

5-9-2014

## Identification of microRNA Biomarkers in HER2-Positive Breast Cancer

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**Identification of microRNA Biomarkers in HER2-Positive Breast Cancer**

by

Hossam Tashkandi

Bachelor of Science  
University of South Carolina, 2011

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

2014

Accepted by:

Hexin Chen, Director of Thesis

Alan Waldman, Reader

Peisheng Xu, Reader

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## **Acknowledgements**

I would like to thank Allah for everything and then my thanks and appreciation to my adviser, Dr. Hexin Chen, for his advice and pushing me to achieving everything I can in my short time with him.

I wish to acknowledge the support and advice from my colleagues, friends, and lab-mates, Dr. Shou Liu, Dr. Nirav Shah, Dr. Ufuk Ozer, Varun Chandrashekar, Sara Johnson, and Yogin Patel.

My appreciation also goes out to Dr. Alan Waldman and Dr. Peisheng Xu for taking time from their busy lives to serve as committee members.

Finally, my sincerest thanks and gratitude to parents, family, and my lovely fiancé for putting up with me at my worst during the stressful parts of my career.

## **Abstract**

Human epidermal growth factor receptor 2 (HER2) is overexpressed in approximately 30% of breast cancer patients with poor prognosis. In addition, microRNAs are small non-coding RNA that have been linked to many cancers. Here we investigate which miRNAs are differentially regulated by HER2 overexpression. Using quantitative reverse-transcription polymerase chain reaction (QRT-PCR) and matching it with the clinical data acquired from Dvninge, we find five candidate miRNAs. When comparing the miRNAs' effect on patient survival, only three miRNAs stand as good predictors of patient survival outcome. These miRNAs are miR-146a-5p, miR-181d, and miR-195-5p. When miR-146a-5p is up-regulated, which is the case in HER2 positive patients, survival of patients drop significantly. In addition, miR-181d and miR-195-5p are down-regulated in HER2. With these two miRNA having low levels, the patient survival drops. Therefore, this data indicates that miR-146a-5p, miR-181d, and miR-195-5p are potential markers for patient survival and that their expression levels are highly associated with HER2 overexpression.

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# Chapter 1

## Introduction

### 1.1: Cancer

For many decades, cancer has been rigorously analyzed in many laboratories and population-based researched throughout the world. Due to the dedication of many scientists, a plethora of information is found where we can peer into cancer. However, even though scientists have been hard at work in understanding cancer, no complete cure has been found for the disease. This leaves us with the fact that we have not understood a lot about cancer. In order to realize what is yet unknown, we must talk about what we already know about cancer.

Cancer occurs in patients due to a multitude of factors that changes the behaviors of cells. There are six hallmarks of cancer that describes what a cancer cell needs to be able to acquire in order to be cancerous. These hallmarks are: evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, and sustaining proliferative signaling (Hanahan & Weinberg 2011). Though diet is considered a major contributor to the formation of cancer cells (Willett 2000), the fundamental change in this formation is a genetic change. This change is a mutation that produces a dominant oncogene that promotes tumor formation and recessive tumor suppressor genes that keep tumors at bay (Hanahan & Weinberg 2000). Oncogenes is a term used to described genes that, when over expressed,

promote tumor formation, whereas tumor-suppressor genes are genes that inhibit tumor formation and are generally down regulated in tumors. With enough accumulation of mutation to oncogenes and tumor suppressor genes, any cell can turn into a cancer cell. These cancer cells would then have the ability to proliferate indefinitely and generate tumors (Lobo et al 2007). Therefore, it is apparent that the scientific community has a large understanding and has progressed in the molecular mechanism of the cellular machinery in tumorigenesis (Reya et al 2001). However, due to the immensely complex nature that is the molecular and biochemical mechanism and pathways that control cell behavior, there is a mass sum of information that has yet to be uncovered to better understand and treat cancer.

## **1.2: Breast Cancer:**

Because of the complex mechanisms that control cell behaviors, cancer can manifest in different types. Among these types are solid tumors that are of epithelial origin. These epithelial tumors account for a significantly large cancer burden derived from many tissues such as breast, colon, ovary, lung, prostate, pancreas, and brain which constitute 80% of cancers (Visvader & Lindeman 2008). In the United States, breast cancer is the number one most common cancer in women and it is a leading cause of death among these women second only to lung cancer (Centers for Disease Control and Prevention [CDC], 2013). Breast cancer is the cause of more than 40,000 cancer-related deaths recorded annually in the U.S. among women (Klonisch et al 2008). In order to understand different types of breast cancer, we must briefly explore the morphology of the breast and the molecules that affect them. The breast is made up of fat and imbedded

in this fat is the mammary gland. The mammary gland is formed of branched ducts terminating in lobular and alveolar units (Klonisch et al 2008). Different cell lineages occupy different parts of the mammary gland. The myoepithelial cells line the basement layer of both the ducts and alveoli. The ducts and the alveoli are formed by the ductal epithelial cells and the alveolar epithelial cells respectively, with the alveolar epithelial cells having the ability to produce milk (Klonisch et al 2008). The breast cells are highly influenced by different signals from the body, such as growth factors and the hormones estrogen and progesterone. On their surface, the cells contain receptors for these signals that they keep at a controlled quantity. Overexpression of surface receptors can cause alternations in the signaling cascade downstream of the receptor. These alterations can cause a shift in cell proliferation rate and intercellular change that can cause genomic instability and the transformation of normal cells into cancerous cells (Hanahan & Weinberg 2000).

Furthermore, the overexpression of the surface receptors is caused by mutations in the gene expression mainly. Therefore, based on gene microarray, it has been deduced that there are five subtypes of breast cancer depending on their gene expression pattern. These subtypes are: luminal A, luminal B, basal-like, normal-like, and human epidermal growth factor receptor 2 (HER2) positive tumors (Perou et al 2000). Luminal A and luminal B breast tumors are estrogen receptor positive (ER+), which means they overexpress the estrogen receptor. However, the difference between luminal A and luminal B tumors is dependent on the tumor grade. Tumor grades are scored from three to nine based on the differentiation of the tumor cells. The differentiation ranges from well-differentiated to poorly differentiated tumors with the former having the best prognosis

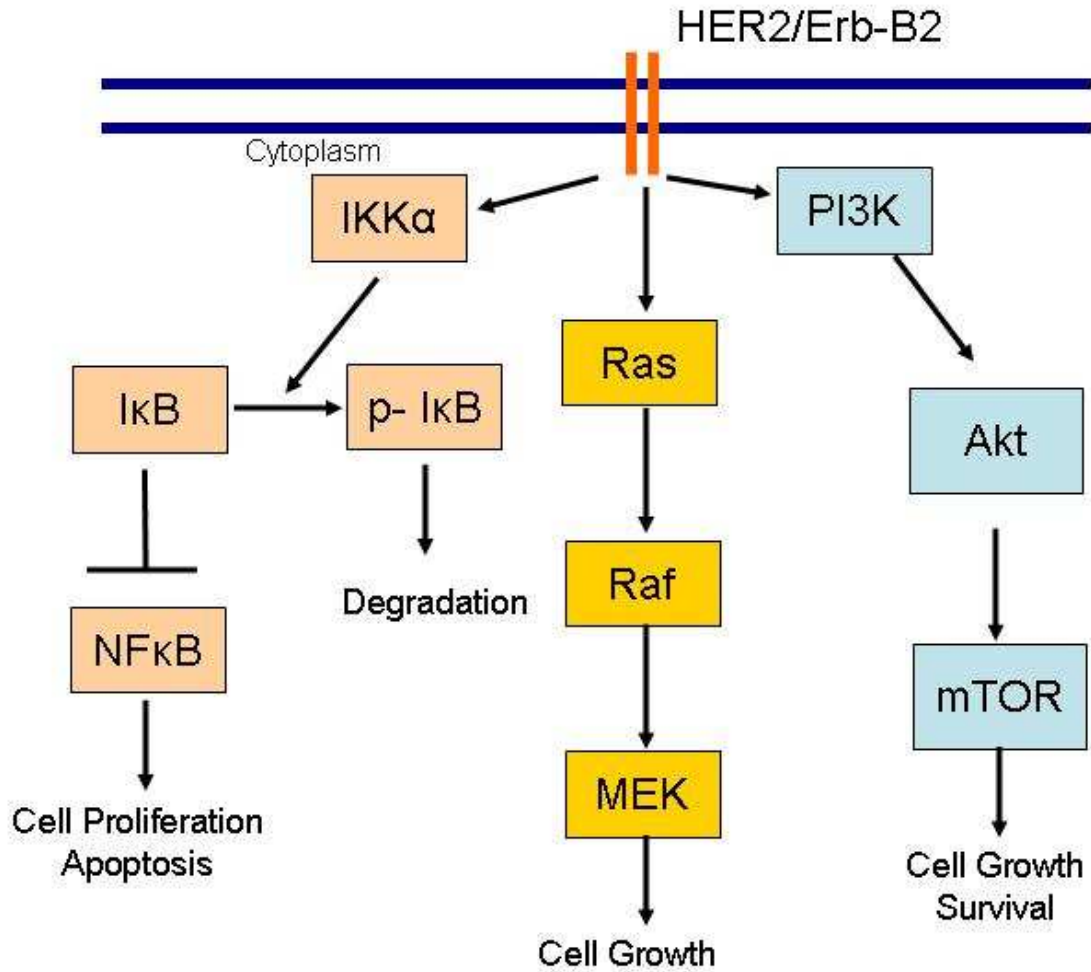
and the latter having the worst. Luminal A is of the well-differentiated grade and luminal B is among the poorly differentiated grade. Among all types of breast cancers, luminal A has the best prognosis for the patient, along with the normal-like tumors (Metzger Filho et al 2011). Basal-like tumors are tumors that are negative for ER, progesterone receptor (PR), and HER2. The basal-like tumors generally are found in 10%-15% of all tumors and carry with them bad prognoses for the patient (Perou 2011). In addition, some of the tumors that are classified under one subtype or the other can carry genetic similarities with tumors that are classified under a different subtype. For example, basal-like tumors can be enriched with HER2 and yet still be under the basal-like tumor type (Perou 2011).

HER2 is one of those receptors, when overly expressed, can cause genomic instability which can transform normal cells into cancer cells in the breast and thus is considered an oncogene. The HER2 gene is located in the long arm of chromosome 17 (17q12). HER2 is named so because it shows high resemblance to the structure of the human epidermal growth factor receptor (HER1). Furthermore, HER2 is also known by other names for different reasons. It can also be called ErbB-2 because it shows similarity to the avian erythroblastosis oncogene B (ErbB) (Coussens et al 1985).

HER2 is a ligand-independent receptor tyrosine kinase that can amplify the signaling pathway from other growth factors (Citri & Yarden 2006). The extracellular domain of HER2 stays in a constitutively active confirmation (Yan et al 2014). HER2 can homodimerize and heterodimerize, and with the constant active confirmation, HER2 becomes the preferred dimerizing partner of HER1, HER3, and HER4. The dimerization causes an autophosphorylation (attachment of a phosphate molecule) to the amino acid tyrosine in the HER2 receptor which initiates the signaling cascade (Olayioye 2001).

