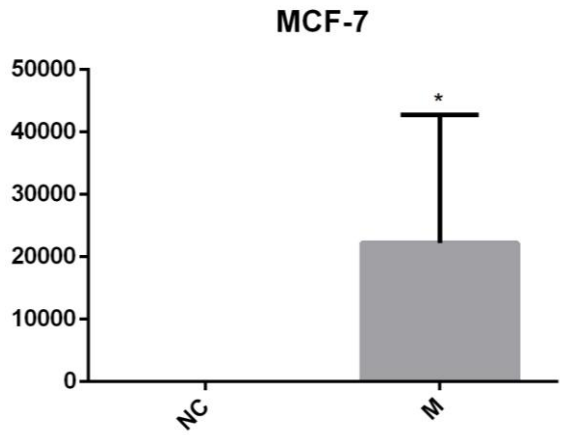

Figure 5b. Transfection efficiency of miR-452 mimics in MCF-7 cells. miR-452 expression was validated by RT-qPCR at 48 hours after transfection with miR-452 mimics and NC. RNU6B was used as an endogenous control. Mann-Whitney test was utilized. Error bars present SEM (n=4) * denotes $p<0.05$. FC: fold change

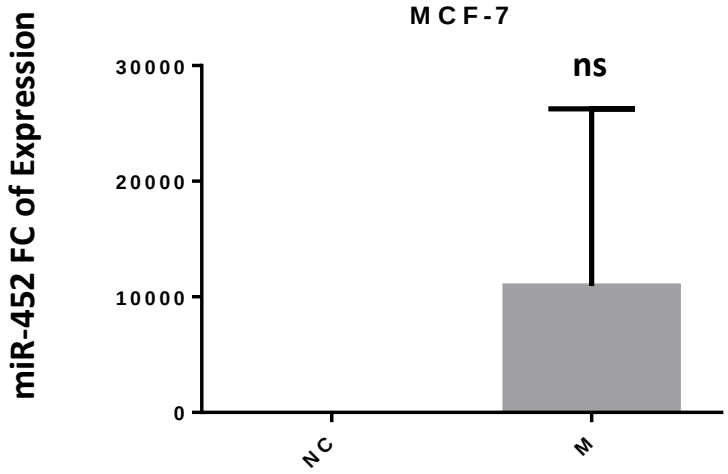

Figure 5c. Transfection efficiency of miR-452 mimics in MCF-7 cells. miR-452 expression was validated by RT-qPCR at 72 hours after transfection with miR-452 mimics and NC. RNU6B was used as an endogenous control. Mann-Whitney test was utilized. Error bars present SEM (n=2) (ns: non-significant) (p=0.3) FC: fold change.

D. miR-452 does not affect proliferation in MCF-7 cell line at the tested time points

In order to determine the effect of overexpression of miR-452 on MCF-7 cells, MTT assay was used. There was no significant effect on proliferation while comparing the proliferation of transfected MCF-7 cells with (M) to the those transfected with the control (NC) at 24, 48 and 72 hours revealing that miR-452 overexpression has no effect on proliferation at these tested time points.

