Expression and function of microRNAs in HER2+ breast cancer

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Master Thesis 60 credits 2012

Breast cancer is not just a disease that strikes women. It strikes at the very heart of who we are as women: how others perceive us, how we perceive ourselves, how we live, work and raise our families-or whether we do these things at all.

-Debbie Wasserman Schultz

Aim of the study

Breast cancer is the most frequent cancer type in women and approximately 20% of breast cancer patients are positive for the epidermal growth factor receptor 2 (ERBB2/HER2). This type of breast cancer is defined by having the chromosome region 17q12-21, where the *HER2* gene is mapped, amplified which leads to overexpression of the HER2 receptor. microRNAs (miRNAs) are small non-coding RNAs that have been shown to play a regulatory role in cancer, including HER2+ breast cancer. Although there has been extensive research on both miRNAs and the HER2+ cancer, little is known about the role that miRNAs play in HER2+ cancer development. In 2011, five miRNAs were mapped within the HER2 amplicon. However, the functional role of these miRNAs has not been evaluated. Therefore, we wanted to study the role of miRNAs in HER2+ breast cancer cell lines, both responsive and non-responsive to Trastuzumab. The specific aims of this study were:

1. Study the genome-wide expression of miRNAs in HER2+ breast cancer cell lines in relation to Trastuzumab response by searching for differentially expressed genes between Trastuzumab responsive and non-responsive cell lines.
2. Investigate the expression level of five novel miRNAs mapped to the HER2 amplicon, and study the functional role of these miRNAs in HER2 breast cancer cells.

The overall aim of the present study was to increase the knowledge about miRNAs and their role in HER2+ cancer.

Acknowledgments

The study was performed at the Oslo University Hospital, Institute for Cancer Research, Department of Genetics as part of the Master program in Biotechnology at the Norwegian University of Life Sciences (UMB), The Department for Chemistry, Biotechnology and Food Science (IKBM).

I would like to direct my gratitude to Prof. Anne-Lise Børresen-Dale for allowing me to join the research group at the Department of Genetics and providing this opportunity for my study. A special thanks goes to my supervisor Dr. Kristine Kleivi Sahlberg for planning a very interesting research project and for support and guidance during the experiments and for support in putting together this thesis. My co-supervisors Hege Edvardsen and Suvi-Katri Leivonen have given me extensive support and guidance, especially during Kristines maternity leave, a big thanks to you both! I would also like to thank my supervisor at UMB Prof. Odd Arne Rognli.

Thanks to Sandra Nyberg and Grethe I Grenaker Alnæs. Sandra for support in the functional studies in the cell lab, you have been great help. Grethe for support and guidance during the qRT-PCR, thanks a lot for your patience! Many thanks to all of the people at the department, for making an inspirational working condition and for making me feel very welcome.

This year would not have been the same without the other master student at the department, you are all fantastic! My boyfriend, friend and family has all been a great support, I would not be here if it weren't for you.

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13.05.2

Sammendrag

miRNAer er små, ikke-kodene RNA som har bliit påvist å regulere mange viktige mekanismer i cellen. miRNA har også blitt knyttet til utviklingen av flere sykdommer, som blant annet kreft. Blant kvinner er brystkreft den vanligste krefttypen, og 20% av alle brystkrefttilfeller er HER2+, en type brystkreft som er forbundet med dårlig prognose. Pasienter som blir diagnisert som HER2+ blir gitt en målrettet terapi, Trastusumab, men halvparten av pasientene responderer ikke på behandlingen eller blir resistente over tid. Lite er kjent om rollen miRNA spiller i HER2+ brystkreft, og om de har enn effekt på Trastuzumab respons.

For å undersøke om miRNA er viktige i forhold til Trastuzumab-respons i HER2+ cellelinjer, ble differesiellt uttrykte miRNA detektert mellom Trastusumab responsive og ikke responsive cellelinjer ved å analysere miRNA ekspresjonsarray data. Fire miRNAer: miR-342-3p, miR-15a, miR-26b og miR-29a ble identifisert som differensielt uttrykt mellom de to gruppene. Tre av miRNAene, miR-15a, miR-26b og mir-29a, har blitt forbundet med utviklingen av kreft.

Fem miRNA ble nylig lokalisert innenfor *HER2* amplikonet, og var derfor ikke tilstede på ekspresjonsarrayet. For å undersøke ekspresjonen av disse fem miRNAene i HER2+ cellelinjer ble det utført qRT-PCR. To av miRNAene i ampliconet, miR-4726-3p og miR-4728-5p, ble detektert i nesten alle cellelinjene. Disse ble derfor valgt til videre funksjonelle studier hvor funksjonen av disse miRNAene i HER2+ cellelinjer ble studert. Ved å hemme funksjonen av disse miRNAene så vi reduksjon i levedyktighet i cellene. Ved å hemme funksjonen til miR-4726-3p førte det til en reduksjon i levedyktighet på ca 20% to cellelinjer som ble testet. Når funksjonen til miR4728-5p ble hemmet førte dette til en reduksjon på ca 15%, i kun den ene cellelinjen.

I dette studiet har vi identifisert differensielt uttrykte miRNA forbundet med Trastuzumab respons og studert den funksjonelle rollen til miRNA i *HER2* ampliconet og vist at miRNAer spiller en rolle i HER2+ brystkreft.

Abstract

miRNAs are small non-coding RNAs that have been found to regulate several important mechanisms in the cells. miRNAs have been linked to the development of several diseases, including cancer. Breast cancer is the most common cancer type in women, and 20% of all breast cancers are HER2+, which are associated with poor prognosis. These patients receive Trastuzumab, a targeted therapy directly inhibiting the HER2 receptor. However, about half of the patients do not respond or become resistant to this treatment. Little is known about the role miRNAs play in HER2+ breast cancers, and their possible association with Trastuzumab response.

To investigate the putative role of miRNAs in relation to Trastuzumab response, miRNA expression arrays were used to search for differentially expressed genes between Trastuzumab responsive and non-responsive HER2+ cell lines. Four miRNAs: miR-342-3p, miR-15a, miR-26b and miR-29a were identified as differentially expressed, and three of the miRNAs: miR-15a, miR-26b and miR-29a, are found to be related to cancer development. There were several oncogenes among the validated miRNA targets, and the target genes were found to be involved in pathways related to cancer.

Five miRNAs were recently mapped within the *HER2* amplicon, and were therefore not present on expression arrays. We used qRT-PCR to study the expression levels of these novel miRNAs in the same panel of HER2+ cell lines. Two of the miRNAs, miR-4728-5p and miR-4726-3p, were found to be expressed in almost all of the cell lines, and these were chosen for knock-down experiments to evaluate whether they were functionally important in HER2+ cancer cells. Knocking down the function of miR-4726-3p led to a reduction in cell viability in two cell lines tested, whereas knocking down the function of miR-4728-5p gave a small growth reduction in only one of the cell lines.

In this study we have identified differentially expressed miRNAs associated with Trastuzumab response and studied the functional role of miRNAs in the HER2 amplicon showing that miRNAs plays a role in HER2+ cancers.

Contents

1 Introduction

1.1 Cancer - a genetic disease

Cancer caused 7.6 million deaths in 2008, accounting for approximately 13% of the deaths in the world. The most common types of cancer in men are lung, prostate and colon cancer, whereas for women the most common are breast, colon and lung cancers. Breast cancer alone caused 460 000 deaths worldwide in 2008 (1;2).

Cancer is a genetic and epigenetic disease which may occur when genes that are involved in the maintenance and homeostasis of normal tissue are impaired. These changes might give growth advantages which can lead to uncontrolled cell growth and tumor development. The tumor cells have the potential to invade the surrounding tissue and may eventually spread to distant part of the body.

The genes involved in mechanisms related to cancer development can be divided into three groups: tumor suppressor genes, oncogenes and repair genes. An oncogene is a gene whose presence can trigger the development of cancer. Most oncogenes code for proteins involved in growth signaling pathways, and in this way they can promote uncontrolled growth of the cell. On the contrary, tumor suppressor genes are genes that have repressive effect on cell proliferation and whose loss or inactivation can lead to cancer (3). Repair genes are genes that codes for proteins involved in the repair of DNA damage. If these genes and their function are altered, damage to the DNA might not be repaired which can lead to the development of cancer.

Cancer is a progressive disease that gradually develops over time, often over many years. Disruption of genes associated with cancer can be caused by point mutations, insertion, deletions, amplification, and translocation or more indirectly by epigenetic changes. These alterations can be caused by replication errors during cell divisions or exposure to mutagens, radiation or viruses. Some mutations in critical genes can also be inherited leading to an elevated risk for cancer development (4).

There are certain essential alterations, also called hallmarks, in the cells physiology which are necessary for tumor development. In 2000, Hannahan and Weinberg

suggested six hallmarks of cancer and published an updated article in 2011 where two emerging hallmarks were included (5;6). They also suggested two enabling characteristics which make the cells achieve and obtain these hallmarks (Fig. 1). The hallmarks of cancer include avoiding growth suppressors, sustaining proliferation signals, inducing angiogenesis, resisting apoptosis and activating invasion and metastasis. The two emerging hallmarks are reprogramming of energy metabolism and avoidance of the immune system. Cancer cells do not work alone to generate the most favorable conditions for further growth and invasion, instead they work together with the surrounding cells. The surrounding stroma of epithelial cells contains various cells such as fibroblasts, endothelial cells and leukocytes that regulate the epithelial cells via paracrine, hormonal and physical interactions. The tumor cells recruit these non-neoplastic cells to obtain the hallmark traits by making a favorable tumor microenvironment. These features are essentials for tumor cell initiation and development (5).

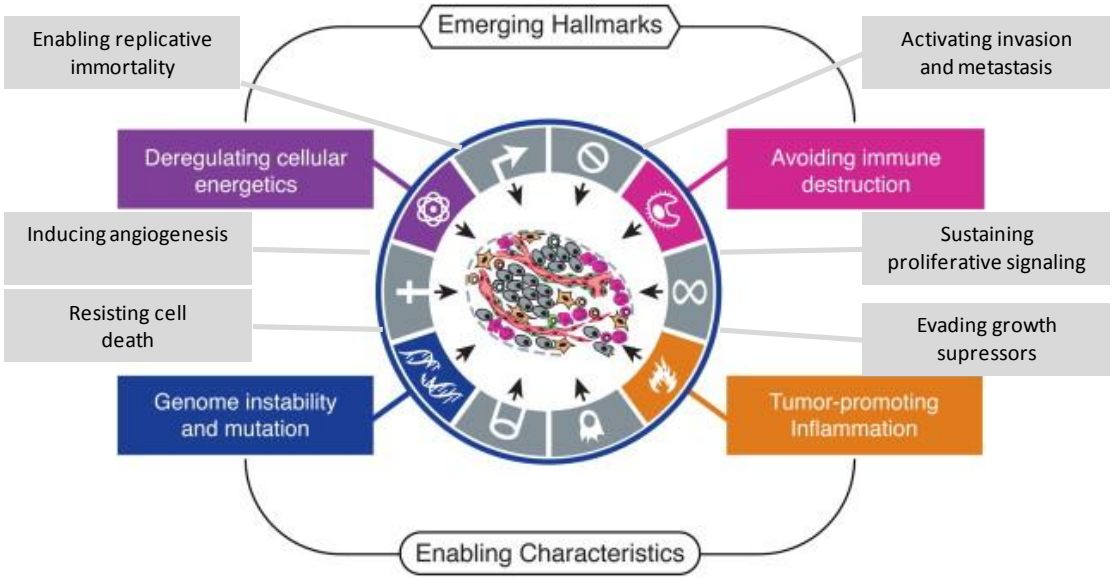


Figure 1. Hallmarks of cancer. The hallmarks of cancer are capabilities the cells need in order to develop into a tumor. A total of eight hallmarks are suggested. The six hallmarks first suggested are in grey boxes and the two emerging hallmarks of cancer and the enabling characteristics of both the core and the emerging hallmarks are presented in colored boxes. Modified from Hanahan and Weinberg 2011 (5).

1.2 Breast cancer

1.2.1 Incidences of breast cancer

In 2009, 2745 new cases of breast cancer were diagnosed in Norway. This makes breast cancer the most commonly diagnosed cancer in women in Norway. The survival rate is high; up to 95% in early breast cancer (stage I) but as low as 18.8% if the disease has spread to distant organs (stage IV) (Table 1) (7). Over the last 50 years, incidences of breast cancer have been increasing, but despite this the survival rate has stayed more or less the same over the years (Fig. 2). The highest increase of breast cancer incidence was from mid-1990 to 2005, which can partly be explained by the increased screening by mammography. From 2005 to 2009, there has been a decline in breast cancer mortality, which most likely reflects the early detection, improvement in surgery and treatment (8).

Table 1. **Stage divided, 5 year relative survival rate**. Five year relative survival rate for Norwegian women with breast cancer year 2005-2009 depending on the stage of disease development (7).