In normal cells, HER2 expression is responsible for cell survival, proliferation, differentiation, migration and apoptosis (Olayioye 2001, Roy & Perez 2009). For that reason, HER2 expression is tightly regulated. In approximately 30% of breast cancer, HER2 is overexpressed, and this type of breast cancer is called a HER2-positive breast cancer (Korkaya et al 2008). This overexpression causes an increase in the homo and heterodimerization which promotes tumorigenic signaling cascade (Yan et al 2014). HER2-positive breast cancer cases tend to have very low survival rates due to the overexpression of HER2 and its effect leading to tumorigenesis, increased invasiveness, and metastasis (Sorlie et al 2001). Some of these HER2 signaling cascades involves pathways that include the phosphoinositide 3-kinase (PI3K/Akt) pathway, the Ras pathway, and the NFκB pathway (Feldman et al 2007, Merkhofer et al 2010) (Figure 1.1)

HER2-positive breast cancer treatments include using antibodies and chemotherapy. Among the antibodies, trastuzumab is well known as having a very significant positive effect on HER2-positive breast cancer patients. Trastuzumab results in an overall response rate of 20%-35% in previously untreated HER2-positive breast cancer, resulting in progression free survival and overall survival. These results are intensified with the combination of chemotherapy along with the trastuzumab treatment (Roy & Perez 2009). However, despite the promising success of trastuzumab, most HER2-positive breast cancer patients do not respond to trastuzumab regardless of whether it is single-agent or combined with chemotherapy. In addition, for those patients who do respond to trastuzumab, a big majority end up developing a resistance to the antibody (Roy & Perez 2009). Understanding the pathway will help in locating better targets for treatment or even better markers for an early diagnosis.



**Figure 1.1: HER2 pathway:**

Depiction of the HER2 pathway affecting three major pathways according to literature. Right branch is PI3K/Akt pathway leading to mTOR. Middle branch is the Ras and Raf eventually leading to the MEK and ERK pathway. Left branch is the pathway eventually leading to the NF $\kappa$ B pathway.

### **1.3: microRNA:**

MicroRNAs (miRNA) are small non-coding regulatory RNA that are, on average, 21 nucleotides long that regulate the expression of proteins post-transcriptionally. There are over a thousand miRNA in human cells that are all produced first in the nucleus as primary miRNA (pri-miRNA) in the shape of a stem-loop (Lee et al 2002). The miRNAs are then cleaved by an RNase III enzyme called Drosha, which produces a ~70 nucleotide product called pre-micro RNA (pre-miRNA) (Lee et al 2003). RNase III enzymes are proteins that are dsRNA-specific endonucleases that produce staggered cuts on both sides of the RNA helix (Filippov et al 2000). Once pre-miRNA is produced, it is shuttled out of the nucleus and into the cytoplasm to be processed by another RNase III enzyme called Dicer. Dicer cleaves the loop from the pre-miRNA producing a ~21 nucleotide double stranded miRNA (Lee et al 2002). The desirable strand of the double stranded miRNA is separated and is placed in the RNA-induced silencing complex (RISC) while the undesired strand – called the passenger strand – is degraded (Okamura et al 2008, Rana 2007). RISC is a ribonucleoprotein complex which contains RNA and proteins and can also be known as miRNP (Schwarz & Zamore 2002) (Figure 1.2).

The proteins from the Argonaute (Ago) protein family are essential for the function of RISC. The Ago proteins contain binding domains that help bind and orient the mature miRNA to interact with the targeted messenger RNA (mRNA). RISC silences mRNA translation by either inhibiting translation or degrading the mRNA. When there is complete complementarity between the miRNA in RISC and the mRNA, the mRNA gets degraded. However, when there is only partial complementarity between the miRNA in RISC and mRNA, there is only silencing of the mRNA and inhibition of translation. The

silencing and degradation occurs in many different ways, including binding to the 3 prime untranslated region (3'-UTR) of the mRNA and/or decapping the 5' cap of the mRNA (Filipowicz et al 2008, Schwarz & Zamore 2002). Therefore, because miRNA are regulatory RNA, they are generally tightly regulated.

When regulation of miRNAs becomes faulty, alterations of miRNA expression can occur and would be associated with many malignant human ailments such as cancer (Bartel 2009). miRNAs' DNA sequences have been reported in many deletion and over-expression sites of other genes. This means that they may be expressed or repressed along with the expression or repression of the DNA where they were located (Calin et al 2004). Likewise, miRNAs have also been suggested to act as tumor initiation miRNAs (oncomirs) or tumor suppressor miRNAs depending on their targeted genes (Esquela-Kerscher & Slack 2006). For example, if a miRNA targets HER2, then it is considered a tumor suppressor miRNA because HER2 is an oncogene and would generally be down-regulated or suppressed in cancer. On the other hand, miRNAs that target p53, a well-known tumor suppressor gene, are considered oncomirs, and their expression is generally up-regulated in cancer. An example of a tumor suppressor miRNA is miR-195-5p that is normally down-regulated and has been observed to show suppressive capabilities in hepatocellular carcinomas (Wang et al 2013). In addition, miRNAs have also been proposed to be used as biomarkers for cancers as a way of identifying the types of cancers that are involved, such as miR-146a-5p as a biomarker for colorectal tumor localization (Iorio et al 2005, Omrane et al 2014).

Though many miRNAs have been studied extensively in many cancers, miRNA regulations in breast cancers – specifically HER2-positive breast cancers – were not

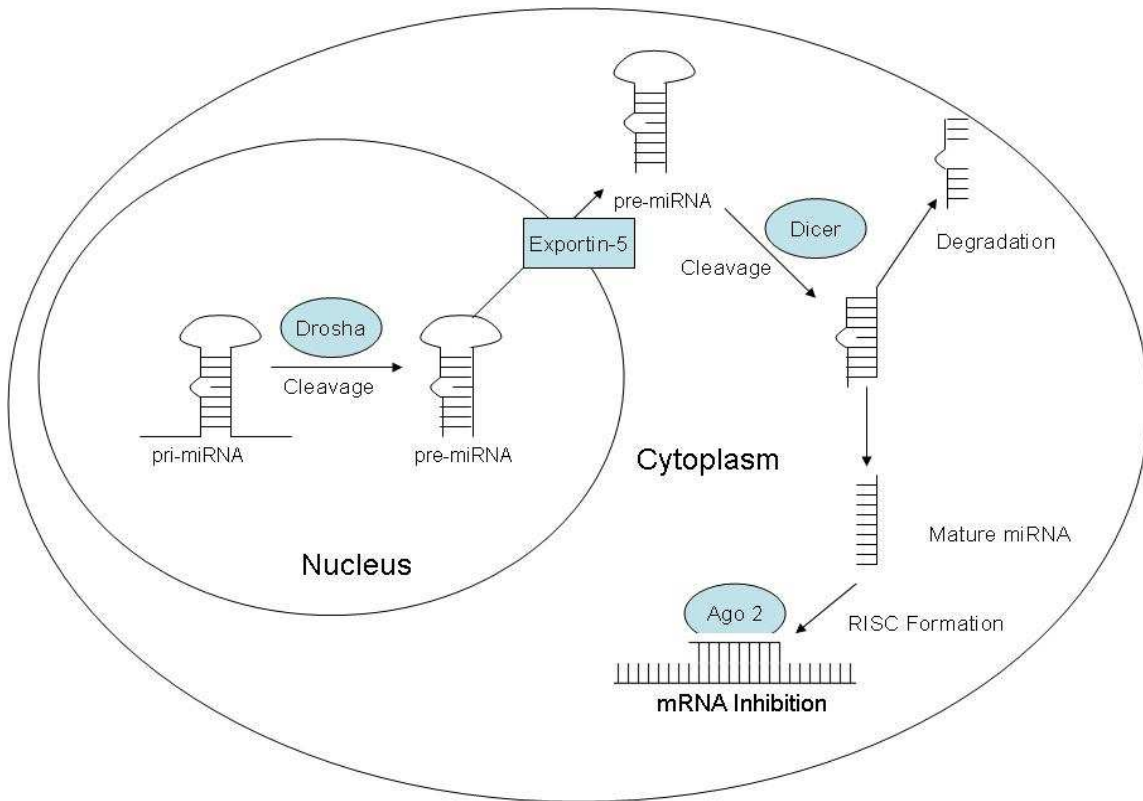


researched as extensively. Consequently, there remains a whole host of miRNAs and their pathways that can be explored to further our understanding of the molecular mechanism by which HER2-positive breast cancer functions. Since HER2 regulates and increases tumor survivability, invasiveness, and metastasis, disrupting the signal cascade that results from the regulation of miRNA may aid in slowing down tumor progression and lead to a better prognosis for patients.

#### **1.4: Clinical implications:**

HER2 positive breast cancer patients are ~30% of the total breast cancer patient population and generally have extremely low prognosis (Korkaya et al 2008). Although some drugs have been established to have some positive effect on HER2 positive cancer, such as trastuzumab, the number of patients that respond to the treatment are low, and the drug activity is diminished after a while (Roy & Perez 2009). Therefore, it is important to better understand the complex HER2 pathway to develop better treatments and drugs that have more specific and effective targets.

Part of understanding the HER2 positive cancer is to understand how miRNAs are involved in the HER2 pathway. It is known that miRNAs are involved in many pathways in cancer and serve as biomarkers for cancer identification. However, miRNAs' involvement in the HER2 pathway has scarcely been studied, and there remain many avenues to discover regarding HER2 and miRNAs. Therefore, we propose that the HER2 overexpression will lead to differential regulation of a number of miRNAs that can help HER2 maintain the cancer's tumorigenicity.



**Figure 1.2: miRNA processing pathway:**

The miRNA processing pathway starts off in the nucleus. Drosha and Dicer are two RNase III enzymes for processing RNA. Pri-miRNA is the primary-miRNA. Pre-miRNA is the prequel-miRNA. Ago 2 is argonaute protein that form the RISC with mature miRNA.

## Chapter 2

### Materials and Methods

#### 2.1: Cell Culture:

MCF10A-Vector and MCF10A-HER2 cells were provided courtesy of Emily Wang, Ph.D. from the Institute of City of Hope. Culturing MCF10A cells requires DMEM/F12 medium from Corning cellgro™. In a 500ml bottle, 25ml of 5% horse serum from Sigma® Life Science is added to the medium along with 100µl of 500µg/ml of cholera toxin for a final concentration of 100ng/ml. In addition, 1ml of 5mg/ml insulin for a final concentration of 10µg/ml, 63µl of 4mg/ml of hydrocortisone for a final concentration of 0.5µg/ml, and 50µl of 200µg/ml of EGF for a final concentration of 20ng/ml are added. When detaching cells from cell culture plate, 0.25% of Trypsin acquired from Sigma® Life Science.

#### 2.2: Western Blot:

Protein extraction was achieved using Triton X. The protein concentration is then quantified and normalized to insure equal loading into polyacrylamide gels. 8% polyacrylamide gels were used to separate the proteins. Antibodies used were acquired from Cell Signaling Technology®.

### **2.3: RNA Extraction:**

MCF10A-Vec and MCF10A-HER2 cells were grown in 6cm cell culture plates to 90% confluency. TRIzol® was used to extract the RNA by adding 1mL per plate. The cells were then scraped off using a scraper and put into a 1.5 ml centrifuge tubes. RNA extraction from this point was done using TRIzol® protocol. RNA concentration was measured using Epoch plate reader (BioTek).