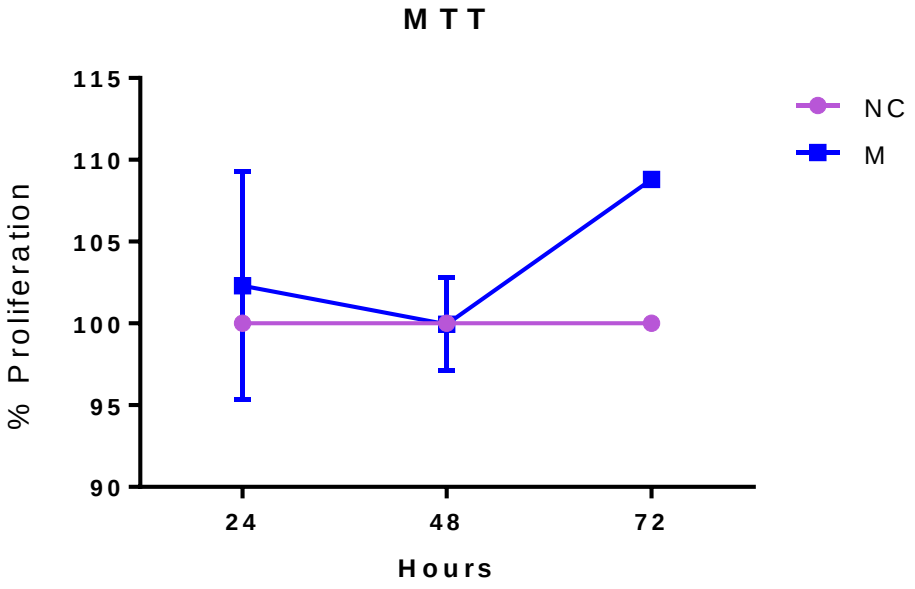

Figure 6. Proliferation of miR-452 mimics transfected MCF-7 breast cancer cells compared to NC transfected MCF-7 cells at 24, 48 and 72 hours post transfection and measured by MTT assay. Mean of two experiments is represented along with bars of standard deviation. Two way ANOVA test was used (t24: n=4; t48 n=4; t72 n=1) ($p>0.05$)

E. No effect on Viability of MCF-7 cells upon transfection with miR-452 mimics and NC

Trypan blue dye exclusion assay was performed in order to check for the viability of miR-452 mimic transfected and NC transfected MCF-7 cells 24 and 48 hours post transfection. There is no decrease in viability upon transfection at the two tested time points. This shows that, miR-452 overexpression has no effect on viability of MCF-7 cells.

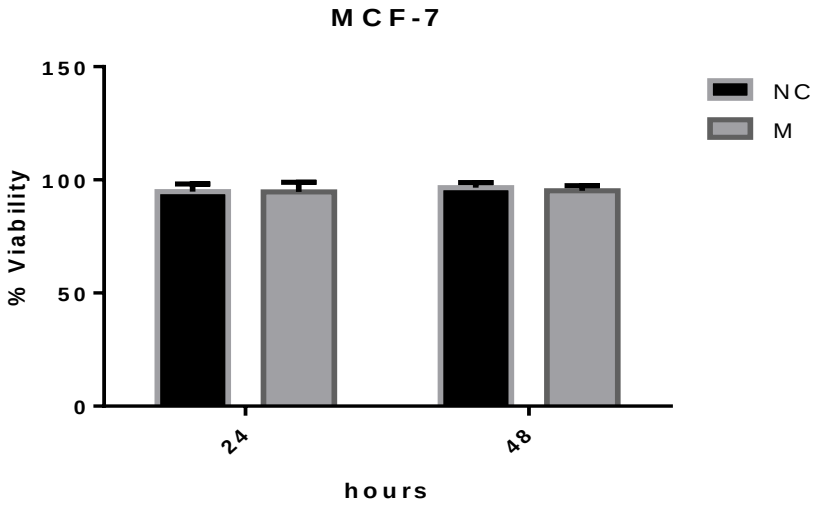

Figure 7. % **Viability of miR-452 mimic and NC transfected MCF-7 cells at 24 and 48 hours calculated using trypan blue dye exclusion assay**. Mann-Whitney test was utilized (n=4) ($p>0.05$)

F. Optimization of Annealing Temperature and Concentration of Primers

All selected target genes were validated by RT-qPCR to be expressed in MCF-7 breast cancer cells. The annealing temperature and the concentration of each primer used were optimized. Analysis of qPCR plate showed that 200nM and 150 nM are the optimized concentrations for *WWP1* and *CDKN1B* respectively while 350 nM is the optimized concentration for *BMI1, HECTD1* and *SOX7*. The annealing temperature selected for all target genes was 60° C except for SOX7 which was 63.3° C (figure 8).