Stage	Survival 2005-2009 %
I	95
II	87
II	69
IV	19
Unknown	87

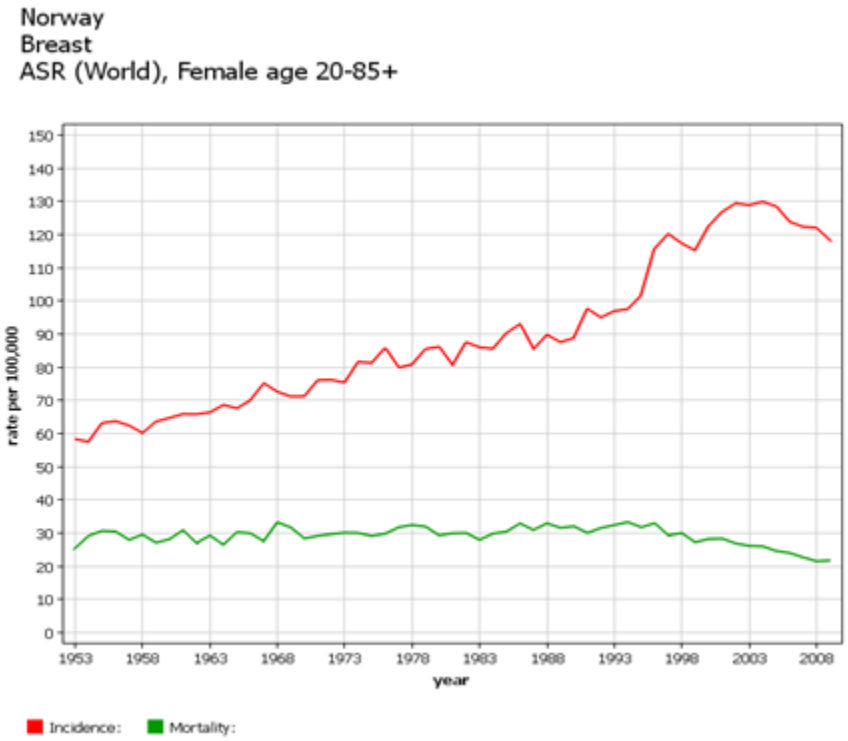


Figure 2. Breast cancer incidences and mortality. Incidences and mortality of breast cancer in Norwegian women from 1950-2008 in the age from 25 - 85+ years old (7).

1.2.2 Risk factors

There are several factors that can contribute to higher risk of breast cancer development. A clear risk factor is inheritance, but still only 5-10% of all breast cancers are familial (4). One known reason for familial breast cancer is mutations in the *BRCA1* and *BRCA2* genes which are tumor suppressor genes. Still these mutations only contribute to 20% of the familial risk (4). Other factors that contribute to a higher risk of developing breast cancer are age, exogenous hormones, reproductive factors such as breast feeding and lifestyle factors and socioeconomic factors such as diet, alcohol, obesity and degree physical activity (9).

1.2.3 Breast anatomy

The human breast is an organ that lies between the second and the sixth rib and consist of approximately 65% gland tissue and 35% fat tissue. In addition, the breast consists of connective tissue, a framework and supporting tissue for the organ, and blood vessels and lymph nodes which are responsible for waste- and nutrition transport. The human gland tissue is composed of terminal ductal lobular units

(TDLU) of lactocyte cells which synthesize milk. These are connected to the ducts which are responsible of transporting the milk to the nipple (Fig. 3) (10).

Breast profile:

A. ducts
B. lobules
C. dilated section of duct to hold milk
D. nipple
E. fat
F. pectoralis major muscle
G. chest wall/rib cage

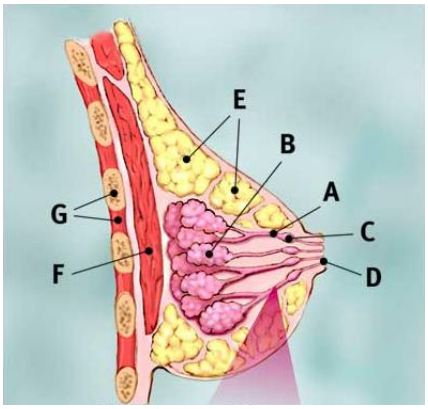


Figure 3. Human breast anatomy. The anatomy of the human breast with illustration of the ducts and lobules. Modified from (11).

1.2.4 Breast cancer development

The development of a tumor from normal epithelial breast cells is through a multistep process. The tissue first develop into a hyperplastic state, an abnormal increase of cells in a tissue, and then into a carcinoma *in situ* (CIS). The CIS is a noninvasive neoplasm that is surrounded by the basement membrane. CIS can further develop into invasive carcinoma (IC) by cells invading the basement membrane and infiltrating the surrounding tissues. This IC may further develop into a metastatic disease where the tumor cells spread to distant organs and develop new tumors (Fig. 4) (12). The majority of deaths from cancer are due to metastasis.

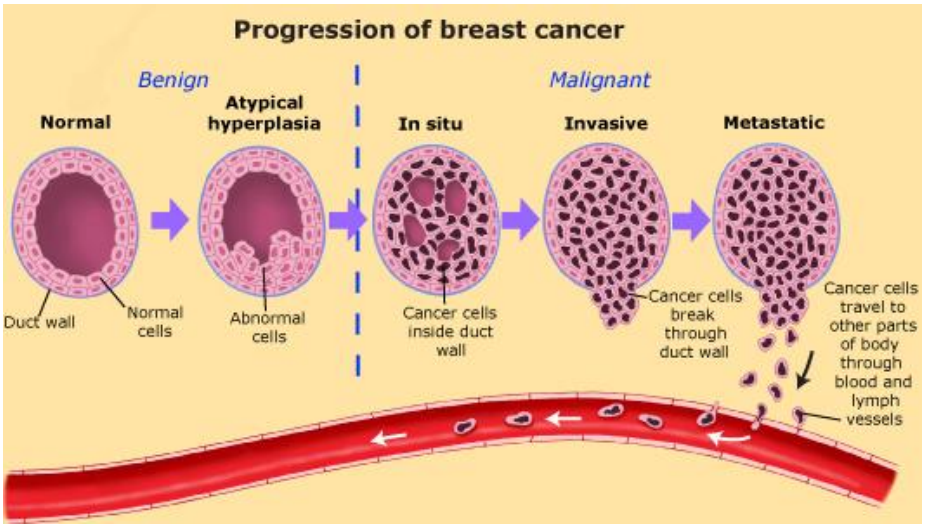


Figure 4. Model for breast cancer progression. Schematic view of breast cancer development showing normal, *in situ*, invasive and metastatic carcinoma progression. Modified from (13).

1.3 Histopathology

Breast cancer is a heterogeneous disease with different characteristics associated with distinctive histological patterns and different biological features which lead to different clinical outcomes. In breast cancer, most of the tumors develop in the ducts and in the lobules and the carcinoma is named lobular or ductal carcinoma depending on where the tumor is located. (14).

Ductal carcinoma *in situ* (DCIS) is non-invasive since the cells have not invaded the basement membrane of the duct, whereas invasive ductal carcinoma (IDC) infiltrates the surrounding tissue of the breast. IDC is the most common invasive breast cancer occurring in 75-80% of the patients. Invasive lobular carcinoma (ILC) accounts for 10-15% of the breast carcinomas and is the second most common type of invasive breast cancer. (15).

To give the breast cancer patient the correct diagnosis and treatment the tumor has to be classified based on prognostic and predictive markers. Prognostic markers provide information about the progression of the disease and the predictive marker provides information about response to a particular treatment. Some factors, such as hormone receptor and HER2 receptor status, are both prognostic and predictive because they give information on which therapy to use and how advanced the disease is. (16).

1.3.1 Histological grade

The histological grade is graded from 1-3 and is defined by three features: degree of nuclear pleomorphism, percentage of tubule formation and the rate of cell proliferation. The grading describes the differentiation of the cells in the tumor and the aggressiveness of the cancer. In grade 1 tumor the cells are well differentiated, the tumor grows slowly and the cancer is not aggressive. On the contrary, in grade 3 tumor the cells are poorly differentiated and the cancer is much more aggressive (17).

1.3.2 TNM stage

While the histological grading classifies the tumor by capturing cellular features, the staging system classifies the state of the disease. TNM (tumor, nodes, metastasis) staging is used to describe the degree of tumor development and it provides a platform for the classification of breast cancer based on tumor size, presence of cancer cell in the lymph node and presence or absence of distant metastasis (18). The TNM staging classifies breast cancer into four distinct stages (Table 2).

Table 2. TNM stages. The TNM method describes the state of the disease by classifying the tumor into four different stages depending on tumor size, lymph node status and metastasis.

Stage	**Tumor**
Stage I	Tumor size of < 2 cm, with no metastasis to neither lymph nodes nor distant organs.
Stage II	Tumor size of 2-5 cm with no metastasis in the lymph nodes or < 2 cm with 1-3 positive lymph nodes.
Stage III	Tumor size > 5 cm with 1-3 positive lymph nodes or < 5 cm with 4-9 positive lymph nodes.
Stage IV	Tumor of any size and lymph node status, but presence of metastasis to distant organs.

1.3.3 Cellular receptor classification

Breast cancer tumors are often classified based on the expression of hormone receptors and HER2 receptor. Breast cancer tumors are defined as estrogen receptor positive (ER+) if the tumor cells have receptors for estrogen on the cell surface. This enables the cells to receive signals from estrogen that can promote cell growth and proliferation. The same features are valid for progesterone receptor positive (PR+) tumors (19). A breast cancer tumor is defined as HER2+ if it has amplification of the

chromosome region where the *HER2* gene is mapped. To determine the HER2 status immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) are done to study the protein- and amplification levels of *HER2* (20).

1.4 Molecular subtypes of breast cancer

The development of microarray technology in the last decade has made it possible to measure the genome wide gene expression patterns. Using this technology breast cancer tumors are classified into molecular subclasses. In 2000, five molecular subclasses of breast cancer were described, and those divided breast cancer in to the subclasses; Luminal-A, Luminal-B, HER2-enriched, basal-like and normal-like subtypes. The different subclasses have shown to have different survival rate. HER2-enriched subtype and basal-like has the lowest survival rate whereas Luminal-A subtype has the highest survival rate (21;22).

1.5 HER2+ cancer

Approximately 20% of all diagnosed breast cancers have amplification of the chromosome region 17q12-21, which leads to overexpression of the HER2 receptor (23). HER2+ breast cancers represent an aggressive form of the disease with significantly shortened disease free- and overall survival.

The *HER2* gene is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors, which consist of the receptors EGFR (HER1), HER2, HER3 and HER4. These receptors are transmembrane molecules and consist of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. When two of these receptors dimerize, the tyrosine kinase domain starts a phosphorylation cacade, which leads to an activation of downstream signaling pathways such as the PI3-K/Akt- and MAPK pathway. These pathways control cell survival, proliferation, adhesion, differentiation and cell migration which are all important features in cancer development. Although HER2 has no known ligand, it is the preferred hetrodimerization partner of the other HER2 family members (Fig. 5) (24).

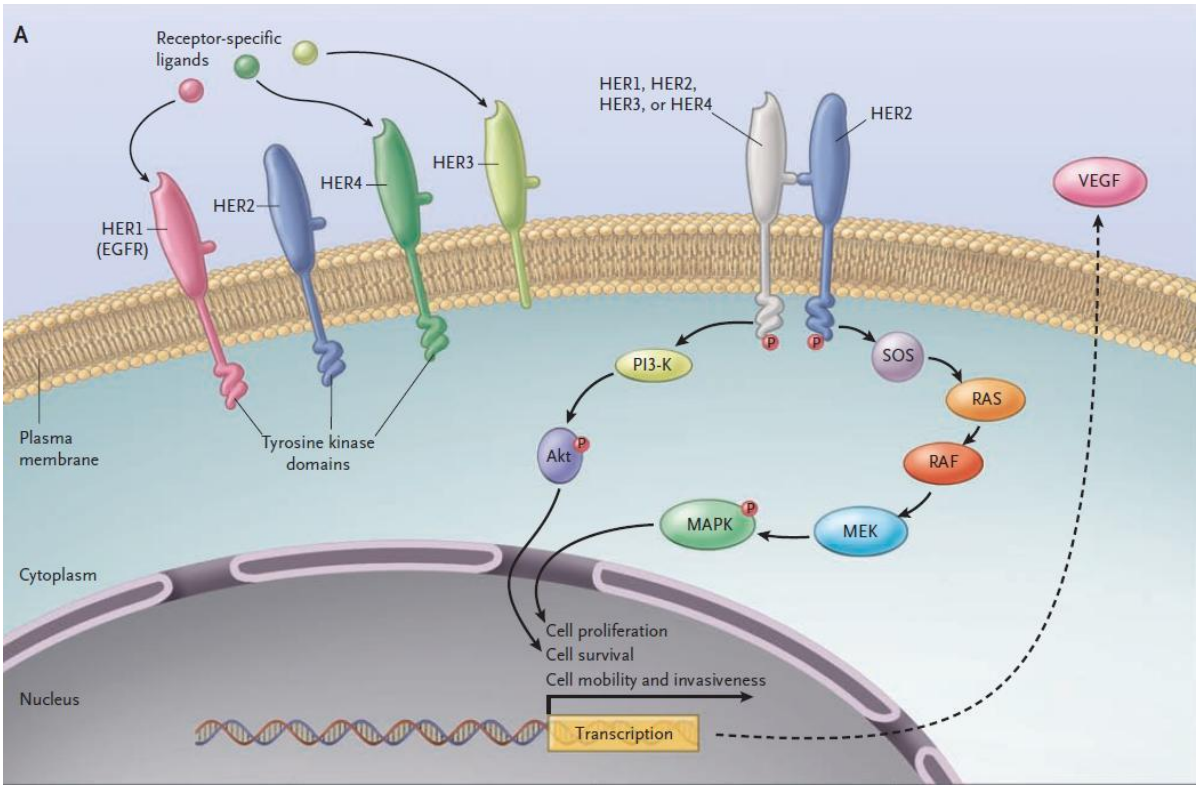


Figure 5. The HER2 pathway. The EGFR family has four family members: HER1 (EGFR), HER2, HER3 and HER4. When the HER2 receptor dimerize with one of the family members it activates downstream signaling pathways trough a phosphorylation cascade. (25).

1.6 Treatment of breast cancer

The treatment of breast cancer patients in Norway is based on the recommendations of The Norwegian Breast Cancer Group (NBCG) and the therapy chosen for the patient depends on the clinical classification of the tumor (26).