### **2.4: Poly-adenylation:**

The RNA that was extracted was poly-adenylated using *E.coli* poly(A) polymerase from New England Biolabs® inc. 2µg of total RNA was added with 1µl of poly(A) polymerase. 1µl of 10mM adenosine-5'-triphosphate (ATP) from New England Biolabs® inc. was also added. 1µl of recombinant ribonuclease inhibitor named RNaseOut™ from Invitrogen was added to the mix. Then, 2µl 10X reaction buffer from New England Biolabs® inc. was added. Finally, fill the reaction mix was filled to 20µl with RNase-free water from Thermo Scientific. The samples were then incubated at 37°C for 1 hour. After the poly-adenylation, 8µl of the poly-adenylated total RNA was mixed with 6µl of RNase-free water. Added to the mix was 2µl of 10mM of dNTP from Bioline, and 8 µl of 2.5µM of polyT adaptor primer to make a total volume of 24µl. The polyT adaptor primer sequence is as follows:

GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTVN

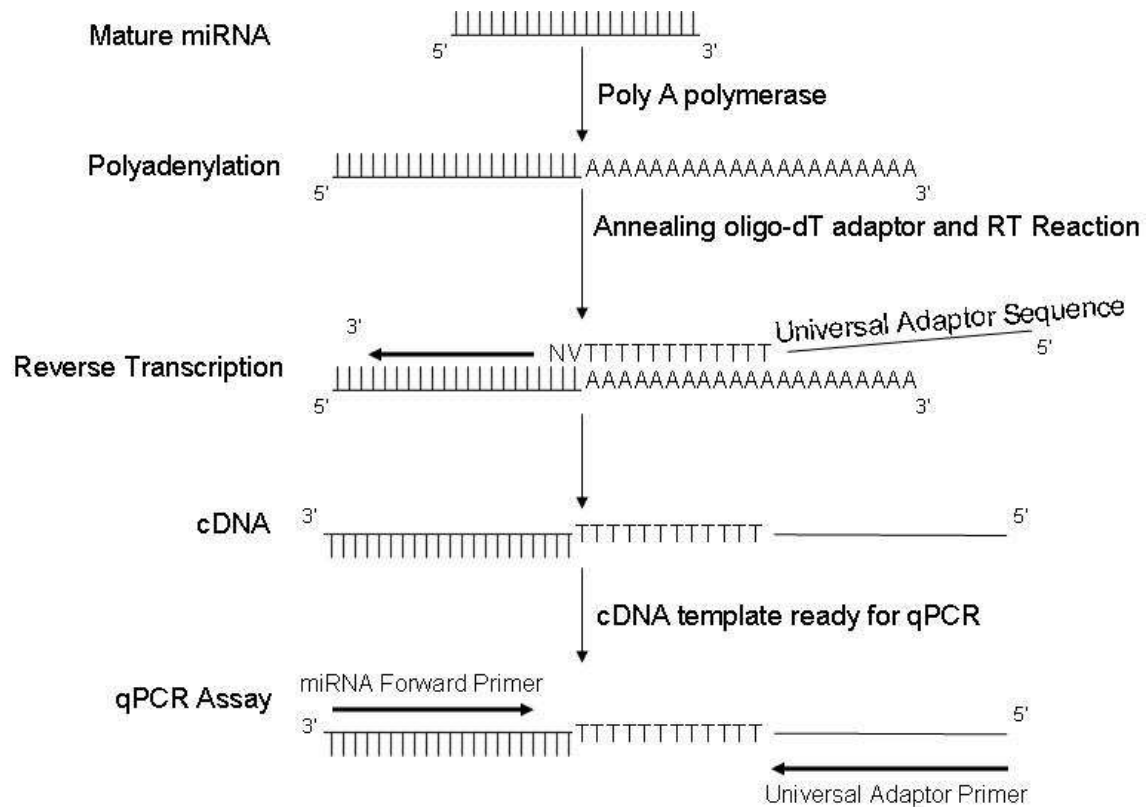
Where N is any nucleotide and V is any nucleotide except thymine (T). The sample was then incubated at 65°C for 5 minutes for the primer to anneal. The samples must then immediately be chilled on ice.

## **2.5: Quantitative reverse-transcription polymerase chain reaction:**

For the reverse-transcription polymerase chain reaction (RT-PCR), the 24 $\mu$ l product from the poly-adenylation step was used and 8 $\mu$ l of 5X first strand buffer from Invitrogen was added to it. Additionally, 4 $\mu$ l of 0.1M DTT and 2 $\mu$ l of RNaseOut™ – both from Invitrogen – was added to the mix for a total of 38 $\mu$ l. The mix was then brought up to 37°C by incubating it for 2 minutes. 2 $\mu$ l of 1X M-MLV reverse transcriptase acquired from Invitrogen brings the final volume of the reaction to 40 $\mu$ l. The reaction was then incubated for 50 minutes at 37°C. The reaction was terminated by incubating the reaction at 70°C for 15 minutes. Finally, the reaction was then diluted by bringing the total volume to 400 $\mu$ l (Figure 2.1). For the quantitative polymerase chain reaction (QPCR), 10 $\mu$ l of RT<sup>2</sup> SYBR® Green ROX qPCR Mastermix from Qiagen was added. In addition, 2 $\mu$ l of cDNA from the previous RT-PCR was added along with 6 $\mu$ l of RNase-free water and 1 $\mu$ l of a universal reverse primer with the following sequence:

GCGAGCACAGAATTAATACGAC

Finally, 1 $\mu$ l of a forward primer specific to the miRNA in question was added to make a total reaction volume of 20 $\mu$ l. The reaction was run in an Applied Biosystem™ qPCR machine with the following parameters: Stage 1: 3 minutes hot start at 95°C. Stage 2: 15 seconds denaturing step at 95°C, followed by 30 seconds annealing step at 60°C, and finally, the extension step was held for 35 seconds at 70°C. This stage was repeated for 45 cycles.



**Figure 2.1: Polyadenylation and QRT-PCR assay:**

Polyadenylation occurs after total RNA extraction. Poly A polymerase adds Adenosine to the end of miRNAs. Annealing of the oligo-dT adaptor to make a cDNA with a universal sequence to all miRNAs. Using miRNA specific forward primer is used on the cDNA to amplify desired miRNAs.

## Chapter 3

### Results

#### **3.1: Many microRNAs are found to be regulated by overexpression of HER2:**

For screening purposes, immortalized human breast cells called MCF10A-Vector and its HER2 positive counterpart was used. To confirm whether the HER2 transformed cells do produce the HER2 receptor, a western blot was performed for evidence (Figure 3.1). The MCF10A-HER2 indeed has a higher expression of the phosphorylated HER2 (p-HER2), which signifies the activation of the HER2 receptor in the cells. The activation of HER2 triggers the activation of the downstream signaling pathway. This downstream pathway activation is affirmed by the phosphorylation of Akt (p-Akt), a direct downstream pathway of HER2, and the phosphorylation of Erk (p-ERK ½), which is downstream of the MEK pathway. In addition, the p65 is a subunit of the NFκB and is activated by phosphorylation when HER2 is overexpressed (p-p65). To further prove that the downstream pathways are dependant on HER2, HER2 was knocked down using siRNA. The result from the knock down shows a decrease in expression of HER2, p-Akt, and p-ERK ½ but not p-p65. NFκB pathway can be regulated by many different pathways other than HER2 which would explain why no change occurs in p-p65.

MCF10A-Vector and MCF10A-HER2 total RNA was used to screen for potential miRNA that are differentially regulated by the overexpression of HER2. 303 miRNA primers were created for 303 different miRNA.

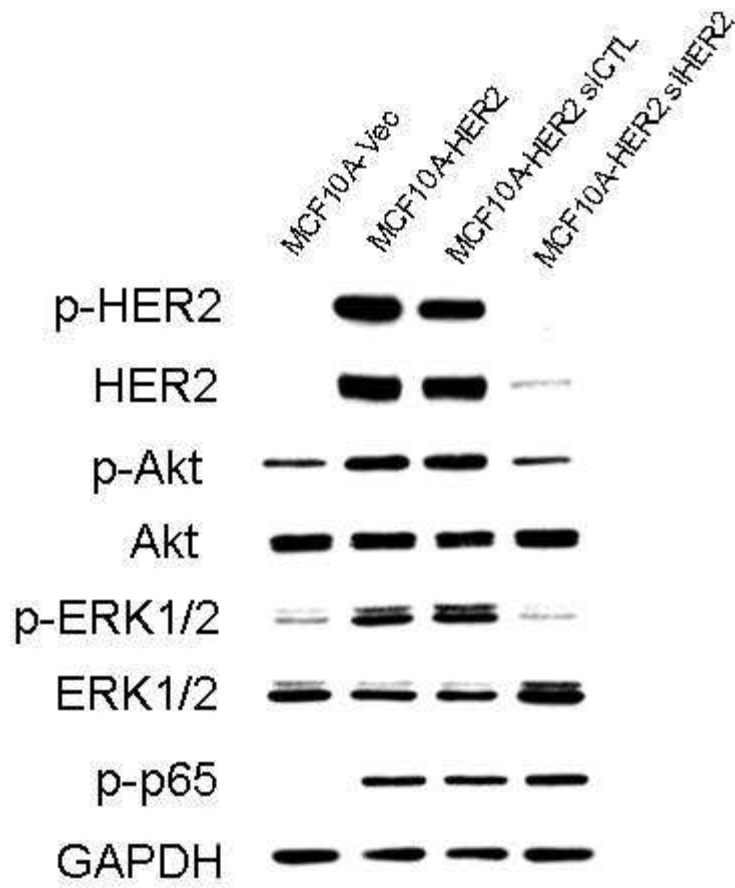
With the cDNA created from the total RNA and the primers, the 303 miRNA expression levels were measured between the MCF10A-Vector and MCF10A-HER2 using quantitative reverse-transcription polymerase chain reaction (QRT-PCR). The results show how many folds the miRNAs have changed from MCF10A-Vector to MCF10A-HER2 cells (Figure 3.2a). However, not all results from the QRT-PCR for the miRNAs are accurate. Normally, the QRT-PCR product is run in an agarose gel to determine if the product of each miRNA primer is correct based on size and number of bands visible. The QRT-PCR product is between sixty five and seventy bases long depending on the size of the mature miRNA being detected. Therefore, the correct size is determined by observing if the product is the correct size, which should be between the fifty base pair (bp) and the one hundred bp markers. The miRNAs in figure 3.2b show sharp bands at the correct size with the exception of miR-22-3p. Therefore, miRNAs that had the wrong size band – higher than roughly one hundred base pairs – or had multiple bands were also eliminated. That is because multiple bands indicate a non-accurate primer that has amplified a multitude of genes. For example, miR-214-3p (Figure 3.2c) shows bands at the wrong size – higher than one hundred base pairs – indicating inaccurate primer. Additionally, miRNAs that show no band, like miR-210-5p (Figure 3.2c), indicate low abundance of those particular miRNAs and are therefore, eliminated. It is important to note that ideally we may be able to visualize a difference between the band in the MCF10-Vector and MCF10-HER2 band like in miR-219-5p (Figure 3.2b). However, the QPCR was run up to forty five cycles and possibly lead to saturation of the amplified miRNAs.