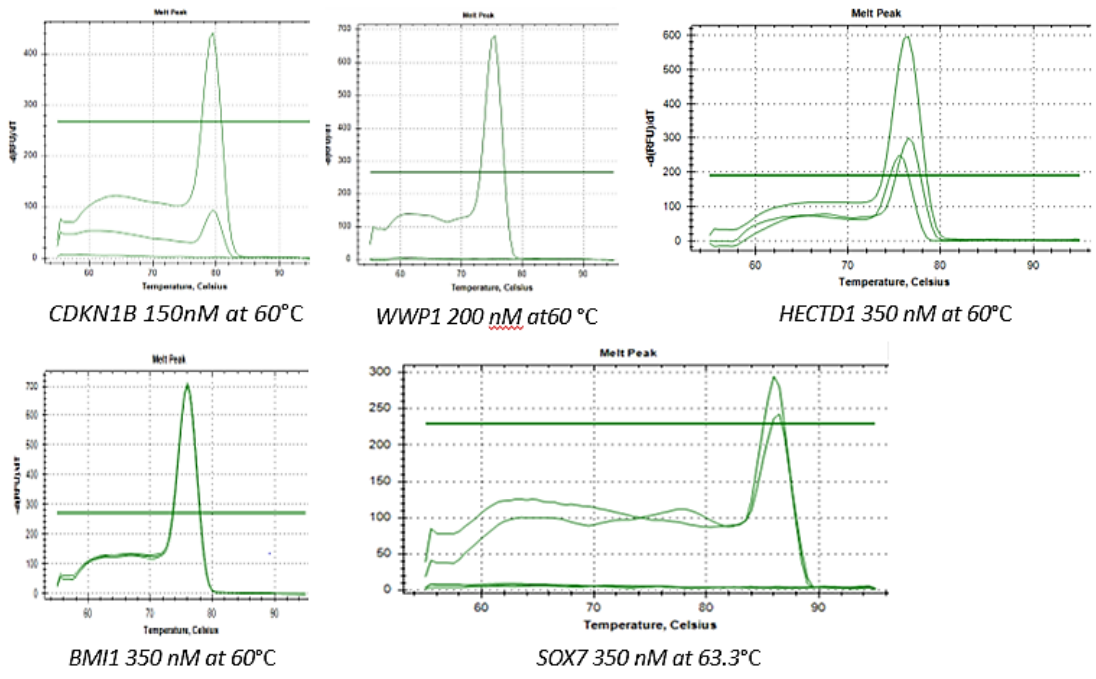

Figure 8: Representation of Optimized target genes Primers showing their melting peaks.

G. miR-452 targets mRNA of BMI1,CDKN1B, HECTD1,WWP1 and SOX7

mRNA of *BMI1,CDKN1B, HECTD1,WWP1* and *SOX7* were assessed using RT-qPCR to check for their downregulation in miR-452 mimic transfected MCF-7 breast cancer cells compared to NC transfected MCF-7 breast cancer cells. *GAPDH* was used as a reference gene. mRNA of target genes *BMI1/CDKN1B /HECTD1* and *WWP1* was significantly downregulated ($p<0.01$ and $p<0.05$ respectively) in MCF-7 breast cancer cells. *SOX7* was slightly downregulated in miR-452 mimics transfected MCF-7 cells (Figure 9).

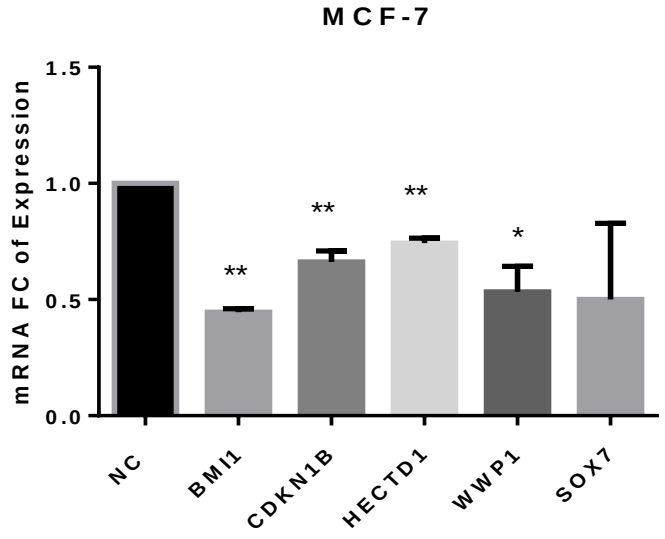

Figure 9. Relative expression of miR-452 targets in MCF breast cancer cel1s upon miR-452 mimics transfection and compared to NC transfected cells. mRNA expression in MCF-7 24 hrs post transfection. GAPDH was used as an internal control. Error bars represent SEM.One Sample t-test was utilized. * denotes $p<0.05$ and ** denotes $p<0.01$ ($n = 3$).FC: Fold Change.