Almost all breast cancer tumors are removed surgically; either by lumpectomy or mastectomy. Lumpectomy is a method where the tumor, including some healthy surrounding tissue, is removed and sometimes also the lymph nodes are removed. When a mastectomy is performed, the whole breast and sometimes also surrounding lymph nodes are removed. If the tumor is too large to operate at the time of diagnosis the patient can be treated with neoadjuvant therapy, which can be radiation, chemotherapy or targeted therapy, to reduce the tumor to operable size. Chemotherapy and radiation are often also used as adjuvant treatment to kill any remaining tumor cells. Patients that are ER+ are treated with Tamoxifen, an estrogen

antagonist that binds to the ER receptor, preventing the estrogen to bind. This leads to inhibition of cell growth signals and cell death. If the female patient is post-menopausal she is treated with aromatase inhibitor, which inhibits the body's own production of estrogen (27).

HER2+ breast cancer patients are treated with Trastuzumab or Lapatinib or a combination of these. In Norway, Trastuzumab is administered to the HER2+ patients adjuvantly in primary or metastatic settings and is given intravenously every 3 weeks for one year (26). Trastuzumab is a monoclonal antibody and works by binding to the extracellular domain of the HER2 receptor and thereby preventing the activation of the downstream pathways. Several mechanisms have been proposed by which Trastuzumab decrease the signalling to this pathway; prevention of the dimerization of the HER2-receptor, increased endocytotic destruction of the receptor, inhibition of the detaching of the extracellular domain, and immune activation which leads to tumor cell lysis. Although Trastuzumab has shown to be helpful for some patients, half of all patients do not respond or become resistant to the treatment over time (25).

Lapatinib is a small molecule that has its function on intercellular part of the HER2 receptors by competing with the ATP binding site and therefore inhibiting the phosphorylation of the receptor and the activation of the tyrosine kinase pathway (28). *In vitro* studies have shown that Lapatinib treatment alone has a minimal inhibitory effect on HER2+ tumor concerning proliferation and survival, as well as associated decreases of the protein levels of Akt, ERK1/2 and HER2 pathway. However, the combination of Lapatinib and Trastuzumab show an enhanced effect down regulating the protein levels and increasing tumor cell apoptosis (29).

1.7 miRNAs

miRNAs were first described in *Caenorhabditis elegans* by the Ambros and Ruvkun in 1993. They showed that a small miRNA *lin-4* could repress the activity of another gene, the *lin-14* gene, which is essential for the normal time control of diverse postembryonic developmental events in *C. elegans* (30). Thereafter many new miRNAs have been found and it is believed that many more will be identified in the

future. According to the miRNA database (miRBase 18) 1527 human miRNAs have been identified today (31).

miRNAs are found in almost all eukaryotes and all over the genome in both non-coding- and coding regions. miRNAs bind mRNAs and regulate translation and mRNA stability relatively late in the gene expression pathway. Several proteins are involved in processing these small RNAs to form the mature miRNAs. Initially, miRNAs are transcribed into longer RNA molecules, called primary miRNAs (pri-miRNAs). These fold into hairpin loops which are converted into mature miRNAs by several proteins. Drosha is a nuclear enzyme that cleaves the pri-miRNAs into smaller hairpin RNAs, precursor miRNAs (pre-miRNAs), which are approximately 70 nucleotides long. These pre-miRNAs are then transported by exportin 5 to the cytosol where the enzyme Dicer cleaves the pre-miRNAs into short RNA molecules about 20-22 nucleotides long which are the mature miRNAs. To become an active miRNA, it assembles with several proteins to form a miRISC. The miRISC inhibits the expression of mRNAs by binding to complementary mRNA sequences and either causes mRNA cleavage or translation repression (Fig. 6). For the miRNA to have an inhibitory effect it is usually required that several different miRISCs binds to different complementary or partly complementary sites on the target mRNA. The mature miRNAs can control the activity of many mRNAs and one mRNA can be controlled by several different miRNAs (32). The miRNA sequence also includes a sequence called the “seed sequence”. This is a conserved heptametrical sequence which is mostly placed at the positions 2-7 from the 5´-end of the miRNA. Even though base pairing of miRNA to its target mRNA does not have to be completely complementary, the “seed sequence” has to be perfectly complementary (33).

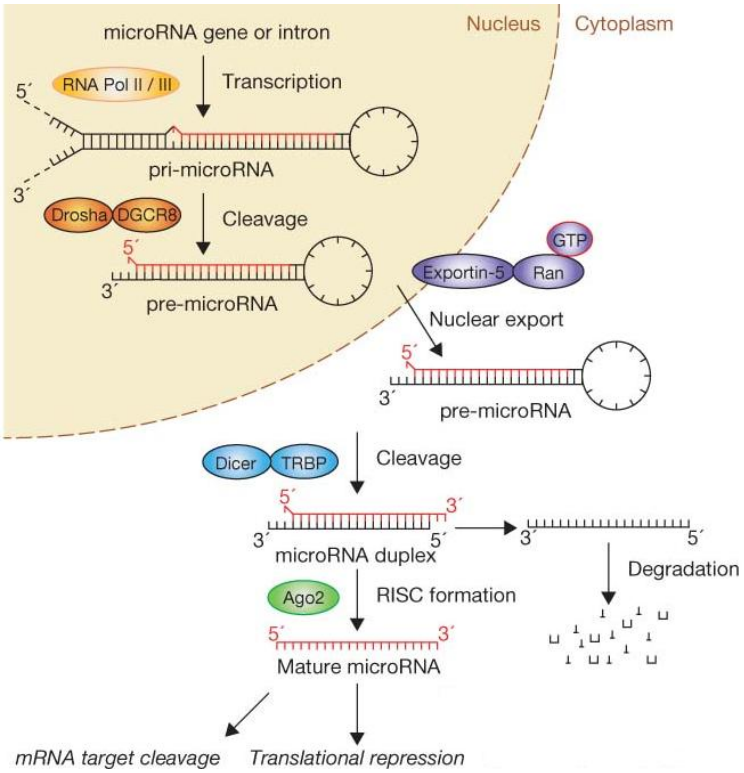


Figure 6. Formation and function of miRNAs. miRNA are transcribed from genes or introns and then transported to the cytoplasm where they evolve to mature miRNA and execute their regulatory functions (34).

miRNAs have been shown to play a role in, for example embryonic development and in the formation of specialized cell types such as immune cells, neurons and skeletal and heart muscle cells (32). To investigate the developmental role of miRNAs, knockout studies in mice have been preformed. One such study was done by Zhao *et al.* (2007) where miR-1-2 was knocked down in mouse embryos. These mice developed severe heart defects leading to half of the mice dying during embryonic development or during the first few months (35).

1.7.1 miRNAs and cancer

miRNAs have been found to be linked to various human diseases such as skin, psychiatric, autoimmune, neurological diseases and cancer (36). miRNAs can play a role in cancer development by regulating certain mRNAs resulting in the activation of oncogenes or inhibition tumor suppressor genes (37). miRNA expression profiles have shown that tumors have a different miRNA profile compared to normal cells from the same tissue. miRNA-expression profiling of human tumors has identified miRNA profiles that are associated with diagnosis, helping to find the primary tumor,

progression, prognosis, staging and response to treatment (38-41). The cause of abnormal miRNA expression is still only partially known. Different mechanisms can cause abnormalities in miRNA expression: the location of miRNAs in cancer-associated genomic regions, regions involved in both deletions and amplification depending on cancer type, epigenetic regulation of miRNA expression and abnormalities in miRNA-processing genes and proteins (38). A study by Lu *et al.* (2005) showed that miRNA expression profiles classify human cancers according to the differentiation state and development lineage of the tumor (39). A similar study by Enerly *et al.* identified through expression profiling, differentially expressed miRNAs between different breast tumor subtypes and between samples that had different levels of proliferation(42) .

miRNA functional studies have shown that a specific group of miRNAs can contribute to the development of cancer when miRNAs are artificially induced in mice. This results in an accelerated rate of cancer development (32). Also in breast cancer many miRNAs have been found to associate with tumor development. One of the key miRNAs in breast cancer is the let-7 family of miRNAs which function as tumor suppressors. It has a unique control mechanism mediated by the protein LIN28 and the expression of let-7 is lost in early stage of breast cancer (43). In addition, miRNAs associated with both TP53 status (44) and estrogen receptor (ER) status (45) have been identified. miRNAs connected to the TP53 status silence anti-proliferation genes and work together with the transcriptional factor TP53 as a fast forward loop to enhance proliferation (44). It has been shown that some miRNAs can down-regulate the ER receptor and thereby inhibit cell growth stimulated by estrogen (45). It has also been found that miR-34b, that is ER and p53 dependent functions as an onco-suppressor miRNA in breast cancer cells (46).

1.7.2 miRNAs in HER2+ cancer

Several studies have shown that miRNAs play a role in HER2+ cancers. Gong *et al.* showed that up-regulation of miR-21 mediates resistance to Trastuzumab therapy in breast cancer (47). A study by Adachi *et al.* showed that *ERBB3* down-regulates miR-205 in HER2+ breast cancers (48). The down-regulation of miR-205 leads to higher expression of cyclin D1, cyclin E, cyclin-dependent kinase 2 (*CDK2*), cyclin-dependent kinase 4 (*CDK4*), and cyclin-dependent kinase 6 (*CDK6*) which leads to

enhanced cell growth (48). It has also been described that miR-205 directly targets the HER3 receptor, one of the HER2 receptor dimerization partners, which leads to inhibition of the activation of the downstream mediator Akt (49).

Scott *et al.* (2007) showed that the expression of *HER2* and *HER3* were down regulated when miR-125a and miR-125b were over-expressed in SKBR3 cells (50). They used retrovirus expressing either miR-125a or miR-125b and this resulted in down-regulation of HER2 and HER3 on both transcriptional and protein level as well as in suppression of the phosphorylation of ERK1/2 and Akt (50). The results of this study suggest that it would be possible to use miRNAs to suppress oncogene expression.

New miRNAs are constantly discovered. In 2011, Persson *et al.* published an study which described the identification of new miRNAs in normal and tumor breast tissue and suggested that some of these miRNAs may play a role in HER2+ cancer (51). They used next-generation sequencing of small RNAs to create an overview of the small RNA expression in breast cancer. This is a new and time efficient method used to find new miRNAs by finding mutations, copy number aberrations and somatic rearrangements in the entire cancer genome at base pair level (52). Ten percent of the new miRNAs they found are located in regions with high-level genomic amplifications in breast cancer. Five of the new miRNAs map to the chromosome region 17q12 where the *HER2* gene is located. The miRNAs mapped in this region are; miR-4726, miR-4727, miR-4728, miR-4734 and Candidate-296. miR-4728 is encoded within the *ERBB2/Her2* gene (Fig. 7) (51). The amplification of this region leads to overexpression of the HER2 receptor and the development of HER2+ cancer. The functional roles of these miRNAs have still not been investigated.

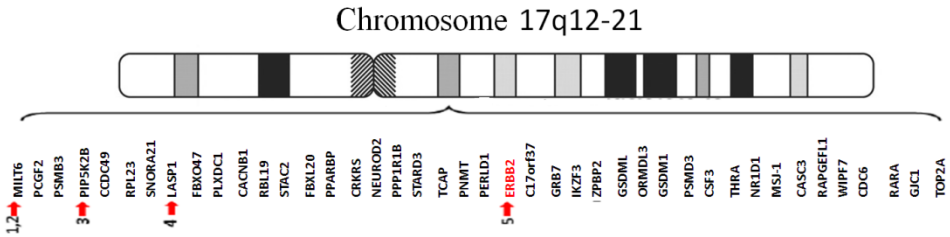


Figure 7. Genes and miRNAs in the HER2 amplicon Chromosome region 17q12-21 were HER2 is mapped and the locations of the five novel miRNAs marker with red arrows: 1) miR-4726, 2) miR-4734, 3) miR-4727, 4) Candidate_296, 5) miR-4728. (Provided by Vesa Hongisto, VTT, Turku, Finland)

1.7.3 miRNAs as therapeutic targets

As miRNAs play an important role in cancer development, it opens an opportunity for use as therapeutic targets to prevent cancer development by inhibiting the function or by inducing the expression. The function of miRNAs can be inhibited by over-expressing so called anti-miRNA oligonucleotides, which are complementary oligonucleotides to the endogenous miRNAs. These anti-miRNAs bind to the miRNAs and thereby inhibit their functions. Many successful anti-miR experiments have been done, one of them on orthotopic xenograft models of metastatic breast cancer, where 4T1 cells were injected into the mammary fat pad of mice and anti-miR-10b was targeted to study the effect this had on the primary tumor and their metastatic capacity. miR-10b targets the transcription factor Hoxd10, and the treatment did not have any effect on the primary tumor but it had a striking effect on reduction of lung metastasis (53).

There are several miRNAs that work as tumor suppressors in normal cells and many of these are down-regulated in tumor cells. Over-expression of these miRNAs can normalize the molecular state of the tumor cell. The use of miRNAs as therapeutic targets has been shown to be successful in cell lines and in cancerous mice models in several studies (54). For instance, the use of miR-34 in replacement therapy has been studied by several different laboratories. miR-34 is transcriptionally induced by TP53, and when *TP53* is mutated it leads to a down-regulation of this miRNA in the cell. miR-34 stimulates apoptosis, G1 arrest and prevents cell migration. It has been found that delivery of the miRNA intratumorally or by tail vein injection inhibits the growth

of non-small-cell lung carcinoma xenografs by reducing proliferation and inducing apoptosis (55). Si *et al.* evaluated the role of miR-21 in tumorigenesis by transfecting breast cancer MCF-7 cells with anti-miR-21 oligonucleotides. They found that anti-miR-21 suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model. They also showed that inhibition of miR-21 was associated with increased apoptosis and decreased cell proliferation. Results suggested a role for miR-21 as an oncogene which can be a potential novel therapeutic target (56). Although miRNAs that are promising therapeutic targets have been found, it is still a major challenge to use miRNAs in therapeutics because the delivery of miRNAs to the tumors without harming healthy tissue is challenging.