As a result, the miRNA screening has shrunk to 129 miRNAs that show a single band in agarose gels at the correct size (Figure 3.3). Furthermore, expression of cDNA is considered unreliable when the expression is detected passed thirty five cycles in QRT-PCR. In addition, it is known that an up-regulation of only two fold change or higher and a down-regulation of negative two fold change or lower are considered a significant change in regulation. That is due to the general low abundance of each miRNA in a cell. Therefore, miRNAs were eliminated that did not show significant up or down-regulation and had detection at thirty five cycles or higher in both MCF10A-Vector and MCF10A-HER2. Thus, out of these the remaining 129 miRNAs, fourteen miRNAs, were selected which are considered the most reliable and strongest candidates (Figure 3.4).

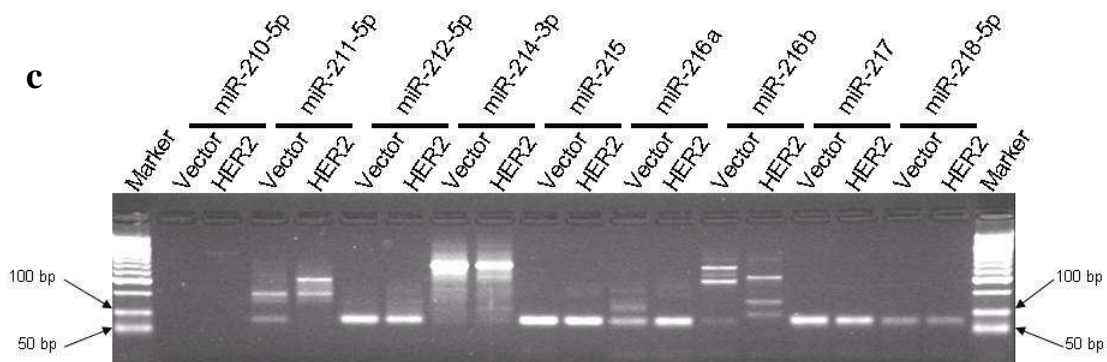
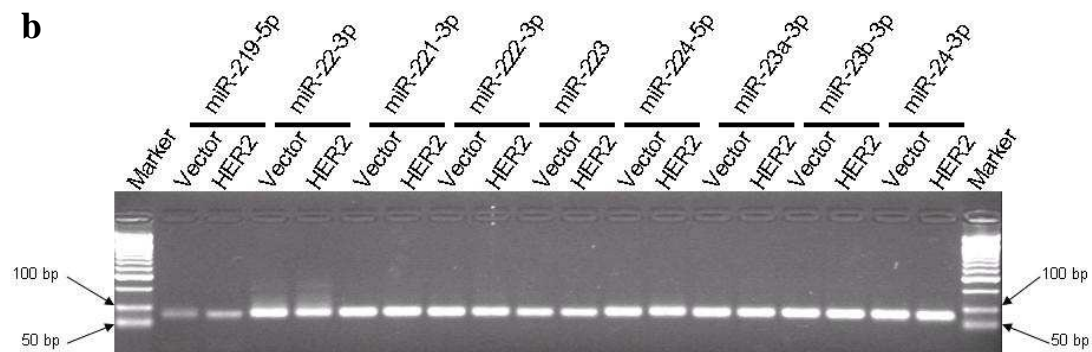
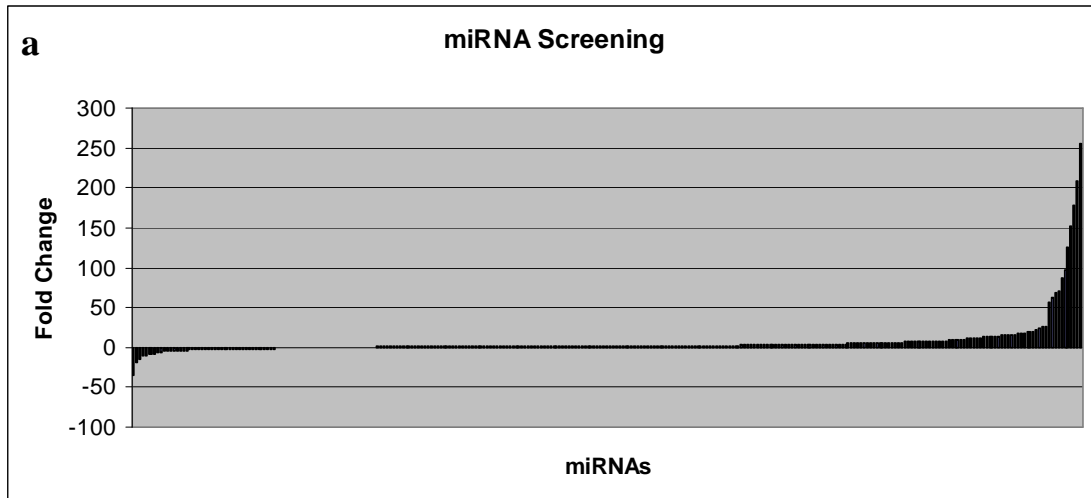
### **3.2: microRNA *in vitro* regulation consistent with clinical data:**

miRNA levels from 1,302 breast tumors were acquired from Dvinge's group where they collected the samples from European tumor banks (Dvinge et al 2013). The data was sorted and selected for patients that have died of breast cancer regardless of HER2 status. Compared with the data in Figure 3.4, only five miRNAs were found to match the differential regulation in that the same miRNAs are either down or up-regulated in both MCF10A-HER2 and HER2 positive tumor samples (Figure 3.5). miR-181d and miR-195-5p show down-regulation in MCF10A-HER2 and in HER2 positive tumor samples as compared with HER2 negative counterparts (Figure 3.5a, 3.5b, 3.5c). In addition, miR-146a-5p shows instead an up-regulation in both the HER2-positive tumor sample and MCF10A-HER2 (Figure 3.5a, 3.5d). Moreover, miR-34a and miR-452-5p show up-regulation in MCF10A-HER2 and in HER2 positive tumor samples (Figure 3.5a,



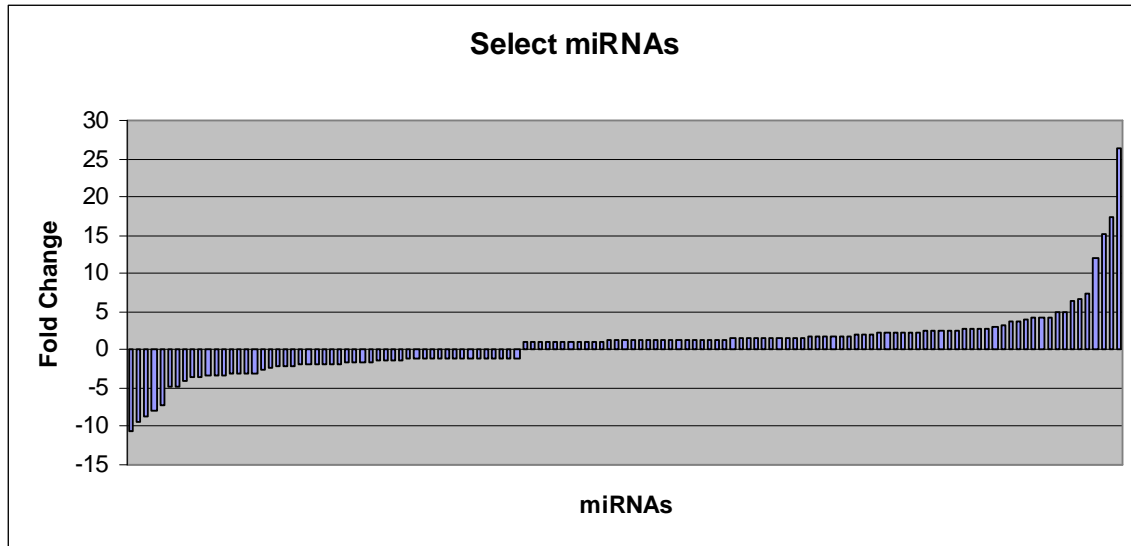
**Figure 3.1: Western blot indicating HER2 overexpression in MCF10A-HER2 compared with MCF10A-Vector and its downstream pathways:**

Western blot analysis indicating the HER2 overexpression in MCF10A-HER2 compared to MCF10A-Vec. The overexpression causes the up-regulation of the downstream pathways of HER2. MCF10A-HER2 siHER2 is knocked down for HER2 and the downstream pathways are knocked down accordingly as compared to MCF10A-HER2 siCTRL.



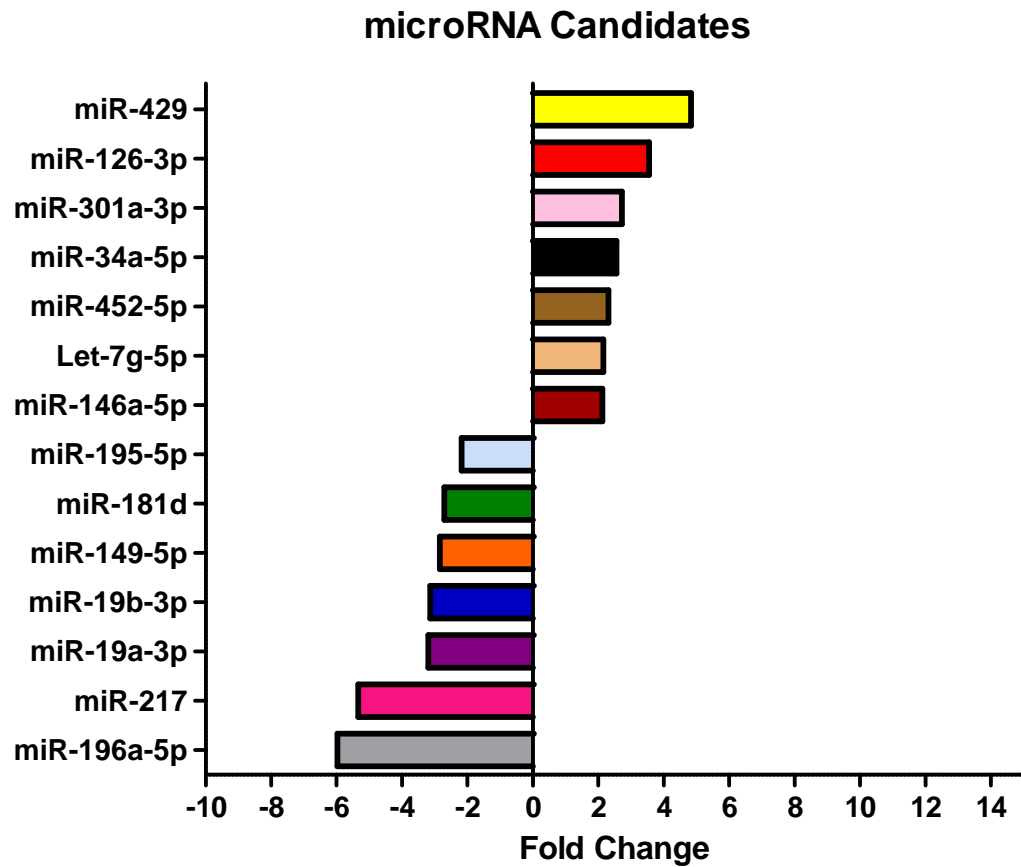
**Figure 3.2: Identification of differentially expressed miRNAs in HER2 overexpressing cells using QRT-PCR analysis:**

MCF10A-Vector and HER2 were used to screen for differentially regulated miRNAs. a) Screening of 303 miRNAs using qRT-PCR and measured according to fold change. b) Agarose gel picture depicting the expression of a sample of qRT-PCR products from the miRNA screening. c) A second agarose gel picture depicting the expression of a sample of qRT-PCR products from the miRNA screening showing ideal size bands.