H. Overexpression of miR-452 decreased the Invasive Capability of MCF-7 cells

Cell invasion capability of MCF-7 cells was assessed by invasion assay 24 and 48 hours following transfection with miR-452 mimic and NC. The invasive ability of MCF-7 was slightly suppressed by the overexpression of miR-452 showing how miR-452 can contribute to metastasis if dysregulated.

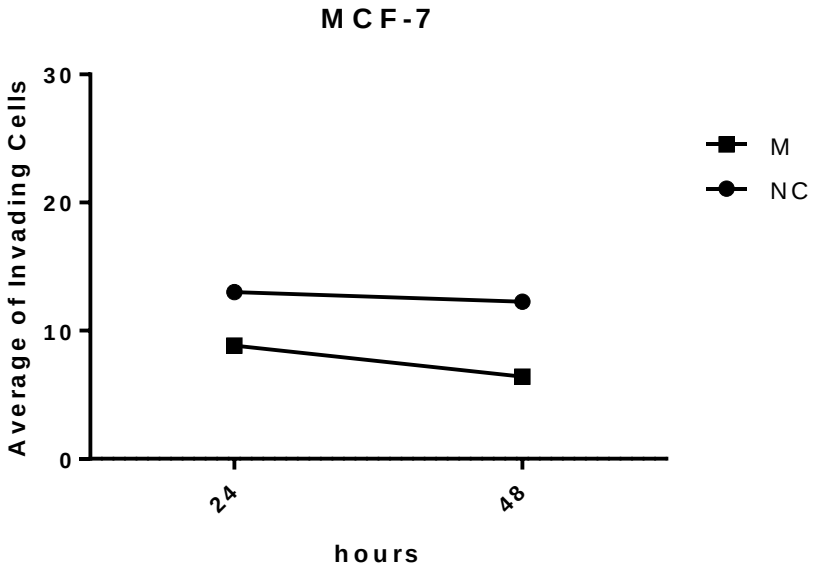

Figure 10. **Decrease in Invasion capability of miR-452 mimic transfected MCF-7 breast cancer cells compared to NC transfected MCF- cells.** Mann-Whitney test was used (n=3) ($p>0.05$).

IV. DISCUSSION

Breast cancer is the most common cancer among women. In Lebanon, breast cancer is the most common malignancy representing 26.7% of all cancer mortality. An early onset of breast cancer among young Lebanese women has been seen accompanied with aggressive state and bad prognosis. Mammography is recommended starting the age of 40, however 22% of breast cancer cases in Lebanon are below 40 and accordingly they develop breast cancer before reaching the recommended age for screening. As mentioned earlier, genetic contribution to breast cancer among which *BRCA1* and *BRCA2* mutations are mostly encountered is minimal [17]. Thus, to understand the mechanisms that may lie behind the early onset of this heterogeneous disease, we were interested in studying the epigenetic factors. We focused on studying miRNA for many reasons: miRNAs act as transcriptional regulators in which one

miRNA may regulate many target genes while one gene can be targeted by many miRNAs [105]. miRNAs are stable in clinical samples such as formalin-fixed paraffin-embedded tissues. They are also present in body fluids such as serum, plasma, urine, and saliva and can serve as potential biomarkers [75]. miRNAs play significant roles in biological cellular processes in which their dysregulation can lead to the initiation and progression of several human cancers including breast cancer. Several miRNAs have been shown to be involved in breast cancer hallmark functions such as invasion, metastasis, proliferation and apoptosis [83]. Analyzing the miRNA and mRNA profile of cancerous and normal adjacent breast tissues in young Lebanese breast cancer patients led to the identification of some miRNAs with a significant differential expression in Lebanese patients' tumor tissues [106]. One of these dysregulated miRNA is miR-452 that was significantly downregulated among young Lebanese patients who were diagnosed with estrogen and progesterone receptors positive early invasive ductal carcinoma. This stimulated our interest to further investigate the function of miR-452 in breast cancer which may ultimately be a diagnostic or prognostic biomarker of this disease. We first checked for the expression of miR-452 in breast cancer cell lines (MCF-7, BT-474, AU-565, MDA-MB-231 and MDA-MB-453) and found it downregulated in all tested breast cancer cell lines. This is in accordance with literature that shows that miR-452 is downregulated in breast cancer except for AU-565 which has not been tested before for the expression of miR-452 [107]. MCF-7 breast cancer cells which have a hormonal receptor profile similar to that seen among Lebanese patients (hormone receptor positive) showed the lowest level of miR-452 expression and was accordingly chosen to be transfected with mimics to overexpress miR-452.