2 Materials and methods

2.1 Cell lines

For the experiments in this thesis 15 HER2+ cell lines was used, and one breast cancer cell line as a control. An overview of the cell lines are found in Table 3.

Table 3. Overview of the cell lines. The 15 HER2+ and MCF7 cell lines used in the experiments. Trastuzumab response, ER/PR status and PI3KCA mutation status are listed for each cell line. NA, not available

Cell lines	Trastuzumab response	ER status	PR status	PI3KCA mutation status
HCC1954	Non-responsive	+	+	mut
JIMT1	Non-responsive	-	-	mut
HCC202	Non-responsive	-	-	mut
SUM255	Non-responsive	-	-	NA
KLP4	Non-responsive	-	+	NA
SUM190	Non-responsive	-	-	NA
SUM206	Non-responsive	NA	NA	NA
HCC1569	Non-responsive	-	-	wt
MDA-MB-453	Responsive	-	NA	NA
EMF192A	Responsive	NA	NA	mut
AU565	Responsive	-	-	wt
SK-BR-3	Responsive	-	-	wt
BT474	Responsive	+	+	mut
HCC1419	Responsive	-	-	wt
MCF7	Control HER2-	+	+	mut

2.2 Cell culturing

The cell lines were grown in 100x20 mm Corning Petri-dish (Corning Incorporated, NY, USA). HCC-202 and HCC-1569 were grown in RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA,USA) medium supplemented with 10 mM Hepes (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 2.5 g/L glucose (45% w/v stock, Kebo Lab, Sweden), 1mM Sodium pyruvate (SIGMA, St. Louis, MO, USA), 2 mM L-glutamine (SIGMA) and 1:100 Penicillin/Streptomycin solution (GIBCO). MDA-MB-453 were grown in DMEM (4,5g glucose) (SIGMA) supplemented with 10% FBS, 2mM L-glutamine (SIGMA) and 1:100 Penicillin/Streptomycin solutions (GIBCO). SKBR-3 were grown in McCoy's 5A (SIGMA) supplemented with 10%

FBS, 1,5 mM L-glutamine and 1:100 Penicillin/Streptomycin and HCC1954 were grown in RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM Sodium pyruvate, 10 mM Hepes, 10% FBS, 1:100 Penicillin/Streptomycin and 2.5 g/L glucose. The isolated RNA from the rest of the cell lines was already available at the Department so there was no need for culturing those cells.

The cells were cultured in NuAire Automatic CO_2 incubator (NuAire, Plymouth, MN, USA) at 37^{o}C and 5% CO_2. Cells were cultured in sterile conditions and the gloves were disinfected with 75% ethanol. Renewal of media and sub-culturing procedures were done in a laminar flow hood (LFH) that was disinfected with 75% ethanol. To avoid contamination there was always only one cell line in the LFH at a time, and the LFH was disinfected before and after bringing a cell line in.

The cells were visually observed in an Axiovert 40 inverted microscope, (Carl Zeiss, Germany) and when they reached 80% confluence they were sub-cultured. The sub culturing was done by removing the old medium and thereafter the plate was washed with Phosphate Buffered Saline (PBS) (Invitrogen) to remove any remains of media. After the cells were washed, 1.5 ml 0.25% trypsine (GIBCO, USA) was applied to detach the cells. The petri-dish was incubated for 4 minutes at 37^{o}C. After incubation, 8 ml of media was added to inactivate the trypsin. The cell suspension was then transferred to a 15ml tube and centrifuged at 800 rpm for 8 minutes with Rotina 420 centrifuge (Hettichlab, Germany). The supernatant was removed and the cell pellet was re-suspended in growth media, volume depending on the amount of cells, and distributed to new petri-dishes with 8 ml media.

2.3 The isolation of RNA from cell lines

2.3.1 Pellet for RNA isolation

The cell lines were harvested as pellets for RNA isolation. The cells were trypsinised and centrifuged as described above. The cells were then washed twice with cold PBS and centrifuged after each washing. The pellet was then resuspended in 1ml of cold PBS and transferred to a cryotube and stored in the -80ºC freezer.

2.3.2 RNA isolation

RNA was isolated with TRIzol (Invitrogen, USA) from the following cell lines; HCC-202, EFM-192A, HCC-1419, AU565, HCC-1569, HCC-1954, JIMT1 and MDA-MB-453. For some of the cell lines, pellets were already collected or RNA isolated at the Department. The cell pellets were taken up from the -80ºC freezer and thawed at room temperature. They were then transferred to marked tubes and centrifuged at 12000g for 10 minutes at 4ºC. The supernatant was then removed and the pellet was re-suspended in 500µl TRIzol which stabilizes the RNA. This suspension was incubated for 5-10 min in room temperature. Thereafter, 100µl of chloroform was added to lyse the cells. The tube was then centrifuged at 12000g for 15 minutes at 4ºC. The upper liquid phase, which now contained the RNA, was removed and transferred to a new RNAse free tube. Thereafter, 250µl isopropylalcohol was added to facilitate the precipitation. The tube was then centrifuged at 12000g for 10 min at 4ºC. The supernatant was removed and the pellet was washed twice with 500µl 75% ethanol and centrifuged at 7500g for 5 minutes at 4ºC. All the liquid was removed and the pellet was air dried for 5-10 minutes in room temperature. The RNA pellets were then resuspended in 50µl RNAase free water. If the pellet was small it was resuspended in a smaller amount of water. The tube was then incubated for 5-10 minutes at 55ºC to denaturize the RNA. The RNA concentration was measured by Nano drop 3300 fluorospectrometer (Thermo Scientific, MA, USA) and the samples were stored at -80ºC. The Nano drop measures the RNA concentration and the purity of the RNA. The RNA will absorb at 260nm and a ratio of 260/280 is used to determine the purity of the RNA. If the ratio is ~2.0 it is generally accepted as pure RNA. If the ratio is lower it can indicate the presence of protein, phenol or other contaminants that absorb at or near 280 nm. Another ratio, the 260/230 ratio, is used as a secondary measurement of RNA purity. This is usually in the range of 2.0-2.2 and if the range is lower it can indicate the present of contaminants that absorb at 230nm (57).

2.4 Measuring RNA quality with Bioanalyzer

The RNA quality was measured using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to control the quality of the RNA. 50ng/µl was used as an input. The RNA 6000 Nano dye concentrate (Agilent Technologies), RNA 6000 Nano marker (Agilent Technologies) and the filtered gel were incubated

protected from light at room temperature for 30 minutes. The RNA Nano Dye concentrate was vortexed for 10 seconds, shortly centrifuged and 1µl of the dye was added to the gel to color the RNA samples. The RNA Nano chip was placed in the "chip priming station" and 9µl of the gel mix was added to the well marked G with black background. The sample was pipetted from the top of the tube to avoid particles at the bottom of the tube. The lid was closed without touching the syringe and the syringe was marked on 1ml. The stamp was pressed down and held down for 30 seconds so that the dye would equally distribute over the chip. Then 9µl of the dye was added to the two other well marked G. Thereafter 5µl of RNA 6000 Nano marker was added in all the wells including the well marked ladder. This was added as a positive control for the analysis. Then 1µl of ladder was added to the well marked ladder and 1µl of the samples were added to the 12 wells and the chip was vortexed for 1 minute at 2400 rpm. The Bioanalyzer electrodes were cleaned with 350µl RNase zap for 1 minute, to remove RNA remains, and then with 350µl RNase free water for 10 second and dried for 10 seconds. The chip was placed in the Bioanalyzer and the analyzed with Agilent 2100 expert software.

2.5 miRNA expression array analysis

To study the miRNA expression in HER2+ cell lines Agilent Technologies SurePrint G3 Human v16 miRNA 244K Microarray data were analyzed. The miRNA expression data from HER2+ cell lines were available at the Department. miRNA expression arrays are used to measure the expression of miRNAs genome wide, and they can be used to study miRNA expression signatures in cancer (58). The miRNA microarray analysis had been performed on all the HER2+ cell lines studied in this thesis, except AU565, and the expression of 152 miRNAs were studied. First the array data were processed using the Gene Spring software GX11.5.1 (Agilent Technologies). The data were normalized using the 90 percentile and then Log2 transformed. The data were controlled to see if the cell lines/arrays were within acceptable values for the quality control. The data were then filtered on gene expression using 100% as upper and 20% as lower cut-off. This was done to exclude the very low expressed (not detected) miRNAs. The data were then filtered on flags. Flags are set by the program Feature Extraction (Agilent Technologies) that reads the signal intensities from the spots into numbers. All flagged miRNAs were removed in

all cell lines. If a miRNA was present in one cell line, the data were kept in all. Then the data were exported from Gene Spring to a txt file.

2.5.1 Significance Analysis of Microarray (SAM)

To study whether there are significant differences in the miRNA expression between two groups of the HER2+ cell lines with different characteristics, SAM excel plug-in was performed on miRNAs array data from these cell lines. SAM gives a score to each gene, and a q-value to determine if there is a significant difference between two groups. A false discovery rate (FDR), estimating the percentage of false positive, is calculated (59). It was investigated whether there was a significant difference between cell lines with different characteristics regarding Trastuzumab response, ER- and PR status and *PI3CA* mutation status.

2.5.2 Ingenuity Pathway Analysis

To search for validated mRNA targets for differentially expressed miRNAs, miRecords (http://mirecords.biolead.org/) was used. The validated miRNA targets were further investigated using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA) to identify pathways and networks overrepresented within the target list. IPA can be used to analyze data derived from several experiments which generates gene lists. From this information it can find the associated networks and functions, list the top canonical pathways and the molecular functions in the gene lists (60). In this analysis the p-value was adjusted using Benjamini-Hochberg method of multiple testing corrections and a 0,005 cut of value was used. This means that the amount of false positives among the significant functions is expected to be less than 0.5%.

2.6 Array comparative genomic hybridization (aCGH)

aCGH data from 244K array (Agilent Technologies) was available for 10 of the 15 cell lines. aCGH is used to measure DNA fragment loss or gain. This is done by applying fluorescently labeled sample DNA and control DNA to a microarray. The sample DNA and the control DNA compete to hybridize on the microarray and the microarray scanner measures the results (61). The aCGH data were used to plot the DNA changes at the HER2 amplicon in the different cell lines. Gain of one

chromosome arm (or region) =0.3, loss of one chromosome arm= -0.3. Amplification is higher than 1.0.

2.7 Quantitative RT-PCR

To run qRT-PCR on RNA the RNA has to be converted to complementary DNA (cDNA) which is done by the Reverse Transcriptase Reaction (RT reaction). The RT reaction converts the RNA to cDNA by using primers and reverse transcriptase enzyme. The RT-reaction was run with an input of 10ng RNA and 1.34µl of RT-product was used into the Real-Time PCR reaction. Polymerase chain reaction (PCR) is a method that enables exponential amplification of short DNA fragments. The DNA is amplified using primers, polymerase and cycles of denaturalization of the DNA and the annealing of primers. When the DNA is denatured at 90ºC the temperature is lowered to 50-60ºC and the primer anneals to its complementary strand. The temperature is raised to 72ºC and the heat-stable Taq DNA polymerase extends the DNA from the primers. These are denatured again at approximately 94 ºC so that a new cycle can start. The amount of RNA is determined by using a standard curve and an endogen control.

Some of the primers were not available from the manufacturer, therefor it was not possible to study the expression of miR-4734, miR-4728-3p and Candidate-296-3p.

2.7.1 RT reaction

The RT reaction was done with TaqMan®MicroRNA Reverse Transcription Kit (PN4366596 Applied Biosystem) and TaqMan microRNAs assay (Applied Biosystem, USA). The kit includes Multiscribe Reverse Transcriptase, which is the enzyme that is needed to convert the RNA to cDNA, dNTP mix, containing nucleotides available to make cDNA, RT buffer to stabilize the reaction and RNase inhibitor to prevent the RNA breakdown by possible RNases. The RNA samples, the primers and the kit were thawed on ice. All of the components, besides the primers and RNA samples, were vortexed and centrifuged. Primers and RNA samples were mixed gently and spinned down. The master mix was made for each assay separately with 20% excess volume as shown in Table 4.

Table 4. TaqMan MicroRNA Reverse Transcription setup

Component	Master Mix Volume/ 15µL reaction	Volume into master mix (+20%)
dNTP mix (100 mM total)	0.15	4.20
Multiscribe™ RT enzyme (50U/µL)	1	28
10 x RT Buffer	1.5	42
RNase inhibitor (20 U/µL)	0.19	5.31
Nuclease free water	4.16	116.48
RT-Primer	3	84
RNA sample (2ng/µl (5ng)	5	
Total	15	420

The master mixes were prepared with all components except the RNA, centrifuged and put on ice. A dilution series was made of the human breast total RNA sample from 0.5 to 10ng (Appendix A). This was used as a standard curve to determine the amount of miRNAs in the cell lines.

The mastermix was then pipetted to a Thermo-Fast®96, Non-Skirted plate (Thermo Scientific) and the RNA samples were added to their respective wells. The total RNA dilution was added to the first row in every plate and then 5 µl of the RNA 1ng/µl from the cell lines were added in the wells and the plate was centrifuged. The plate was put in the Gene Amp® PCR system 2700 (Applied Biosystem), the volume was set to 15µl and the RT reaction generating cDNA, was started. At 16 ºC the primers bind and hybridize to the RNA and the optimal reaction temperature for the enzyme is 42ºC and this temperature is held for 30min before the enzyme is inactivated at 85 ºC. (Table 5)

Table 5. RT-program

Temperature (◦C)	Time (min)
16	30
42	30
85	5
4	∞

2.7.2 Real Time PCR reaction

The components in Table 6, except the product from RT reaction, were mixed for each assay and added to a Thermo-Fast®96, Non-Skirted plate (Thermo Scientific). Thereafter the product from the RT reaction was added to the wells. This mix (10µl) was then added to a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystem) with Barcode in triplicates for each sample.