**Figure 3.3: Selected miRNAs based on qRT-PCR single correct product band size and visibility:**

From the 303 miRNA in the original screening, miRNAs that did not show a band, did not have correct band size, and showed multiple bands were all eliminated. This leaves us with 129 miRNAs that show a single band in the correct size.

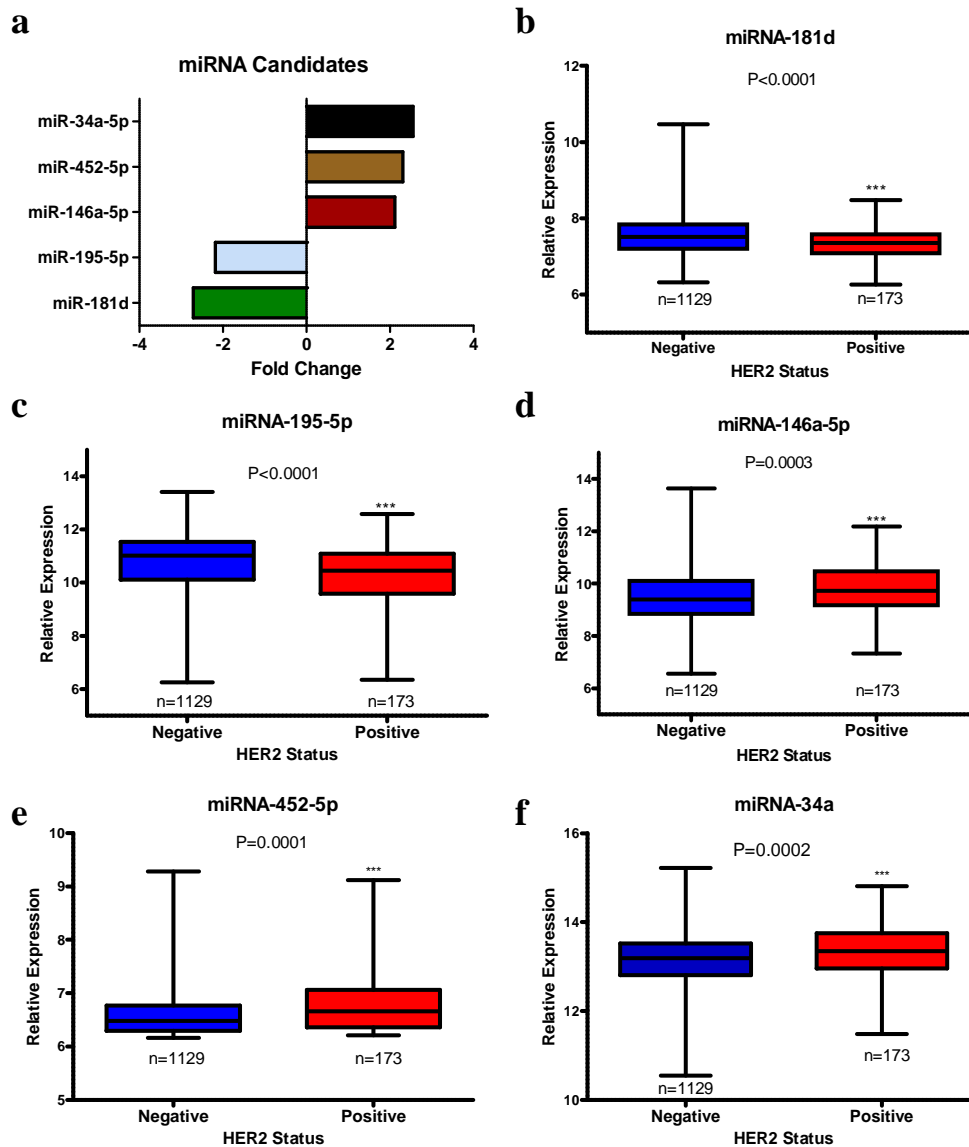


**Figure 3.4: qRT-PCR of selected candidate miRNAs:**

Differential expression of the selected candidate miRNA in MCF10A-HER2. The miRNAs that have negative expression levels are considered down-regulated and have lower levels in MCF10A-HER2 as compared with MCF10A-Vector. miRNAs that have positive expression levels are considered up-regulated and have higher levels in MCF10A-HER2 as compared with MCF10A-Vector.

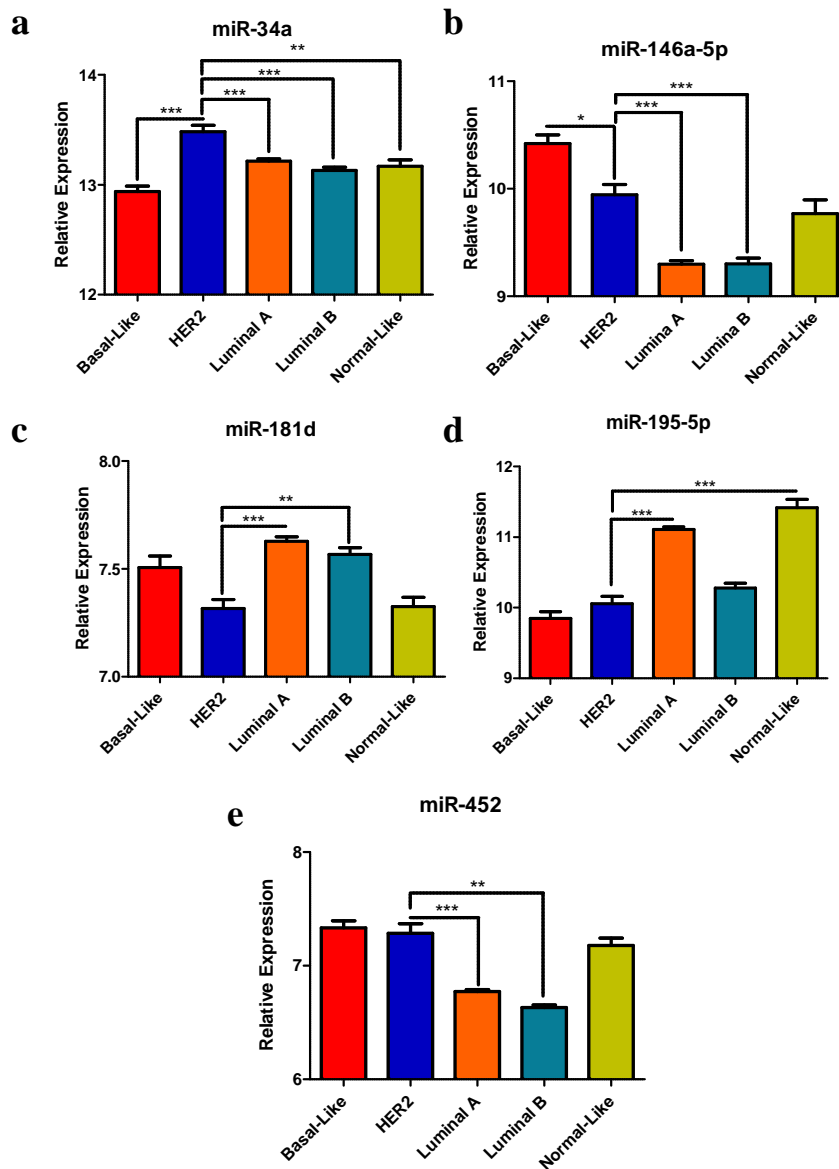
3.5e, 3.5f). Furthermore, the changes in the HER2-positive tumor samples are statistically very significant for all five miRNAs as designated by their P-value.

After narrowing down the miRNAs in five candidates, these five miRNA expression levels were stratified and sorted for each breast cancer subtype. In HER2 positive patients, miR-34a shows high levels of up-regulation, and, consistently, its expression levels are highest in HER2 subtypes in comparison with the other breast cancer subtypes (Figure 3.6a). The second up-regulated miRNA from the five candidates is miR-146a-5p. It is observed that between the five different subtypes, miR-146a-5p expression levels are highest in HER2, basal-like, and normal-like breast cancer subtypes which could be possible due to their similar morphology (Figure 3.6b). The next miRNA is miR-181d, and it is down-regulated in HER2 positive patients. In comparison with the subtypes, miR-181d is down-regulated in HER2 subtypes compared to the luminal subtypes. However, it shows no significant difference between the basal-like and normal-like breast cancer subtypes (Figure 3.6c). The second down-regulated miRNA in HER2 positive patients is miR-195-5p. In comparison with the normal-like and luminal A breast cancer subtypes, the expression level of miR-195-5p is down-regulated significantly (Figure 3.6d). However, miR-195-5p expression levels show no difference between HER2, luminal b, and basal-like subtypes. Finally, miR-452 is up-regulated in HER2 positive patients and is therefore also up-regulated in HER2 breast cancer subtype compared to luminal A and B (Figure 3.6e).



**Figure 3.5: Relative expression levels of the five candidate miRNAs in HER2 positive and negative patient samples:**

Expression levels in both MCF10A-HER2 and HER2 positive tumor tissue of five candidate miRNAs. a) Shows the expression levels of these miRNAs in MCF10A-HER2 using QRT-PCR. b) Relative expression level of miR-181d between HER2 negative and positive tumor tissues (P-value < 0.0001). c) Relative expression level of miR-195-5p between HER2 negative and positive tumor tissues (P-value < 0.0001). d) Relative expression levels of miR-146a-5p between HER2 negative and positive tumor tissues with P-value = 0.0003. e) Relative expression levels of miR-452-5p in HER2 negative and positive tumor tissues (P-value = 0.0001). f) Relative expression level of miR-34a in HER2 negative and positive tumor tissues (P-value = 0.0002).



**Figure 3.6: Relative expression of selected candidate miRNAs in breast cancer subtypes of tumor samples are differentially regulated in HER2 compared to luminal subtypes:**

Relative expression of miRNA distributed according to breast cancer subtype. Expression levels of each miRNA in HER2 subtype is compared against each other subtypes. a) miR-34a expression level. b) miR-146a-5p expression levels. c) miR-181d expression levels. d) miR-195-5p expression levels. e) miR-452 expression levels.