RT-qPCR results demonstrated that miR-452 level was indeed upregulated in MCF-7 cells upon transfection with miR-452 mimics with a significant increase 24 and 48 hours post transfection. However, although, overexpression of miR-452 was not accompanied by a decrease in cell proliferation and viability of transfected cells at the tested time points, it slightly decreased the invasion of MCF-7 cells. It has been previously shown that miR-452 overexpression significantly inhibited the migration capacities of MCF-7 cells by targeting RAB11A[107]. This was also true in other types of cancer. Moreover, in pancreatic cancer, miR-452 overexpression inhibited the migration and invasion capacity of cancer cells [108].

In Silico tools were used to select the targets of miR-452 in breast cancer. In this study, we are interested to assess how miR-452 can act on breast cancer cells by targeting specific genes that were previously shown to play a role in breast cancer, have a direct correlation with miR-452 in other types of cancer but have not been shown to be regulated by miR-452 in breast cancer. The selected target genes *WWP1, BMI1, HECTD1, SOX7* and *CDKN1B* were first validated by RT-qPCR for their expression in MCF-7 cells in particular. Notably miR-452 overexpression was shown to downregulate these target genes in MCF7 cells. To our knowledge, this is the first time *WWP1, HECTD1, SOX7 and CDKN1B* were shown to be potential target genes of miR-452 in breast cancer. Our results are based on the inverse correlation we observed between miR-452 and these targets mRNA. However, we still need to check the effect of miR-452 overexpression on the protein level of these targets and to prove a direct interaction between the miRNA and mRNA targets in breast cancer cells.

WWP1 regulates a variety of cellular biological processes including protein trafficking and degradation, signaling and transcription. Target Scan database predicted that miR-452 binds to the 3' UTR region of *WWP1* [100]. Moreover, overexpression of *WWP1* was observed in breast, prostate and hepatocellular carcinoma[109, 110]. miR-452 targeted the expression of *WWP1* in prostate cancer and the knockdown of WWP1 inhibited the migration and the invasion of prostate cancer cells [100]. Our findings highlighted WWP1 as new potential target for miR-452 in breast cancer.

BMI1 proto-oncogene has been shown to play a role in a number of biological processes including cell cycle, apoptosis and proliferation. Li et al showed that *BMI1* was the direct target of miR-452 in pancreatic cancer in which the overexpression of miR-452 inhibited the migration and invasion of pancreatic cancer by knocking down *BMI1* expression [108]. Moreover, it has been shown that the overexpression of miR-452 inhibited the migration and invasion capacities in non-small lung cancer cells by targeting *BMI1* expression [111]. This is also consistent with our data where we showed an inverse correlation between miR-452 and *BMI1* in breast cancer .

CDKN1B has been shown to play a significant role in the cell cycle. Gan et al showed that miR-452 may serve an essential role in the occurrence and progression of Lung Squamous Cancer Cells (LUSC) by targeting *CDKN1B. CDKN1B* has been also shown to be downregulated upon overexpression of miR-452 in hepatocellular carcinoma[112]. Here, we show that there is also an inverse correlation between miR-452 and CDKN1B in breast cancer.

HECTD1 regulates the dynamics of adhesive structure. Interphase condensins I and II modulate estrogen-regulated enhancer activation by recruiting HECTD1. No previous data was shown for the correlation between miR-452 and HECTD1[113]. This is the first report of any inverse correlation between miR-452 and HECTD1.

SOX7 is a transcription factor mediating several biological processes and has been identified as a direct target of miR-452 in hepatocellular carcinoma[114]. We showed that SOX7 transcripts are also downregulated upon the overexpression of miR-452 in breast cancer.