Table 6. Real-Time PCR reaction setup

Component	**Volume (µl)**	**Volume (µl) for 4x no. of samples (X) reactions (x)**
TaqMan miRNA assay (20x) (Applied Biosystems)	0.5	X*0.5
Product from RT reaction	1.34	(5.36 pr. sample pool (4X))
TaqMan 2x Universal PCR Master Mix, No ampErase UNG (Applied Biosystems)	5	X*5
Nuclease free water	3.16	X*3.16
Total volume	10	X*10

The plate was covered with Micro Amp optical Adhesive Film (Applied Biosystems) and centrifuged. The plate was run with 7900HT Fast Real Time PCR system according to the program showed in Table 7 and data were analyzed with software SDS 3.2 (Applied Biosystems).

Table 7. Standard Real-Time program, 7900HT.

Step	**Temperature**	**Time**	**Cycles**
Optional AmpErase® UNG activity	50°C	2 min	1 cycle
Enzyme activation	95°C	10 min	1 cycle
PCR	95°C	15 sec	40 cycles
PCR	60°C	60 sec	

2.7.3 Student's t-test

To study whether there was a difference in miRNA expression between the cell lines that are responsive to Trastuzumab and those that are not, a t-test was performed using SPSS program (IBM, NY, USA) on the miRNA expression data from the qRT-PCR experiment. A t-test can be used to test if the average value of a normalized dataset is significantly different from the average value of another dataset (62).

2.8 miRNA inhibition

2.8.1 Optimization of cell lines

From the miRNA expression data, two cell lines were chosen for miRNA transfections to study the functional role of the miRNAs. These cell lines have different proliferation rates so optimization of cell number was needed. Optimization of cell amount on white 384 well microplates with clear bottom (Greiner Bio-one, Germany) for three days incubation is important, in order to reach optimum confluency for each cell lines.

To get the right amount of cells in the plate wells, the number of cells in the cell solution was counted using a hemocytometer. 10 µl of cell suspension was applied to the hemocytometer and covered with a cover slip. The cells were counted visually from three of the chambers in the hemocytometer under the microscope. The average of the cell number in the three chambers was used to calculate the total cell number in the suspension. Using this equation:

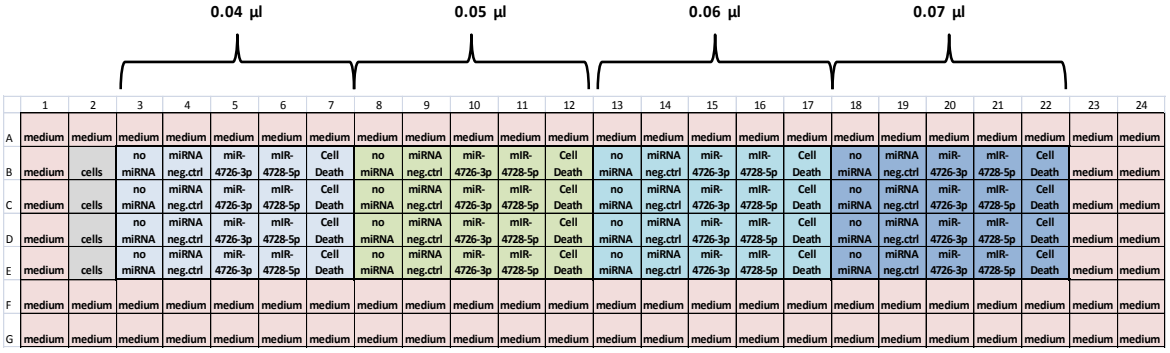
$C1*V1 = C2*V2$

Dilutions were made and 50µl of the suspension was pipetted manually on to the 384 well plate starting on B2. In the surrounding wells, media was added to prevent evaporation. Both cell lines were optimized in a range from 1500 to 2500 cells with 250 intervals and 16 replicates for each cell number.

2.8.2 miRNA transfection

miRNA transfection was performed in SKBR3 and HCC1954 cell lines in 384 well plates by lipid transfection with mirVana miRNA inhibitors miR-4728-5p and miR-4726-3p (Ambion, Life Technologies). The miRNA inhibitors are called anti-miRs and are complementary to the miRNAs studied and inhibit their effect in the cells by binding to them. siAllStar Cell Death(Qiagen, Hilden, Germany) was used as a positive control and mirVana miRNA inhibitor negative control (Ambion) as a negative control. The setup was as shown in Figure 8 with four wells of each miRNA and four with no miRNA, where the transfection lipid reagent (Silentfect, Bio Rad, CA, USA) were applied, and four with cells only where Silentfect were not applied. The Silentfect might have a toxic effect on the cells if the concentration is too high. The toxic effect is controlled in the wells where only Silentfect is applied.

The anti-miR was transfected into the cells to study whether the miRNA inhibition can have an effect on growth and proliferation. The transfection was done with different amounts of Silentfect to determine the amount that would be most efficient without having a toxic effect and the experiment was repeated twice with the optimal amount. (Fig 8)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium
B	medium	cells	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	medium	medium
C	medium	cells	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	medium	medium
D	medium	cells	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	medium	medium
E	medium	cells	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	no miRNA	miRNA neg.ctrl	miR-4726-3p	miR-4728-5p	Cell Death	medium	medium
F	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium
G	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium

Figure 8. Plate setup for miRNA tansfection. The miRNA were transfected in 384 well plates with different amount of Silentfect and with a positive and a negative control.

The miRNA inhibitors were diluted with miRNA dilution buffer containing, 100 mM Potassium Acetate, 30 mM HEPES-KOH, 2 mM Magnesium Acetate; pH 7.4, to a concentration of 250 nM. In the experiment, 5µl of 250nM miRNA were applied into the wells, resulting in a final concentration of 20nM. The Silentfect was diluted in Opti-MEM (GIBCO, Invitrogen) and incubated for 10 min at room temperature. The Silentfect dilutions were thereafter added to the wells and the plate was centrifuged

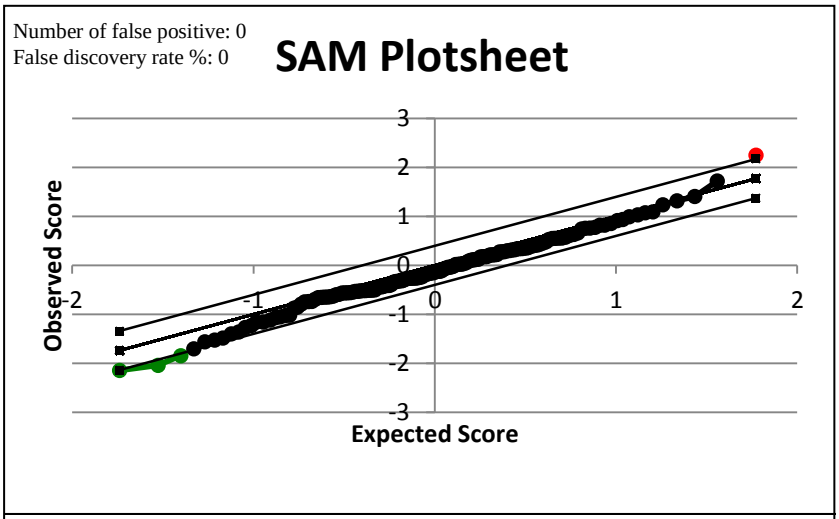
and incubated for 1 hour at room temperature. During this incubation the cells were prepared. The procedure was the same as when the cells where optimized and the amount of cells needed to make the appropriate dilution were calculated. The suspension was prepared and 35µl of the suspension was added to the wells. To prevent vaporization, media was added in the surrounding wells. After 72 hours the number of viable cells was measured with CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA). The CTG measures the amount of viable cells by generating a luminescence proportional to the ATP amount. The CTG lyses the cells and provides necessary reagents to measure ATP amont using a bioluminescent reaction (63).The luminescence was then measured with the Wallac 1450 MicroBeta TriLux luminescence counter (PerkinElmer, Finland).

3 Results

3.1 miRNA expression in relation to Trastuzumab-response

Significance Analysis of Microarray (SAM) was performed on miRNA expression array data to study whether there was difference between miRNA expression in HER2+ cell lines that are responsive to Trastuzumab and those that are not. Four significant miRNAs with a q-value and false discovery rate at 0 were found; miR-342-3p, miR-26b, miR-15a were down-regulated in responsive cell lines and miR-29a was up-regulated in responsive cell lines (Fig. 9 and Table 8). The validated targets for these miRNAs were identified using the miRecords database.

SAM-analysis was also performed on the array data in order to search differentially expressed miRNAs in relation to PR- and ER-status and *PIK3CA* mutation status, but no significant differentially expressed miRNAs were found between these groups.



miRNAs down-regulated in responsive cell lines			
miRNA ID	**Fold Change**	**Score(d)**	**q-value (%)**
hsa-miR-342-3p	0,29461676	-2,14497252	0
hsa-miR-26b	0,469896507	-2,041362856	0
hsa-miR-15a	0,427781038	-1,845703347	0
miRNAs up-reguleted in responsive cell lines			
miRNA ID	**Fold Change**	**Score(d)**	**q-value (%)**
hsa-miR-29a	5,058700425	2,246972393	0

Figure 9. Differentially expressed miRNAs between Trastuzumab responsive and non-responsive cell lines identified by SAM.. SAM analysis identified four miRNAs as differentially expressed between the responsive and non- responsive cell lines. The 3 miRNAs down-regulated in Trastuzumab responsive cell lines are showed in green and the miRNA up-regulated in Trastuzumab responsive cell lines in red.

Table 8. The validated miRNA targets from the differentially expressed miRNAs found in SAM. The validated targets found in miRecords for the four miRNAs that were found in SAM.

miRNA ID	**Targets**
hsa-miR-342-3p	no targets found
hsa-miR-26b	*SERBP1*
hsa-miR-15a	*ACTR1A, ASXL2, BLC2, BMI1, C10orf104, C14orf109, C17orf80, C2orf43, C4orf27, CADM1, CARD8, CCDC111, CCDC76, CCND1,CDC14B, CENPJ, CEP63, CREBL2, DMTF1, ECHDC1, FAM122C, FAM69A, GOLGA5, GOLPH3L, GTF2H1, H3F3B, HACE1, HDHD2, HERC6,HRSP12, HSDL2, HSP90B1, HSPA1A, JUN, LOC339804, MCL1, MSH2, MYB, NPAL2, NT5DC1, OMA1, OSGEPL1, PDCD4, PDCD6IP, PHKB, PMS1, PNN, PRIM1, PWWP2A, RAB21, RAB9B, RAD51C, RHOT1, RNASEL, SKAP2,SLC35A1, SLC35B3, TIA1, TPI1, UGDH, UGP2, VEGFA, VPS45, WIPF1, WT1, ZNF559*
miRNA ID	**Targets**
hsa-miR-29a	*BACE1,CD276,CDC42,CDK6,COL3A1,*
	CXXC6,DNMT3A,DNMT3B,MCL1,PIK3R1 and SPARC

3.2 Pathways for the validated targets

Ingenuity Pathway Analysis (IPA) software was used to identify pathways overrepresented within the miRNA target gene list. The top canonical pathways found with IPA shows what well-characterized cell signaling and metabolic pathways are most relevant in the gene list. The top canonical pathways found with a threshold of 0.005 on the Benjamini-Hochberg corrected p-value and the genes in the gene list that are associated with these pathways are listed in Table 9. The p-value shows the probability that the association between the genes in the dataset and the canonical pathway is explained only by chance.

Table 9. The top canonical pathways. The top canonical pathways of the miRNA targets identified with IPA and the genes associated with the pathways.

Top Canonical Pathways	**p-value**	**Genes from the gene list associated with the different pathways**
p53 signaling	4.14 E-3	*BLC2, CCND1, JUN, PIK3R1, WT1*
Pancratic adenocarcinoma Signaling	4.14 E-3	*BLC1, CCND1, CDC42, PIK3R1, VEGFA,*
ILK signaling	4.14 E-3	*CCND1, CDC42, JUN, PIK3R1, RHOT1, VEGFA,*
PI3K/Akt signaling	4.23 E-3	*BCL2, CCND1, HSP90B1, MCL1, PIK3R1*
Ovarian Cancer Signaling	4.33E -3	*BCL2, CCND1, MSH2, PIK3R1, VEGFA*
Renal Cell Carcinoma Signaling	4.33E -3	*CDC42, JUN, PIK3R1, VEGFA*
Small Cell Lung Cancer Signaling	4.33E -3	*BCL2, CCND1,CDK6, PIK3R1*
HER-2 Signaling in Breast Cancer	4.45E -3	*CCND1, CDC42, CDK6, PIK3R1*
Glioplastoma Multiform Signaling	4.45E -3	*CCND1, CDC42, CDK6, PIK3R1, RHOT1*
Prostate Cancer Signaling	4.45E -3	*BCL2, CCND1,HSP90B1, PIK3R1,*
Colesteral Cancer Metastasis Signaling	4.45E -3	*CCND1, JUN, MSH2, PIK3R1, RHOT1, VEGFA*
Molecular Mecanism og Cancer	4.45E -3	*BCL2, CCND1, CDC42, CDK6, JUN, PIK3R1, RHOT1*

3.3 The HER2 amplicon size

aCGH data was available for 11 of the 14 cell lines and, and were used to detect the amplicon size in the HER2+ cell lines. The positions of the five miRNAs were plotted in to study whether the representative cell line has the region where the different miRNAs are mapped amplified (Fig. 10). The plot shows great variation in the amplicon size in these cell lines and shows that only BT474 and HCC202 have an amplification region that contains all of the miRNAs; miR-4726, miR-4734, miR-4727, miR-4728 and Candidate-296.