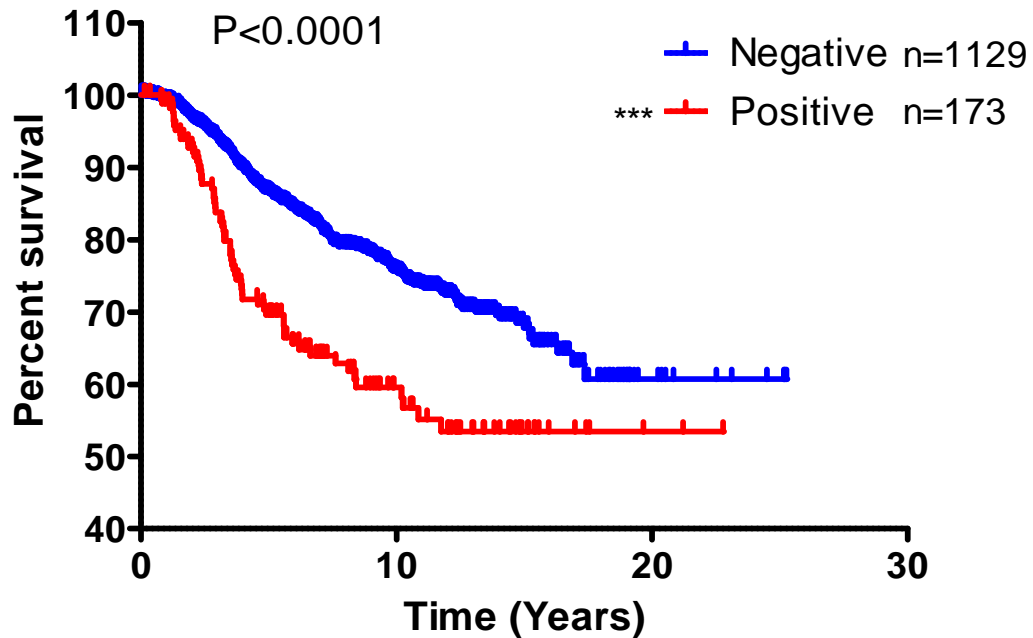
### **3.3: miR-34a and miR-452-5p have no correlation to HER2 positive patient survival:**

Consistent with previous research regarding aggressiveness of HER2 positive cancers, patients whose tumor samples tested positive for HER2 have shown a decrease in survival as compared with HER2 negative tumor samples (Figure 3.7). This affirms the importance of understanding and treating HER2 positive cancers. On the other hand, when it comes to the miRNAs that are affected by HER2 expression levels, miR-34a and miR-452-5p do not show a significant effect on patient survival. In HER2 negative patients, higher levels of miR-34a increases patient survival percentage (Figure 3.8c). However, miR-34a does not seem to have any impact on overall patient survival (HER2 positive and HER2 negative patients) or even HER2 positive patients. In other words, there is no statistically significant percent survival difference between patients with higher levels of miR-34a and patients with lower levels of miR-34a (Figure 3.8a, 3.8b). In addition, miR-452-5p seems to have no impact on breast cancer patient survival. There is no significance of percent survival in general patients, HER2 positive patient percent survival, or even HER2 negative patient percent survival depending on miR-452-5p levels (Figure 3.9). However, this does not mean that these two miRNAs do not have a significant role in HER2 overexpressing cancers. This just means that they do not have a significant effect on patient survival.

### **3.4: Patient survival is significantly affected by three miRNAs:**

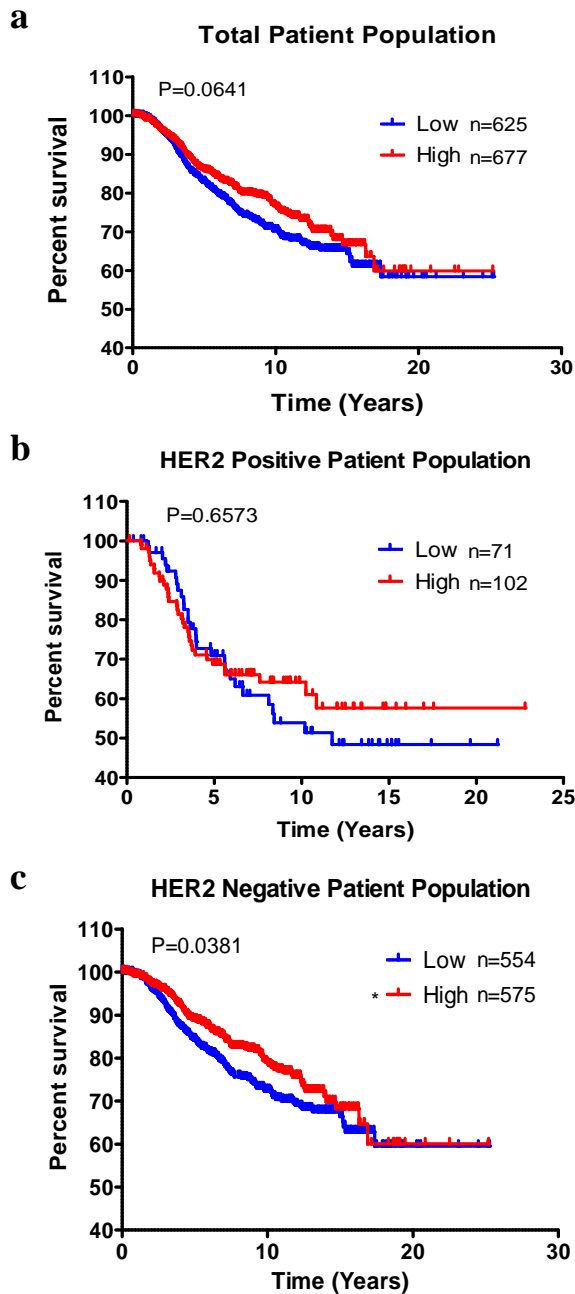
Out of the five miRNAs that were differentially regulated in the same pattern in both MCF10A-HER2 and HER2 positive tumor samples, only three showed a significant impact on patient survivability. The first of these miRNAs is miR-181d. Although miR-

## Survival of Patients Based on HER2 Status



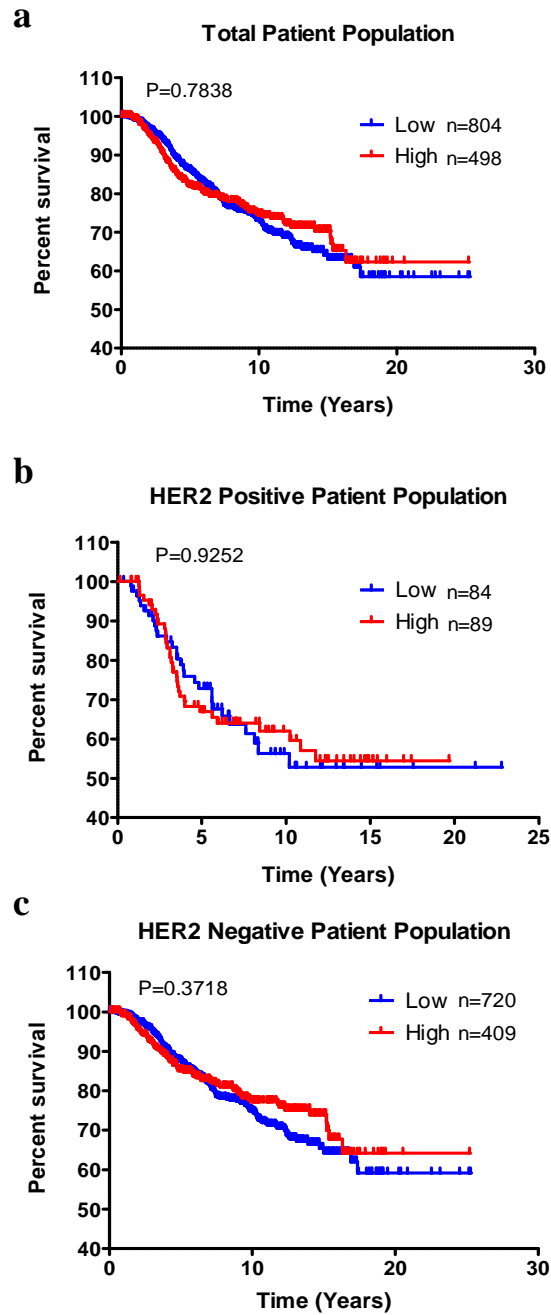
**Figure 3.7: Kaplan-Meier survival curve based on their HER2 status:**

Blue survival curve shows 1129 patients with HER2 negative tumors. 173 Patients with HER2 positive tumors are represented with the red survival curve. The curve is measured in percent of patient survival versus the time in years. P-value <math>< 0.0001</math> for the survival plot.



**Figure 3.8: Kaplan-Meier survival curve according to miR-34a levels:**

Blue survival curve shows patients with lower than average level of miR-34a. Red curve shows patients survival with miR-34a levels that are higher than average. The curve is measured in percent of patient survival versus the time in years. a) General patients' percent survival based on the level of miR-34a (P-value = 0.0641). b) HER2 positive patient's percent survival dependent on miR-34a levels (P-value = 0.6573). c) Survival of patients with HER2 negative tumors based on miR-34a levels (P-value = 0.0381).



**Figure 3.9: Kaplan-Meier survival curve based on miR-452-5p levels:**

Impact of miR-452-5p levels on patient survival. Blue survival curve shows patients with lower than average level of miR-452-5p. Red curve shows patients' survival with miR-34a levels that are higher than average. The curve is measured in percent of patient survival versus the time in years. a) General patients' percent survival dependent on miR-452-5p levels (P-value = 0.7838). b) HER2 positive patients' percent survival based on miR-452-5p expression levels. c) HER2 negative patients' percent survival based on miR-452-5p levels.

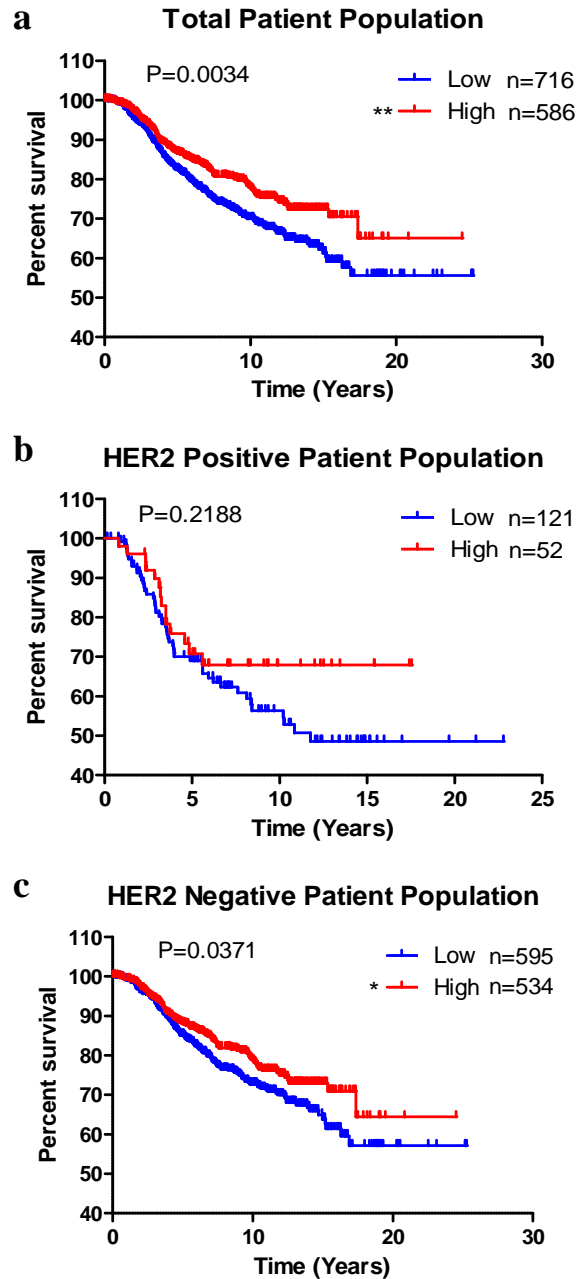
181d shows no significant impact on HER2 positive patient survivability (Figure 3.10b), it does show statistically significant impact on the survival of the general population of patients and in HER2 negative patients' survivability (Figure 3.10a, 3.10c). With miR-181d, the higher its levels are, the higher the percentage of patient survival through time for both HER2 negative patients and the general patient population. Therefore, this indicates that miR-181d does have a significant role or target in the HER2 pathway that affects patient survival positively. Therefore, when HER2 is overexpressed, it down-regulates miR-181d causing it to be unable to function as efficiently. It is also important to note that the number of patients that express higher than average miR-181d in HER2 positive patients (Figure 3.10b) is at a lower ratio than the number of patients expressing higher than average miR-181d in the general patient population and in the HER2 negative patients (Figure 3.10a, 3.10c). In the general patient population and in the HER2 negative patient population, the number of patients in lower and higher than average expression of miR-181d is nearly equivalent as opposed to the HER2 positive patients. This is due to the fact that in HER2 positive patients, the overexpression of HER2 causes a significant down-regulation of miR-181d to be below average levels where having a higher than average expression level is an outlier.