Hence, this study has helped in identifying new targets for miR-452 in breast cancer. However, additional functional tests have to be done to examine the effects of miR-452 overexpression on breast cancer cells (MCF-7 as well as other breast cancer cells) proliferation, clonogenic activity in 3D culture, cell cycle and invasion and to validate the direct interaction between miR-452 and these potential targets. Finally, miR-452 can be further investigated as a promising potential biomarker for breast cancer early screening and detection especially among early onset patients.

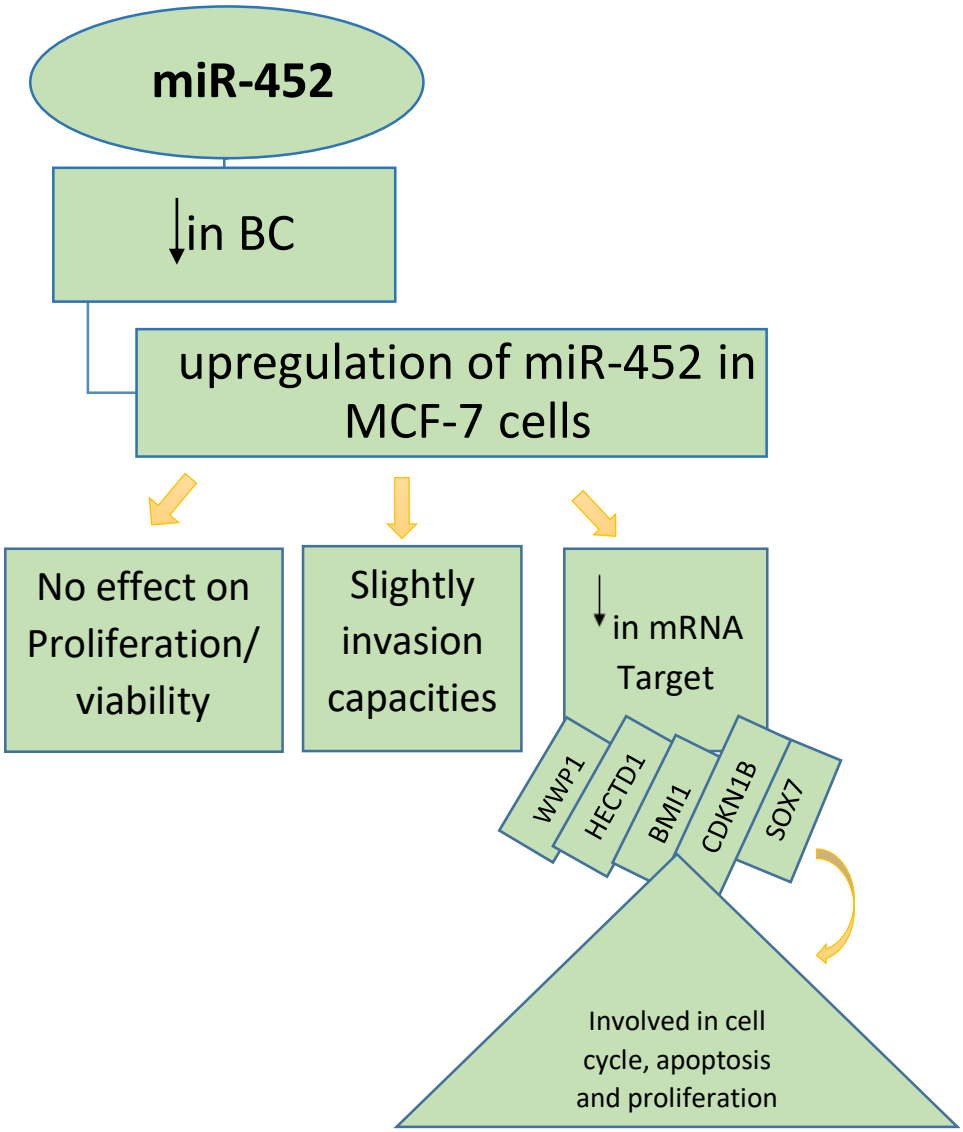

Figure 11. Summary of Results

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