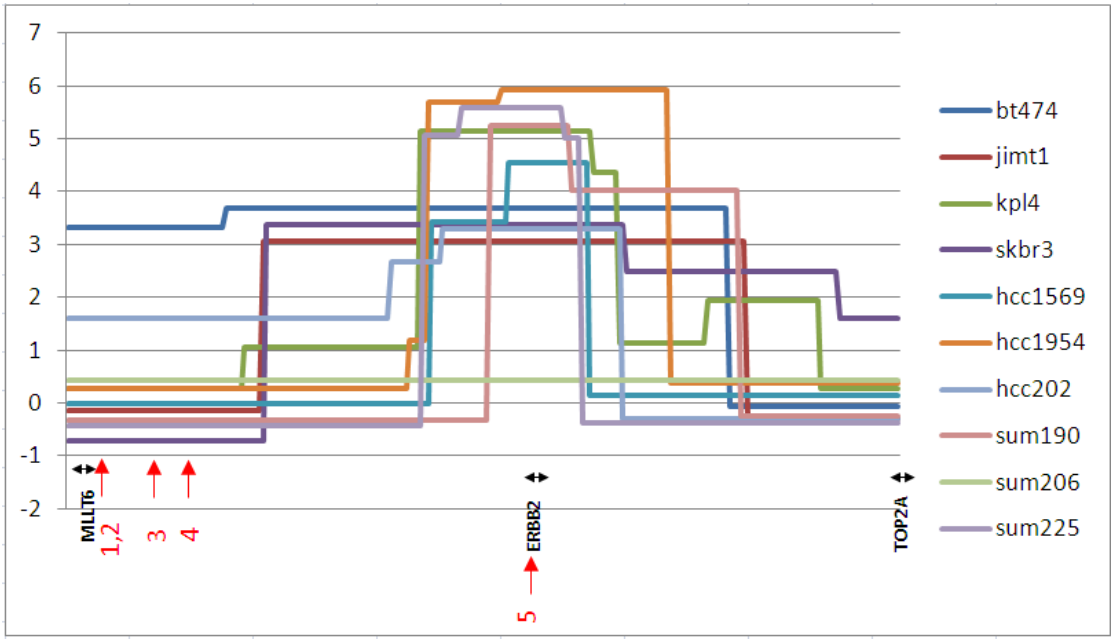


Figure 10. aCGH data showing the amplicon size in the cell lines. The localization of the five novel miRNAs are schematically showed with red numbers: 1) miR-4726, 2) miR-4734, 3) miR-4727, 4) Candidate_296, 5) miR-4728. The gene names are shown to illustrate which chromosomal region is shown.

3.4 Expression of the miRNAs in the HER2 amplicon

Measurement of the isolated RNA with Bioanalyzer showed that the RNA had good quality. Quantitative RT- PCR was performed on 14 HER2+ cell lines, and MCF7 as control, to study the expression of the five miRNAs in the HER2 amplicon. Results from the RT-PCR from are shown in Table 10.

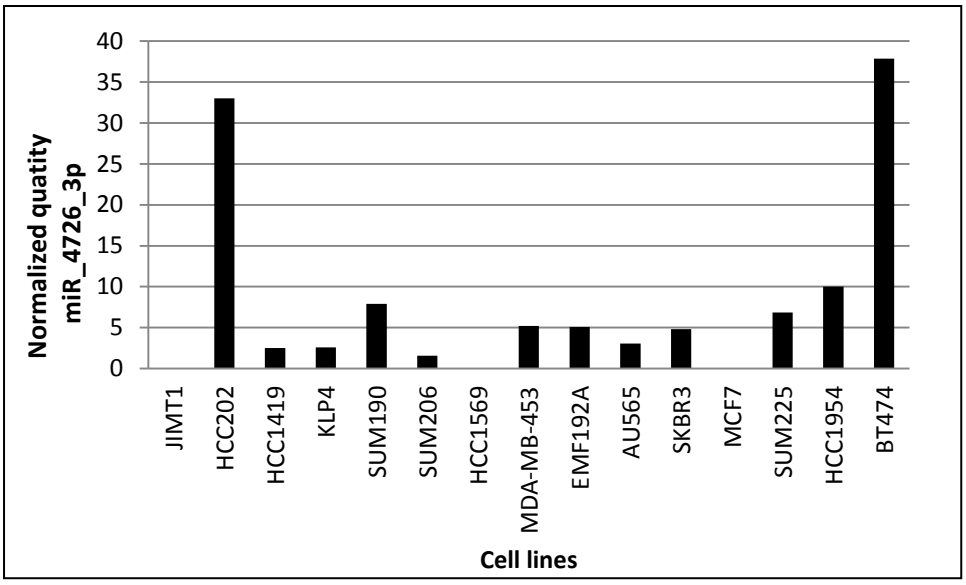
miR-4728 was expressed in all of the cell lines tested. This was expected since this miRNA is mapped within the *HER2* gene. miR-4726-3p was expressed in all cell lines except JIMT1 and HCC1569. The other four miRNAs were expressed in only a few of the cell lines tested. Therefore, miR-4726-3p and miR-4728-5p were chosen for further study. miR-4726-5p and Candidate-296-5p were not detected in the totRNA used for standard curve so absolute quantity could not be detected for these miRNAs, but expression of miR-4726-5p was detected in BT474, EMF192A and MCF7, and Candidate-296 was detected in KLP4 and BT474. The Trastuzumab response data for the cell lines were also available, and is presented in Table 10, so it was possible to determine whether there was a difference in the expression level of the different miRNAs in cell lines responsive and non responsive to Trastuzumab.

Table 10. Expression of miRNA in the HER+ cell lines. The miRNA expression in a panel of 14 HER2+ cell lines and control MCF7. These data give an overview on the expression of the miRNAs in these cell lines. ND-Not detected, D-Detected

	miRNA						
Cell lines	**Response to Trastuzumab**	miR_4726_5p	Normalized quantity miR_4726_3p	Normalized quantity miR_4727_5p	Normalized quantity miR_4727_3p	Normalized quantity miR_4728_5p	Candidate_296_5p
HCC1954	Non-responsive	0	10	0	1	1700	0
JIMT1	Non-responsive	0	0	0	0	2,16	0
HCC202	Non-responsive	0	33	0,909	0,81	15,56	0
KLP4	Non-responsive	0	2,6	0	0,278	8,066	D
SUM190	Non-responsive	0	7,92	0	0,32	2,92	0
SUM206	Non-responsive	0	1,57	0	0	0,119	0
HCC1569	Non-responsive	0	0	0	0	11,32	0
SUM255	Non-responsive	0	6,85	0	0	8,41	0
MDA-MB-453	Responsive	0	5,2	0	0	1,31	0
EMF192A	Responsive	D	5,08	1,909	0	5,06	0
AU565	Responsive	0	3,07	0	0	10,22	0
SK-BR-3	Responsive	0	4,87	0	0	5,28	0
HCC1419	Responsive	0	2,5	0	0,409	30,93	0
BT474	Responsive	D	37,85	0,267	0	0,8	D
MCF7	Control HER2-	0	0,17	0	0,329	1,58	0

The expression of the two miRNAs, miR-4728-5p and miR-4726-3p which are expressed in most cell lines are presented in charts in Figure 11.

a)



b)

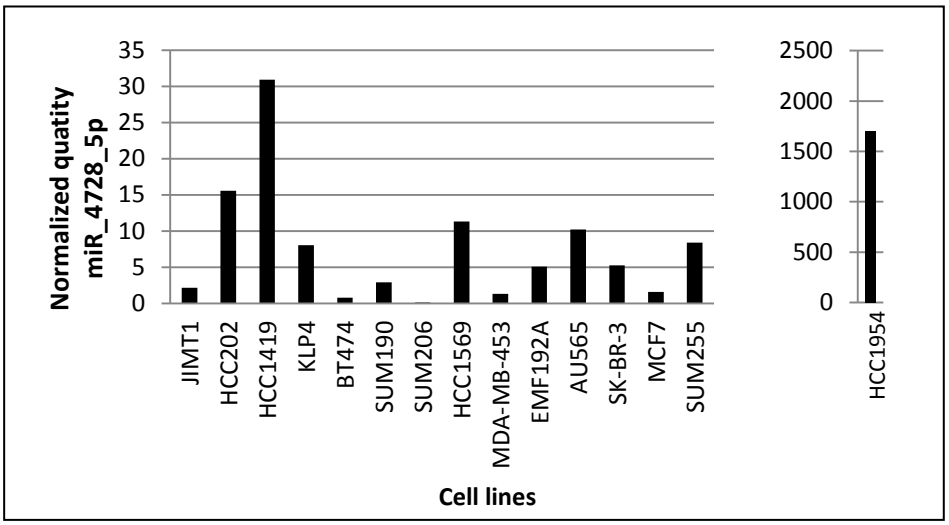


Figure 11. Normalized quantities of the miRNAs in cell lines. a) Normalized quantities of miR-4726-3p in the cell lines b) Normalized quantities of miR-4728-5p in the cell lines.

It was studied whether there was a significant difference in miRNA expression levels in the cell lines that had amplification in the region where miR-4726, miR-4734, miR-4727 and Candidate_296 are mapped and those that are not amplified in this region. This was done to study whether the qRT-PRC results correlate with the aCGH data. So the mean expression levels of these miRNAs were calculated for the two cell lines

that have this region amplified, HCC202 and BT474, and for the rest of the cell lines that do not have this region amplified (Table 11).

Table 11. Comparison of the expression level of miRNA. Comparison of the expression levels of miRNAs lines that showed amplification in the region where miR-4726, miR-4734, miR-4727 and Candidate-296 are mapped compared with the cell lines that did not show amplification in this region.

Mean quantity of the miRNAs in the amplified region, HCC202	**Mean quantity of the miRNA in the amplified region, BT474**	**Mean quantity of miRNA in the cells without this region amplified (n=12)**
11.57	12.7	1.4

A T-test was performed to study whether there was a significant difference in miRNA expression between responsive or non responsive cell lines. The test was run with a 95% confidence interval but no significant difference between the groups was observed (Table 12).

Table 12. T-test comparing miRNA expression between responsive and non-responsive cell lines.

miRNA	**Response status**	**N**	**Mean**	**Std. derivation**	**P-value**
miR-4726-3p	Non-responsive	9	6.9	10.48	0,65
	Responsive	6	9.7	13.8	
miR-4727-5p	Non-responsive	9	0.2	0.42	0,63
	Responsive	6	0.36	0.76	
miR-4727-3p	Non-responsive	9	0.3	0.37	0,17
	Responsive	6	0.06	0.16	
miR-4728-5p	Non-responsive	9	194.3	564.6	0,44
	Responsive	6	8.9	11.29	

3.5 The effect of miRNAs on the proliferation of HER2+ cell lines

3.5.1 Optimization of cell amount

For the functional studies it was desirable to choose two cell lines that had high expression of the two miRNA that showed expression in most of the cell lines; miR-4726-3p and miR-4728-5p. One cell line responsive and one non-responsive to Trastuzumab were chosen. The cell lines were SKBR-3, which is responsive to Trastuzumab, and HCC-1954 which is non-responsive to Trastuzumab. SKBR3 and HCC1954 were optimized in a 384 well plate with 72 hours of incubation. The optimal cell amount was determined visually with a microscope. The cells were visualized with 5X magnification and the optimal cell amount for SKBR3 was determined to be 2500 and for HCC1954 1750 because they showed 80% confluence after 3 days (Fig. 12).

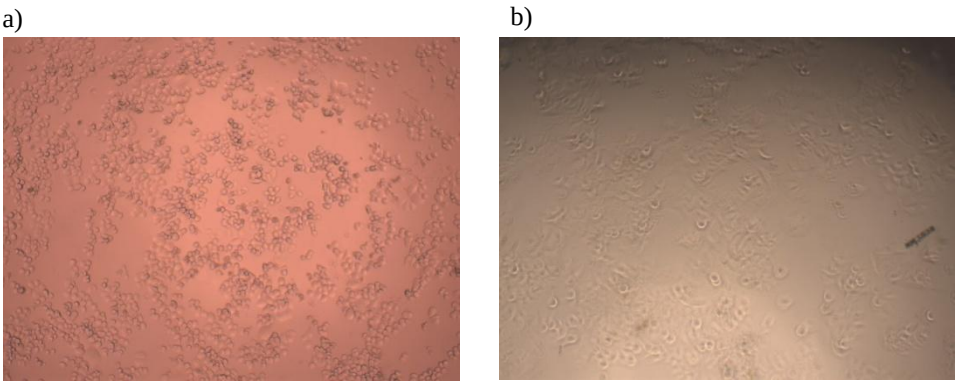


Figure 12. Visualization of cell lines. The chosen cell number after optimization: a) SKBR3 with a cell number of 2500 b) HCC1954 with a cell number of 1750.