The second miRNA that has a significant impact is miR-195-5p. miR-195-5p shows no significant statistical impact on the percent survival of HER2 positive patients (Figure 3.11b). However, the effect of miR-195-5p is visible when looking at the general population of patients and HER2 negative patients (Figure 3.11a, 3.11c). As shown, miR-195-5p expression levels are directly proportional to patient survivability in the general patient population and in HER2 negative patients. Higher levels of miR-195-5p increases

patient survival and lower levels of miR-195-5p decrease it. These results are similar to that of miR-181d and would produce the same speculation that miR-195-5p targets an oncogene. When HER2 is overexpressed, it down-regulates miR-195-5p, causing it to be unable to efficiently inhibit the translation of the oncogene's mRNA. Likewise, it is also important to note the sample numbers for each population of patients. Unlike miR-181d, miR-195-5p's general patient population is not distributed in equal ratios between the above and below average miR-195-5p expression (Figure 3.11a). The population of HER2 negative patients is also not distributed evenly between miR-195-5p that is above or below average expression levels (Figure 3.11c). However, when looking at the population distribution in HER2 positive patients, we are able to notice that the number of patients that are expressing lower than average miR-195-5p is more than the number of patients expressing above average miR-195-5p (Figure 3.11b). This trend also follows that of miR-181d in HER2 positive patients where the number of patients expressing lower than average miR-181d is more than the patients expressing above average miR-181d levels (Figure 3.10b). Like miR-181d, the reason for this distribution of patients in HER2 positive patients is because HER2 overexpression causes a down-regulation of miR-195-5p in most patients that an above average expression level is an outlier.

Finally, the third miRNA that is significantly impacting patient survivability is miR-146a-5p. HER2 positive patients show no significant impact due to the levels of miR-146a-5p (Figure 3.12b). However, as with the previous two miRNAs, the impact of miR-146a-5p on survivability is most evident in the general patient population and HER2 negative patients (Figure 3.12a, 3.12c). For both the general patient population and HER2 negative patients, the higher the levels of miR-146a-5p, the lower the patients' percent

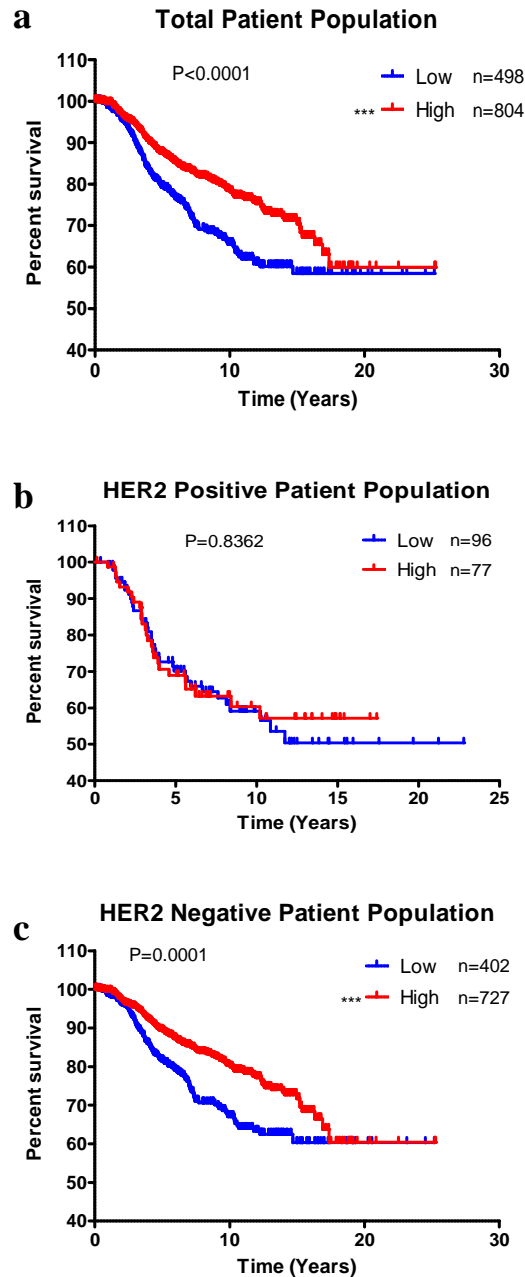
survival, making miR-146a-5p inversely proportional to patient survivability. Opposite from what is observed in miR-181d and miR-195-5p, the number of patients expressing above average miR-146a-5p is larger than the number of patients expressing lower than average miR-146a-5p in HER2 positive patients (Figure 3.12b). That is due to the overexpression of HER2 where it up-regulates miR-146a-5p in the majority of patients and that patients that have lower than average miR-146a-5p expression are outliers. However, the patient distribution between above and below average miR-146a-5p expression in the general patient population shows a shift towards the lower than average expression and HER2 negative population shows an even distribution.



**Figure 3.10: Kaplan-Meier survival curve based on miR-181d levels:**

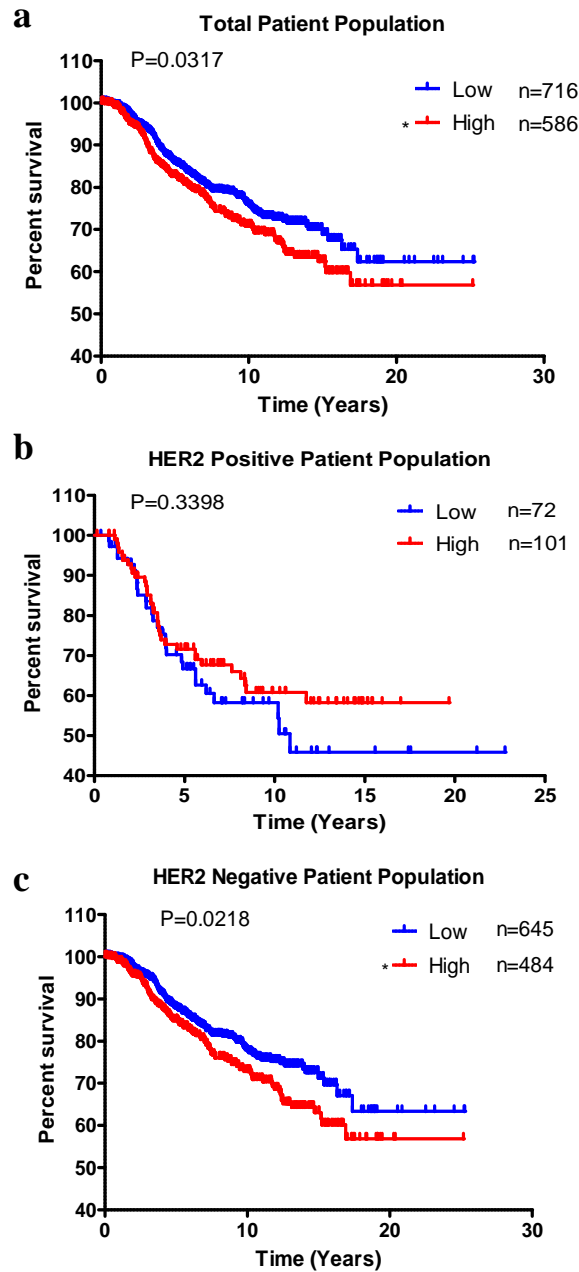
miR-181d's levels' effect on patient survival. Blue survival curve shows patients with lower than average level of miR-181d. Red curve shows patients' survival with miR-34a levels that are higher than average. The curve is measured in percent of patient survival versus the time in years. a) General patients' percent survival based on the level of miR-181d (P-value = 0.0034). b) Survival of patients with HER2 positive tumors based on miR-181d levels (P-value = 0.2188). c) HER2 negative patient's percent survival dependent on miR-181d levels (P-value = 0.0371).





**Figure 3.11: Kaplan-Meier survival curve according to miR-195-5p levels:**

miR-195-5p's levels' effect on patient survival. Blue survival curve shows patients with lower than average level of miR-195-5p. Red curve shows patients' survival with miR-195-5p levels that are higher than average. The curve is measured in percent of patient survival versus the time in years. a) General patients' percent survival based on the level of miR-195-5p (P-value < 0.0001). b) Survival of patients with HER2 positive tumors based on miR-195-5p levels (P-value = 0.8362). c) HER2 negative patients' percent survival dependent on miR-195-5p levels (P-value = 0.0001).



**Figure 3.12: Kaplan-Meier survival curve according to miR-146a-5p levels:**

miR-146a-5p's levels' effect on patient survival. Blue survival curve shows patients with lower than average level of miR-146a-5p. Red curve shows patients' survival with miR-146a-5p levels that are higher than average. The curve is measured in percent of patient survival versus the time in years. a) General patients' percent survival based on the level of miR-146a-5p (P-value = 0.0317). b) Survival of patients with HER2 positive tumors based on miR-146a-5p levels (P-value = 0.3398). c) HER2 negative patients' percent survival dependent on miR-146a-5p levels (P-value = 0.0218).

## Chapter 4

### Discussion

Since their discovery in 1993, miRNAs have been extensively studied for over two decades with many exciting results (Lee et al 1993). However, there are over a thousand human miRNAs that have so far been discovered and only a small portion has been studied. Furthermore, within the small portion of miRNAs that have been studied, there remains many areas of each single miRNA to be studied whether in regulation of human physiology or the impact of these miRNAs on any type of cancer and its related hallmarks. Typically, the most recently discovered miRNAs are the most extensively studied due to their high abundance in cells. This high abundance is the reason for these miRNAs' early detection as compared to miRNAs that are detected much later in the years. Moreover, miRNAs in HER2 positive breast cancers have not been intensively studied leaving much information still unknown about HER2 positive breast cancer and its interaction with miRNAs.

Starting off, it is important to note that our data shows the significance of how overexpression of HER2 affects patient survival. In confirmation with many previous research on HER2, it is shown in this population sample that overexpression of HER2 does indeed decrease patient survival with a very high statistical significance. MCF10A cells are known as immortalized breast epithelial cells that can serve as non-cancerous control cells. By overexpressing HER2 in MCF10A, the effect of overexpression can be

studied as this overexpression is the only significant variable. As part of the screening, many miRNAs are observed to be differentially regulated based on the HER2 expression levels in MCF10A cells. However, not all miRNAs' differential expression based on HER2 translates into cancerous cells or tumor samples. Therefore, only a small number of miRNA – specifically five – maintain their trend of expression. In other words, miRNAs that are up-regulated in MCF10A-HER2, such as miR-146a-5p, miR-34a, and miR-452-5p, are indeed up-regulated in HER2 positive tumor samples as compared with HER2 negative tumor samples; and in addition, miRNAs that are down-regulated in MCF10A-HER2 such as miR-181d and miR-195-5p, are in fact down-regulated in HER2 positive tumor samples as compared with HER2 negative tumor samples. Thereby, the miRNAs from the original screening are narrowed down from around 303 miRNAs to only five potential targets.