3.5.2 Transfection of miRNA inhibitors

The cell lines SKBR3 and HCC1954 were transfected with antimiR-4728-5p and antimiR-4726-3p. An anti-miR negative control and siAllStar Cell Death positive control siRNA were used as controls. The number of viable cells after 72 hours was measured with CellTiter-Glo® Luminescent Cell Viability Assay. The mean value of the four transfected wells were calculated and then normalized to the mean of the wells with anitmiR negative control. This was done to eliminate any toxic effect the Silentfect would have on the cells, so the results only reflects the effect the anti-miR has on the proliferation. After normalization the data show that transfection of miR-

4726-3p lead to a growth reduction of 20% in both cell lines. Transfection of miR-4728-5p shows a growth reduction of approximately 15% only in the HCC1954 cell line. The cells transfected with positive control, siAllStar Cell Death, had a reduction of over 90%, which shows that the transfection has been successful in all of the experiments (Fig. 13)

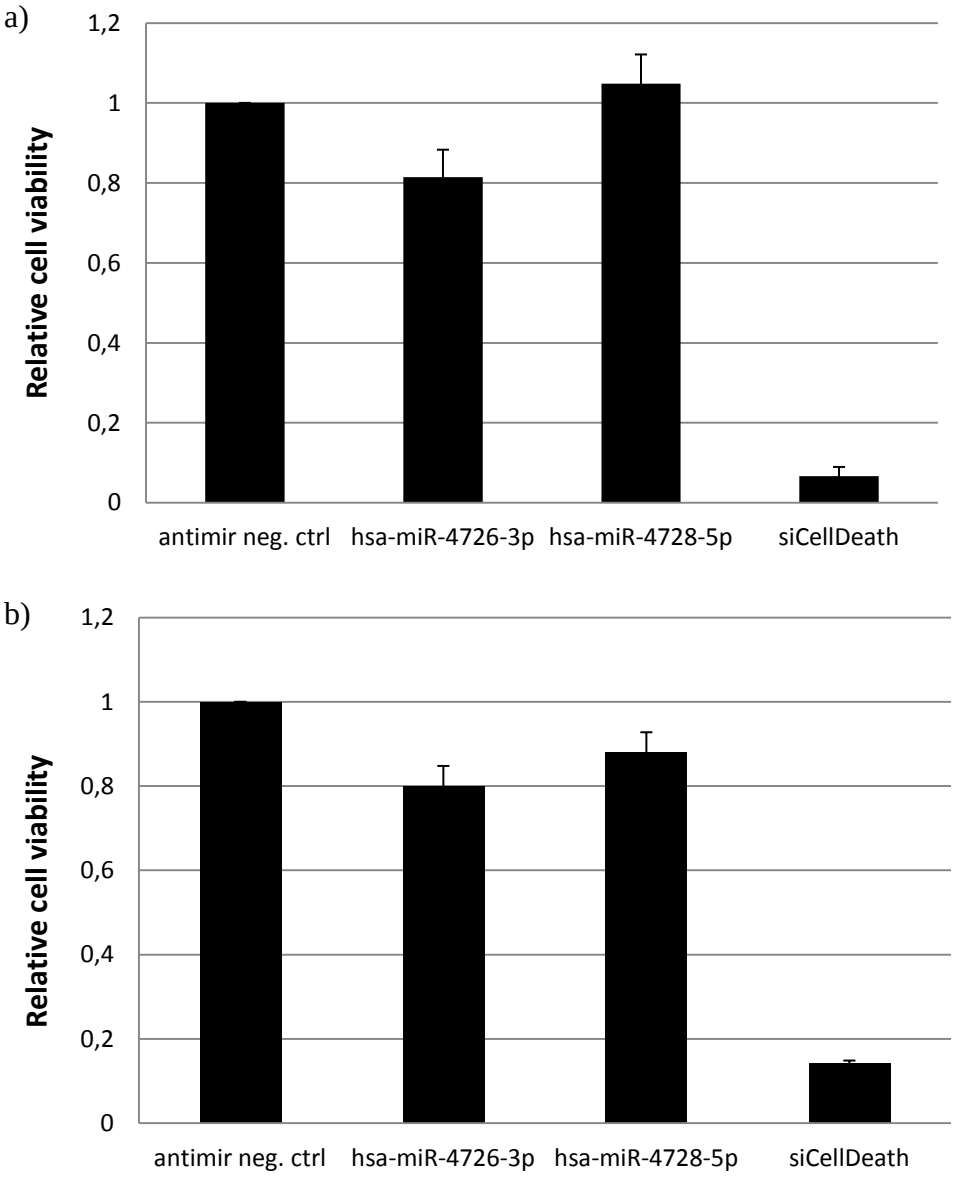


Figure 13.Normalized data, average for two transfection experiments for the cell lines. a) Normalized data SKBR3. The results shows that the cells where miR-4726 was knocked down has an approximately 20% growth reduction compared to the negative control. b) Normalized data for HCC1954. The results shows that the cells where miR-4726 was knocked down has an approximately 20% growth reduction compared to the negative control. The cells where miR-4728-5p were knocked down had an approximately 15% growth reduction compared to the negative control.

4 Discussion

During the last years, extensive research on the expression and function of miRNAs has been performed to evaluate their role in cancer. Both the miRNA expression profiles and their functional role have been investigated in normal tissues and disease state, in addition new miRNAs are continuously identified. Many miRNAs have been found to play a role in tumorgenesis some acting as oncogenes, whereas others functioning as tumor suppressors (37). miRNAs related to specific types of cancer have been found, and extensive research is ongoing to evaluate the role of these miRNAs and to evaluate the potential for these as therapeutic targets. Breast cancer is one of the most frequent cancer types in women and about 20% of all breast cancers are HER2+. Therefore, we studied whether miRNAs play a role in HER2+ cancer by investigating miRNA expression levels and by functionally inhibiting over-expressed miRNAs in the HER2 amplicon in breast cancer cell lines.

Breast cancer cell lines have been shown to be good models for cancer research since they have similar genomic profiles as primary tumors (64). Therefore, experiments that are not possible to perform with patients due to ethical reasons can be performed in cell lines. However, the microenvironment is different *in vivo* as compared to *in vitro*. In *in vitro* conditions the micro environment cannot affect the cancer cells, compared to *in vivo* conditions where the cells in the micro environment can inflict the cancer cells trough cell signaling (12). Therefore, the *in vitro* findings have to be validated *in vivo* to determine whether the results are comparable.

4.1 Expression of miRNAs in relation to Trastuzumab response

The genome-wide miRNA expression levels in HER2+ cell lines were studied in order to search for differentially expressed miRNAs in relation to Trastuzumab response. Differentially expressed miRNAs in cell lines responsive and non-responsive to Trastuzumab were detected using SAM analysis on miRNA expression array data. The SAM-analysis identified four miRNAs that were differentially expressed between the cell lines responsive and non-responsive to Trastuzumab: miR-342-3p, miR-26b and miR-15a were down-regulated in responsive cell lines whereas miR-29a was up-regulated. The validated targets of these miRNAs were searched in

miRecords, which is a resource for miRNA-target interactions. miR-29a, which was statistical significantly up-regulated in the responsive cell lines, has been shown to control the innate and adaptive immune responses during infection by targeting interferon-γ (65). This miRNA is also involved in the inhibition of cell proliferation and induce cell cycle arrest in gastric cancer by targeting the *p42.3* gene, which has been related to mitosis regulation (66;67). Several oncogenes were also found with miRecords, such as cell division cycle 42 (*CDC42),* coding for a GTP binding protein, and cyclin-dependent kinase 6 *(CDK6),* (Table 8) that were directly targeted by miR-29a. This shows that miR-29a might play a role in cancer development by targeting oncogenes and thus having a restraining effect on proliferation in cancer cells when up-regulated. The miRNAs that were down-regulated in the responsive cell lines also target several oncogenes, such as the apoptotic regulator *BCL2* and cyclin D1 (*CCND1).* In the non-responsive cell lines these miRNAs are down-regulated, and the miRNAs that are down-regulated in the responsive cell lines are up-regulated in the non-responsive cell lines. miR-15a was found to be down-regulated in the responsive cell lines as compared to the non-responsive and miRecords suggested more than 60 validated targets for this miRNA. miR-15a has been shown to be down-regulated in several types of cancer and its over-expression inhibits cell proliferation and promotes apoptosis in cancer cells by targeting several oncogenes such as *BLC2,* the anti-apoptotic *MCL1*, *CCND1* and *WNT3A,* which encodes for a secreted signalling protein (68). Also miR-26b was found to be down-regulated in the Trastuzumab responsive cell lines. This miRNA has previously been reported to induce apoptosis in MCF7 breast cancer cell lines by targeting SLC7A11, a protein involved in aminoacid transport, and has been found to be down-regulated breast cancer tumors and cell lines (69). The targets of the miRNAs found to be differentially expressed in SAM-analysis are only the validated targets. These miRNAs most likely have many targets that are still not validated. There are also several miRNAs working together to inhibit the function of one target and without the full information about the miRNA targets the complete role of these miRNAs in HER2+ breast cancer and Trastuzumab response is difficult to determine.

To further study the miRNA target genes and to find which pathways and networks they are involved in, pathway analysis was run. Among the most significant pathways were 12 canonical pathways including many cancer signaling pathways such as;

integrin-linked kinase (ILK) signaling, TP53 signaling, and PI3K/Akt signaling pathway. ILK has been found to be increased in several types of cancer, such as prostate cancer and glioblastoma, and it regulates several important pathways in tumor development, such as the PI3K/Akt pathway. Therefore, ILK has been proposed as an attractive target for cancer therapy (70). The PI3K/Akt pathway has been shown to be important in HER2+ cancer and in Trastuzumab response. The HER2 receptor activates the PI3K/Akt pathway and when the HER2 receptor is inactivated by Trastuzumab, the activation of the PI3K/Akt pathway is inhibited (25;71). Oncogenic mutants of *PIK3CA* have been found to be associated with Trastuzumab resistance. Patients with this mutation have been associated with poor prognosis after Trastuzumab therapy (72). Mutation in the *TP53* gene is one of the most frequent mutations in cancer and the accumulation of the mutant TP53 protein enhances cancerous qualities of the cells (73;74). The fact that the differentially expressed miRNAs were detected to be involved in these pathways might emphasize the importance of the miRNAs in cancer development. They have been found to target several oncogenes, and are most likely involved in these pathways by inhibiting them. In view of the fact that we found differentially expressed miRNAs in responsive vs. non-responsive cell lines it is interesting to see that some of the target genes of these miRNAs, *CDK6,* phosphoinositide-3-kinase, regulatory subunit 1 (*PIK3R1*) and *MCL1,* are found in relation to pathways that are involved in Trastuzumab response. Levels of *CDK6* activity are suppressed by Trastuzumab (75), Trastuzumab treatment reduces *MCL1* expression in the cell (76) and mutations of *PIK3R1* are involved in Trastuzumab resistance by affecting the function of p85, a regulatory subunit, which leads to the induction and constitutive activation of the PI3K/Akt pathway (77).

Although the target genes of the miRNAs seem to be important in cancer, future functional studies are needed to determine whether these miRNAs actually play a role in Trastuzumab response. Although the present study was a small scale study with few cell lines, the four miRNAs identified in SAM showed statistical significance with a low q-value and a FDR=0, increasing the likelihood that these might be valid in larger datasets. Since this analysis was performed in cell lines, it should also be validated in patient material to determine if these findings also are true in *in vivo* tumor cells. However, Trastuzumab is a relatively new treatment in the clinic, and it is

difficult to get fresh frozen tumors and miRNA expression data from Trastuzumab treated patients with follow-up data.

4.2 Expression of the miRNAs in the HER2 amplicon

We also studied the expression of the five novel miRNAs that had recently been mapped within the *HER2* amplicon (51). As they were recently discovered, they were not present on the miRNA expression array. Therefore, their expression levels were evaluated by qRT-PCR in this study. The hypothesis was that these miRNAs might be over-expressed and might play a role in HER2+ cancer. The expression of the miRNAs were evaluated with qRT-PCR in a panel of 14 HER2+ cell lines and a control HER2- breast cancer cell line. We observed that the miRNAs in the HER2 amplicon had differential expression levels among the cell lines studied. In addition, the different miRNAs had differential expression levels in the same cell line, although some of them are mapped very close in the genome. Methylation, miRNA processing/degrading might be an explanation for this. (78).

Copy number changes on DNA level were available for 10 of the 14 HER2+ cell lines and gave a schematic overview of the amplicon size among the different cell lines (Fig. 10). Only BT474 and HCC202 have an amplicon "window" that includes all of the five miRNAs. Some of the cell lines actually show DNA loss in the region where several of the miRNAs are mapped. The aCGH data therefore shows that HCC202 and BT474 would have the highest expression of miRNAs miR-4726, miR-4727 and Candidate-296 since these were the only cell lines that showed amplification at the DNA level where these miRNAs are located. On the other hand, this might not be entirely reflected in the qRT-PCR results since the factors mentioned above and the expression of the proteins processing the miRNAs in the cell might have an effect on the miRNA expression levels.

When comparing the aCGH data and the qRT-PCR we observed that the results from the qRT-PCR do not entirely reflect what was seen in the aCGH plot. miR-4726-3p has much higher expression level in BT474 and HCC202, compared to the rest of the cell lines, but at the same time, miR-4726-5p shows no expression in HCC202. miR-4727-3p shows no expression in BT474 whereas it is expressed in other cell lines.

Although, when miRNAs are processed, normally one form, 3p or 5p, dominates which leads to expression of only one mature form. The mean expression of miR-4726, miR-4727 and Candidate-296 was higher in the cell lines which were amplified in the region where the miRNAs were located. This show that the expression of the miRNAs in the HER2 amplicon can reflect to some extent the amplification rate but, might also be regulated by several other factors in the cells. We also tested whether there was a statistically significant difference in the miRNA expression levels between cell lines responsive and non-responsive to Trastuzumab. However, the T-tests showed no significant differences between the groups.

The qRT-PCR experiment was run once for each cell lines, which is not the routine for qRT-PCR experiments. To ensure that the qRT-PCR data are correct, it should be repeated until there are 3 runs with the same results. This was not done in this project, so the data only gives an overview of the expression levels of the miRNAs. In order to get the accurate expression levels of the miRNAs, the experiment should be repeated two more times.We observed that qRT-PCR is a very sensitive method, where even small differences in pipetting can have a severe effect on the results, which can make validating the results, a challenge.

4.3 Inhibition of miR-4726-3p and miR-4728-5p function affects cell growth

The qRT-PCR results indicated that miR-4728-3p was expressed in all of the cell lines and miR-4726-3p in all except two. Therefore, these miRNAs were chosen for functional inhibition studies. Based on the results from the qRT-PCR we chose two cell lines that had high expression of miR-4728-5p and miR-4726-3p. One of the cell lines was responsive, SKBR3, and one non-responsive, HCC1954, to Trastuzumab. The reason one responsive and one non-responsive cell line was chosen for the experiment was that they might have different molecular properties which may affect the transfection result. The functions of miR-4726-3p and miR-4728-5p were inhibited, to study if these miRNAs are involved in proliferation in HER2+ cell lines, by transfecting anti-miRs into the cells. Anti-miRs are oligonucleotides which inhibit miRNA function by binding complementarily to their corresponding miRNAs. The transfection was performed using a lipid transfection reagent, Silentfect, which forms a lipophilic capsule around the anti-miRNA molecule and assists in delivering the

anti-miRNA into the cells. In the cytoplasm the anti-miRs are released and they bind to the miRNAs and thereby inhibit their function.

Although both of these cell lines are HER2+ and have amplification of this region, they grow differently and show a difference in their proliferation rate, in addition to having different response to Trastuzumab. This further indicates that they have different genomic backgrounds with differentially regulated genes. The optimal cell amount was visually determined in the microscope after optimization and was determined to be 1750 cells for HCC1954 and 2500 cells for SKBR3. We decided that visual inspection was accurate enough to determine what start amount of cells would reach optimal confluence, about 80%, after 72 hours.

The optimization and the transfection were done in 384 well plates. By using 384 well plates the amount of reagents is minimized which makes the experiment more cost efficient. However, it is challenging to work with live cells in this plate format due to the difficulties of accurate pipetting of the small volumes manually, and the fact that all cells do not grow as well in such small volumes. Evaporation of nutrient medium is also more serious when using small volumes. Evaporation can be prevented by adding media in the surrounding wells, and putting the plate in an open plastic bag containing a moist paper. Media is added to the surrounding wells also to prevent the edge effect which means that cells do not grow as well in the wells near the edges of the plate as they do in the rest of the plate. CellTiter-Glo (CTG) reagent, which measures the amount of viable cells, was used as an end-point. The assay is based on measuring the amount of ATP, which correlates with the amount of live cells. This end point was chosen because viability and proliferation is an important hallmark in cancer. Other end point such as the amount of active caspases, that play an important role in apoptosis, could also be used.

siAllStar Cell Death was used as a positive control to control that the transfection had been successful. The siAllStar Cell Death is a mix of anti-proliferative siRNAs targeting genes essential for cell survival. By transfecting siAllStar Cell Death into the cells, the cells will undergo apoptosis. It can be difficult to transfect some cell lines, but we observed that the transfection was successful in all of the experiments due to the severe growth reduction of more than 90% of the cells in the wells where

siAllStar Cell Death was transfected. As a negative control we used a miRNA inhibitor negative control, which is a miRNA which has been tested to have no effect on the growth of a large panel of cell lines. As negative controls we also included four wells with no miRNA, only transfection reagent and cells, and four with only cells. The wells with cells only were to control any toxic effects the Silentfect might have on the cells. We chose the amount of Silentfect for each cell line, which was effective, but which did not have any toxic effect on the cells. In the wells where the cells were transfected with miR-4726-3p inhibitor a reduction in growth of approximately 20% of cells was observed compared to the control miRNA transfected cells. Knock-down of miR-4728-5p inhibited only HCC1954 cell growth by approximately 15%. From this it can be assumed that miR-4726-3p is involved in proliferation in these cell lines. It would be interesting to study, whether this miRNA is essential also for other HER2+ cell lines. Since miR-4728-5p inhibition only showed an effect in one of the cell lines it is possible that this miRNA only regulates proliferation in some cell lines. miR-4728-5p showed an effect in the non-responsive cell line and not in the responsive cell line. Based on that we could speculate that miR-4728-5p is involved in Trastuzumab response. However, this should be validated with a larger panel of cell lines.

The reduction in growth was not as severe for the cells transfected with the anti-miRNAs compared with the cells transfected with siAllStar Cell Death. This is expected because siAllStar Cell Death is a combination of several siRNAs damaging the cell. We might have seen a more severe growth reduction with higher concentrations of the anti-miRNAs, especially in HCC1954 where the amount of miR-4728-5p is very high. The effect of 20% is a small effect and might be explained by the fact that several miRNAs often work in clusters to inhibit target function and by knocking down only one of the miRNAs the effect is not that severe. Combination experiments by knocking down both miR-4726-3p and miR4728-5p or several other miRNAs in the same cells, might therefore improve the results. Still there are often several pathways controlling for example proliferation, and by targeting only one or two of the miRNAs regulating targets connected to pathways the cell growth is not completely inhibited. A more efficient knock-down experiment might be to knock-down genes with siRNA in addition to miRNAs or do the knock-down in combination with drug treatment.

This study indicates that miR-4726-3p is involved in the regulation of proliferation in the breast cancer cell lines SKBR3 and HCC1954 and that miR-4728 has the same effect in HCC1954. A similar effect has been shown in studies where other miRNAs have been knocked down in cancer cells. A study by Hiyshi *et al.* evaluated the role of miR-21 in proliferation and invasion in esophageal squamous cell carcinoma. They transfected the cells with anti-miR-21 and found that the transfected cells had a proliferation reduction of 20-50% compared to the control (79) which is a similar reduction as in our findings. This emphasizes that a more severe reduction by miRNA knock-down might not be seen when only one miRNA is inhibited.

Genes in amplicons have been shown to have oncogenic effect by regulating cancerous processes in the cells. As mentioned above, the function of the miRNAs mapped within the HER2 amplicon has not been evaluated, but an article by Kao and Pollack showed two genes, *GRB7* and *STARD3*, in this amplicon which contributed to proliferation levels of breast cancer cells (80). This shows that *HER2* does not work alone as an oncogene in this amplicon and support the hypothesis that both the genes and the miRNAs in this amplicon play a role in the development of HER2+ breast cancer. Genes mapped within other amplicon related to breast cancer have been found, and they have been showed to act as oncogenes. A chromosome region that is frequently amplified in breast cancer is the 8p11-12 chromosome region where Bernard-Pierrot *et al.* found two genes, *PPAPDC1B* and *WHSC1L1*, that played a role in regulating the cell survival and cell transformation (81).

Studies have found that not only the genes, but also the miRNAs in amplicons can have an oncogenic effect on the cell. Li *et al.* localized two miRNAs, miR-517c and miR-520g, in an amplicon on chromosome 19q13.41, which is often detected in brain tumors. These miRNAs showed oncogenic activity both *in vivo* and *in vitro* (82). In contrast to our study they chose to transfect cell lines that had a low expression of these miRNAs to study if the overexpression of the miRNAs would affect the proliferation. By over-expressing the miRNAs in normal cells the genomic factors are different than in cells with an amplicon, which might affect the results.

Although the results in this thesis gives a very good indication that miR-4728-5p and miR-4726-3p might contribute to the proliferation of HER2+ cell lines, the results have to be validated in several other cell lines before it can be concluded that these findings are accurate. It would also be interesting to see if these miRNAs have an effect on Trastuzumab response by knocking down miR-4728-5p and miR-4726-3p in the presence of Trastuzumab. Further studies are needed in order to examine whether these miRNAs have an effect on Trastuzumab response and to evaluate if they can be used as therapeutic targets in the clinic.

There are several concerns regarding the use of miRNAs as therapeutic target in the clinic. Performing miRNA knock-down *in vitro* using cell lines is less complicated compared to knock-down on primary tumors *in vivo.* This is due to the fact that when performing knock-down in cell lines it is possible to use high concentration of anti-miR without the ethical and safety concern that the anti-miR might affect normal cells which can lead to severe damage in the patient.

However, there are successful *in vivo* studies using miRNAs as therapeutic targets. One miRNA that shows great promise as therapeutic target in breast cancer treatment is miR-10b. This miRNA is highly expressed in metastatic breast cancer tumor cells, and it has been shown that by knocking down this miRNA in tumor bearing mice, breast cancer metastasis can be suppressed (83). However it is a great challenge to deliver the miRNA into the tumor without harming healthy tissue. In the earlier approaches miRNAs were delivered with viral or non-viral vector systems. Recently tumor-specific, ligand-targeting, self-assembled, nanoparticle–DNA lipoplex system that is designed for gene therapy in cancer has been developed (84). This system has shown promising results by efficiently and specifically targeting tumors, but not normal tissue, *in vivo*. These nanovectors have successfully been used for *TP53* gene therapy and HER2-siRNA therapy and provide a promising tumor targeting delivery system for RNA-based therapies, such as miRNA therapy. This system has been approved by the FDA in the USA and was in a phase-1 clinical trial in 2009 (84). miRNA therapy might be promising and might give cancer patients better prognosis and prolonged life time. This therapy might be seen in the clinic in the future if the delivery and specificity problems are overcome.

5 Conclusions and future perspectives

In this project, the relation between miRNAs and HER2+ breast cancer has been studied. The first part of this study shows that there are four miRNAs that are differentially expressed between HER2+ cell lines responsive and non-responsive to Trastuzumab. The targets of these miRNAs are involved in pathways connected to cancer development. The role of these miRNAs in relation to Trastuzumab response should further be evaluated functionally to study whether they are involved in Trastuzumab response.

We also studied the expression of miRNAs in the *HER2* amplicon and found that their expression varied in the 14 HER2+ cell lines. Two miRNAs, miR-4726-3p and miR-4728-5p, were most frequently expressed in these HER2+ cell lines. We observed that the amplicon size varied among the HER2+ cell lines.

In the last part of the study we observed that knock-down of miR-4728-5p led to a growth reduction of approximately 15% in one cell line and that knock-down of miR-4726-3p led to a growth reduction of approximately 20% in both of the cell lines tested. However, these results have to be validated in a larger panel of HER2+ breast cancer cell lines in order to further study the role of these miRNAs. In addition, knock-down experiments should be done with the remaining miRNAs mapped in the amplicon. By doing knock down experiments in combination with Trastuzumab it could be explored if these miRNA are involved in the Trastuzumab response in HER2+ cancer. Knock-down experiments where several miRNAs are knocked down at the same time can be done to investigate if this can give a more severe effect growth reduction.

Future studies *in vitro* and *in vivo* are needed to understand the complete role of miRNAs in relation to HER2+ breast cancer. Both targets and functional roles have to be determined before the miRNAs in the *HER2* amplicon can be evaluated as potential therapeutic targets.

6 Appendix

Appendix A: Standard curve qRT-PCR dilution

Table A.1 Standard curve: Dilution of Human Breast total RNA (Ambion, Cat# 7952, Conc. 1mg/ml):

1ng/µl: 1000ng/µl x Xµl = 1ng/µl x 1000µl

1µl Human Breast total RNA 999 µl Nuclease free water

5 ng/µl: 1000ng/µl x Xµl = 5ng/µl x 1000µl

5µl Human Breast total RNA 995 µl Nuclease free water

Well no.	totRNA in RT reaction, ng	Stock conc. Human Breast Cancer totRNA, ng/µl	µl Human Breast Cancer totRNA	µl Nuclease free water	µL in RT reaction
1	0	1	0	5	5
2	0.5	1	0.5	4.5	5
3	1	1	1	4	5
4	2	1	2	3	5
5	4	1	4	1	5
6	6	5	1.2	3.8	5
7	8	5	1.6	3.4	5
8	10	5	2	3	5

Appendix B: Reagents and equipment

Table B.1: Reagents and equipment used in the cell lab

Name	Vendor	Catalog number
Corning® 100x20mm Petri Dish with Cover	Corning	70165-102
Fisherbrand* Sterile Polystyrene Disposable Serological Pipettes with Magnifier Stripe	Fischer Scientific	13-676-10J
Serological pipettes, 5 mL	Sarstedt	86.1253.001
Serological pipettes,25 mL	Sarstedt	86.1685.020 *
Reagent and centrifuge tube, 15 mL	Sarstedt	62.554.502
Bürker Chamber		
Dulbecco's Modified Eagles Medium	Carl Roth GmbH	T730.1
McCoy's 5A Modified Medium	Sigma-Aldrich	D5546
GIBCO® Distilled Phosphate Buffered Saline (PBS)	Sigma-Aldrich	M8403
GIBCO®Pencillin Streptomyces (Pen-Strep)	Life Technologies	1490
Hepes (1M stock)	Life Technologies	15630-056
GIBCO® Fetal Bovine Serum (FBS)	Life Technologies	15140-122
GIBCO® 0,25% Trypsin-EDTA	Life Technologies	26140-079
L-Glutamine (200 mM)	Life Technologies	25200-056
miRVana microRNAs inhibitor miR-4728-5p	Ambion	4464084
miRVana microRNAs inhibitor miR-4726-3p	Ambion	4464084
miRVana microRNAs inhibitor negative control	Ambion	4464076
AllStar HS Cell Death control siRNA	Qiagen	1027299
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	G7570
µCLEAR-PLATE,WHITE,384WELL 127,8/86 MM TC, W/LID	Greiner Bio One	781098

Table B.2: Reagents used in RNA isolation

Name	Vendor	Catalog number
Trizol Reagent	Invitrogen	15596-026
Isopropylalcohol		
75% Ethanol		
Chloroform		

Table B.3: Reagents and equipment used in qRT-PCR

Name	Vendor	Catalog number
Taqman MicroRNA Assay hsa-miR-4726-3p	Applied Biosystems	465253_mat
Taqman MicroRNA Assay hsa-miR-4726-5p	Applied Biosystems	463408_mat
Taqman MicroRNA Assay hsa-miR-4727-3p	Applied Biosystems	463732_mat
Taqman MicroRNA Assay hsa-miR-4727-5p	Applied Biosystems	464016_mat
Taqman MicroRNA Assay hsa-miR-4728-5p	Applied Biosystems	461811_mat
Taqman MicroRNA Assay Candidate_296-5p	Applied Biosystems	Custom made
Taqman Universal master mix II with UNG	Applied Biosystems	4440038
Taqman MicroRNA Reverse Transkriptase kit 200 reactions	Applied Biosystems	4366596
MicroAmp optical 384 well reaction plate with barcode	Applied Biosystems	4309849
MicroAmp optical Adhesive Film	Applied Biosystems	4311971
ThermoFast 96 Non-skirt	Thermo Scientific	AB-0600/G
Damed Cap Strip	Thermo Scientific	AB-0602

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