To further confirm the differential regulation of these miRNA based on HER2, we can see in figure 3.4 that these miRNA are expressed in the same profile in HER2 breast cancer subtype compared with luminal A and luminal B subtypes. It is worth noting that miR-34a is the miRNA that shows the most significant difference in HER2 subtypes compared to all other breast cancer subtypes. This indicates that miR-34a is possibly the miRNA that is most affected by HER2 expression as the major regulator. Another interesting miRNA when speaking about the breast cancer subtypes is miR-146a-5p. In the HER2 subtypes, miR-146a-5p is significantly higher when compared with the luminal subtypes as is expected. In addition, the luminal A and B subtypes show a surprisingly very low expression level. This could be possibly due to the fact that the luminal subtypes are ER positive. It has been shown that estrogen expression down-regulates the activation

of NFκB by enhancing IκB levels (Xing et al 2012); and that with NFκB is positively correlated with the expression of miR-146a-5p (Taganov et al 2006). However, the basal-like breast cancer subtype shows an even higher expression level than that of the HER2 subtypes. This may be due to the fact that some basal-like tumors, though they are triple-negative, are HER2 enriched (Perou 2011). With this HER2 enrichment – coupled with the absence of ER – miR-146a-5p expression is further amplified. The last three miRNAs are miR-195-5p, 181d, and 452-5p, and they show their expected expression profile when compared with the luminal subtypes. However, when compared with the basal-like and normal-like subtypes, due to the fact that basal-like is enriched with HER2 and the morphological resemblance and aggressiveness of normal-like, the HER2 subtype miRNA expression levels show no difference. The major possibility is, due to the similarity between these three subtypes, cross regulation from other pathways can affect the expression of these three miRNA resulting in no significant difference.

However, these five miRNAs are nothing more than potential targets which need further testing to validate as legitimate factors in patient survival. As such, when searching through the data from the Dvinge group in regards to patient survivability, two miRNAs begin to show their insignificance to patient survival between HER2 positive and HER2 negative patients. The first of those miRNAs is miR-452-5p. Although miR-452-5p shows up-regulation in MCF10A-HER2 and in HER2 positive tumor samples, its effect on the patients from which the tumor samples were taken from is statistically insignificant. This insignificance is evident in all of miR-452-5p on patient survival, including general patient population survival based on miR-452-5p expression levels. The second of those miRNAs is miR-34a. Even though miR-34a generally follows the

same trend as miR-452-5p, it does have a statistically significant effect on HER2 negative patient survival. However, miR-34a does not show any significant effect on patient survival in both the general patient population and on HER2 positive patient survival. This does not mean that its effect on the HER2 negative patient survival can be ignored; it can simply be that it is not the major player of patient survival but a co-contributor. Finally, while these two miRNAs do not have a major effect on patient survival, they can have other effects, such as tumor initiation or tumor progression.

The next three miRNAs from the original five are observed to have a very statistically significant impact on patient survival. The first of these three miRNAs is miR-146a-5p. The fact that there have been many reports of miR-146a-5p being up-regulated in many cancers, including breast cancer, helps support the fact that miR-146a-5p can have a significant impact on patient survival in HER2 positive breast cancer patients (Labbaye & Testa 2012). In this study, miR-146a-5p is indeed up-regulated in HER2 positive breast cancer patients and in MCF10A-HER2. This indicates that miR-146a-5p is up-regulated directly or indirectly by HER2 overexpression.

Furthermore, it is observed that the up-regulation of miR-146a-5p decreases patient survival in both HER2 negative patients and in the general patient population. This means that miR-146a-5p is an oncomir should target a tumor suppressor gene that would control the tumor aggressiveness, for example. However, miR-146a-5p does not cause a significant change in HER2 positive patients. The reason for this is because in HER2 positive patients generally observe above average miR-146a-5p levels due to the overexpression of HER2. Therefore, any increase in up-regulation is not going to have a higher decrease in patient survival because miR-146a-5p is already above the threshold.

The second miRNA that has considerable impact on patient survival is miR-195-5p. Like miR-146a-5p, miR-195-5p has been extensively studied, especially in breast cancer, as it was found to be down-regulated (Ouyang et al 2014). It has also been suggested as a possible diagnostic target for breast cancer (Luo et al 2014). Thus, miR-195-5p is solidified as an important target in breast cancer and supports the data in this study. In accordance with previous research, miR-195-5p is down-regulated in MCF10A-HER2 and also in HER2 positive. These results thus supports the idea that miR-195-5p is regulated by the level of HER2 expression. However, it is unknown how this regulation occurs whether it is direct or indirect.

Moreover, miR-195-5p is shown to be directly proportional to the percent survival of the general patient population and the HER2 negative patients – the same two samples as miR-146a-5p. This means that the lower the levels of miR-195-5p, the lower the chance for patient survival. This further means that miR-195-5p is a tumor suppressor miRNA that targets an oncogene that would normally have an adverse effect on the patient. Like miR-146a-5p, miR-195-5p shows no significant effect on patient percent survival in HER2 positive patients. The reason is also similar to miR-146a-5p in that miR-195-5p is down-regulated below the threshold with the overexpression of HER2 in HER2 positive patients; and that when the levels are already below the threshold, any change in the levels would not have any more impact than what has already been observed.

The final and third miRNA that has a significant effect on patient survivability is miR-181d. Unlike the previous two miRNAs, miR-181d has not been extensively studied. The amount of information and connection between miR-181d and cancer is scarce, and

it is even more so in HER2 positive breast cancers. However, it has been observed that the miR-181 family (miR-181a, miR-181b, miR-181c, and miR-181d), more precisely miR-181c, is activated by HER2 expression (Lowery et al 2009). Even though miR-181c is possibly co-expressed or repressed with miR-181d, there has not been much research done regarding this co-expression or repression. Therefore, it is plausible that miR-181d is down-regulated with the overexpression of HER2 in both MCF10A-HER2 and HER2 positive patients.

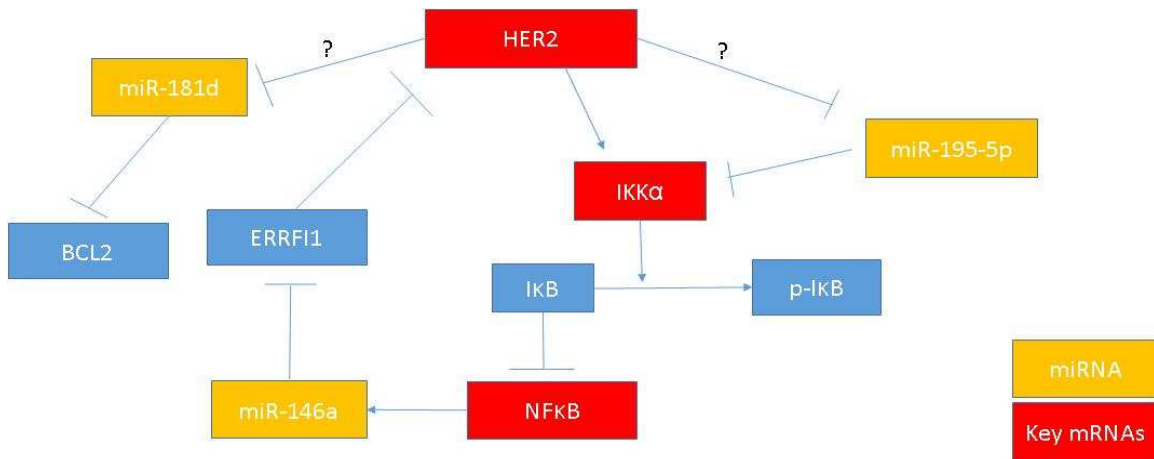
Like miR-195-5p, miR-181d is shown to be directly proportional to patient survival in both HER2 negative patients and the general patient population. These two populations are again the same two populations affected by the two miRNAs that have been previously discussed. Along the same previous lines, the higher levels of miR-181d the higher the chances for patient survivability. Therefore, miR-181d can be classified as a tumor suppressor miRNA targeting an oncogene. Similar to the two previously discussed miRNA, miR-181d shows no significant effect on patient survival in HER2 positive patients. The explanation for this phenomenon is the same with miR-195-5p in that miR-181d is down-regulated below the threshold with the overexpression of HER2 in HER2 positive patients; and that when the levels are already below the threshold, any change in the levels would not have any more impact than what has already been observed.

Finally it is important to understand how all these miRNA tie in together in the HER2 pathway and how they affect the behavior of HER2 positive breast cancer. From previous research, HER2 has been discovered to directly activate the NF- $\kappa$ B independent of PI3K/Akt protein by activating I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ). Activation of IKK $\alpha$  causes the



phosphorylation of I $\kappa$ B, which stops its inhibitory activity on NF- $\kappa$ B. Interestingly, induction of miR-146a-5p is positively dependent on the activation of NF- $\kappa$ B (Taganov et al 2006). Furthermore, ERBB receptor feedback inhibitor 1 (ERRFI1) which can target and inhibit HER2 (Segatto et al 2011), is suggested to be a potential target of miR-146a-5p (Garcia et al 2011). Although this potential target needs experimental confirmation, if it is true, then this interaction can explain why HER2 positive cells can keep overexpressing HER2 without hindrance; and that would be due to miR-146a-5p inhibiting ERRFI1. However, the complex pathway does involve at least one more component and that is miR-195-5p. Research has shown that miR-195-5p down-regulates the NF- $\kappa$ B pathway by targeting and thereby inhibiting IKK $\alpha$  (Ding et al 2013). Therefore, it would down-regulate the expression of miR-146a-5p. Unfortunately, miR-195-5p is down-regulated and has no effect on the expression of miR-146a-5p in HER2 overexpressing tumors. However, there are two unknown factors in this pathway we propose (Figure 4.1). These two factors are how both miR-195-5p and miR-181d are regulated by HER2, whether it is directly or indirectly by HER2's many down-stream proteins.

In conclusion, with better understanding of the HER2 pathway, we are better able to treat HER2 positive tumors. With the understanding that miR-146a-5p, miR-181d, and miR-195-5p are differentially regulated by HER2, it is possible to use these miRNAs as potential targets for drugs designed to inhibit miR-146a-5p for example. It is also possible to hinder tumor progression by inducing the production of miR-195-5p or miR-181d. Finally it is possible to use any of these miRNA as possible biological markers for tumors that can be detected early.



**Figure 4.1: Proposed regulation model of HER2 pathway by the selected miRNAs:**

HER2 pathway involving miR-195-5p, miR-146a-5p, and miR-181d. Inhibition of miR-195-5p by HER2 directly or indirectly is unknown. Inhibition of miR-181d directly or indirectly is unknown. miR-195-5p targets IKK $\alpha$  which eventually effects miR-146a-5p.

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