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A characterization and comparison of the microRNA expression profile in breast cancer cell lines and exosomes

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Summary

Breast cancer is the leading cause in cancer deaths among woman worldwide, and one in ten women will experience the disease during their lifetime. Breast cancer accounted for 23% of the total new cancer incidences and 14% of the total cancer deaths in Norway in 2008. One way to potentially improve long-term cancer survival statistics is earlier detection. That includes the discovery and characterization of minimally invasive and unique breast cancer biomarkers to aid early diagnosis. The presence of circulating microRNAs (miRNAs) in blood components (including serum and plasma) has been repeatedly observed in cancer patients as well as healthy controls. Since the deregulation of miRNA is associated with cancer development and progression, profiling of circulating miRNAs has been used in a number of studies that aim to identify novel miRNA biomarkers. MiRNAs are small RNA molecules that regulate gene expression post-transcriptionally. They play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. Hence, altered miRNA expression contributes to the development and progression of human disease, including cancer.

In this thesis, we used a strategy of small RNA profiling by Applied Biosystem's next-generation sequencing system (SOLiD) to analyze the different genome-wide miRNA expression profiles in breast cancer cell lines and exosomes originating from breast cancer cell lines. We found a number of key miRNAs that were highly expressed in both the breast cancer cell lines and exosomes; miRNAs that could have potential as biomarkers for early breast cancer detection. We also sequenced miRNA from 14 breast cancer cell lines of different subtypes and miRNA from exosomes from 9 of those cell lines. This was done to investigate the potential differences in the miRNA expression patterns, both between the different subtypes of breast cancer cell lines and the exosomes originating from the cell lines. We identified miRNAs with a consistent high expression among all the cell lines and exosomes and miRNAs that were differentially expressed between the cell lines and exosomes. Finally, a comparison of the miRNA expression pattern between the exosomes and the cell lines revealed that the miRNA profiles in exosomes did not reflect the miRNA profiles observed in the parental cells.

Abbreviations

ATCC – American Type Culture Collection

Ago – Argonaute (1-4)

BRCA1 – Breast Cancer, Early onset 1

cDNA – Complementary DNA

CNAs – Copy number abberations

CLL – Chronic Lymphocytic Leukaemia

CSCs – Cancer Stem Cells

dsDNA – Double stranded DNA

dsRNA – Double Stranded RNA

dsRBD – Double Stranded RNA binding domain

DMEM – Dulbecco´s Modified Eagles Medium

dUTP – Deoxyuridine-Triphosphate

DNA - Deoxyribonucleic acid

DUF283 – Domain of unknown origin 283

ECC – Exact Cell Chemistry

ER – Estrogen Receptor

EMT – Epithelial mesenchymal transition

ETS-Pathway – Erythroblast Transformation Specific Pathway

Exp-5 – Exportin 5

ESCRT – Endosomal Sorting Complex

FOS1/FOSI1 – Fos Related Antigen 1

GRB7 – Growth factor receptor bound protein 7

Hur – Human Antigene R

HER-2 – Human epidermal growth factor 2

IGF1 – Insulin-like growth factor 1

IntClust – Integrative Cluster

LncRNA – Long non coding RNA

mRNA – Messenger RNA

miRNA – MicroRNA

miRISC – MicroRNA Induced Silencing Complex

MVB´s – Multi Vesicular Bodies

npcRNA – Non protein coding RNA

nt – Nucleotides

NPM1 – Nucleophosmin 1

NRF2 – Nuclear Factor Erythroid 2 – related factor 2

piRNA – Piwi-interacting RNA

PR – Progesterone receptor

Pri-miRNA – Primary miRNA

Pre-miRNA – Precursor miRNA

P-bodies – Processing bodies

Pol II – RNA Polymerase II

Pol III – RNA Polymerase III

PAZ-Domains – Piwi-Argonaute-Zwille Domains

PABPC – Cytoplasmic Poly A-Binding protein

RPM – Reads Per Million

rpm – Rotations per minute

Oct-4 – Octamer-Transcription Factor 4

RISC – RNA Induced Silencing Complex

Ran-GTP – Ran-Guanosine-Triphosphate dependent transporter

RPMI –Roswell Park Memorial Institute Medium

RIN – RNA integrity number

RNase – Ribonuclease

RNA - Ribonucleic acid

snoRNA – Small nuclear RNA

siRNA – Small interacting RNA

Snai1 – Zinc finger protein 1

PTEN – Phosphatase And Tensine Homolog

PCR – Polymerase Chain Reaction

PACT – Protein Activator of the Triphosphate Dependent Transporter

PCR – Polymerase Chain Reaction

rRNA – Ribosomal RNA

SOCS1 – Suppressor of Cytokine Signalling 1

tRNA – Transfer RNA

Tot-RNA – Total RNA

Tp 53 – Tumor protein 53

TRBP – TAS RNA Binding Protein

XRN1 – Exoribonuclease enzyme 1

ZEB 1/2 - Zinc finger E-box-Binding Homeobox 1

µl – micro liters

1 Introduction

1.1 Breast Cancer

Worldwide, breast cancer is the most common invasive cancer in women and the leading cause of cancer deaths in females, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths in 2008 (1). In 2010, 2839 women and 13 men were diagnosed with breast cancer in Norway and based on normal life expectancy; one of ten women will develop breast cancer. Detection at an early stage significantly increases the 5-year survival rate, and although debated, regular mammography is considered the most important method today for detecting breast cancer at an early stage (2).

1.2 Breast cancer and screening

In 1996 the pilot screening program was implemented in Norway, and women in the age range of 50-69 years old was offered regular screening every other year. The program was gradually implemented in the rest of the country, and in 2004 it was countrywide. Several preliminary studies have shown that the targeted reduction mortality has not yet been attained, causing a big debate in Norwegian media (3). The screening program is currently under evaluation by the Norwegian Research council, aiming to look at whether a reduced mortality is being achieved. Despite this widespread screening, breast cancer remains a leading cause of cancer death in women, in part because screening mammography displays high rates of false-negative results and because many women decline to have routine mammograms. The development of sensitive and specific assays for detection of breast cancer biomarkers would certainly facilitate screening, improve detection and early diagnosis and contribute to therapeutic monitoring and surveillance for recurrence (4).

1.3 Classification of breast cancer

Breast cancer is a heterogeneous group of neoplasms originating from the epithelial cells lining the milk ducts. The diversity between and within the tumors as well as among cancer bearing individuals, determine the risk of disease progression and therapeutic resistance. This heterogeneity among breast tumors has been known for a long time, and the difference have served as the basis for disease classification (5).
Relatively recently, the traditional, mainly pathology-driven classification has been refined and at times replaced by molecular classifications. However, the vast heterogeneity in cancer cell phenotypes combined by the dynamic plasticity of the tumor microenvironment makes tumor categorization a demanding task, especially in relation to therapeutic response and disease progression. During the last 10 years, five subtypes of breast cancer have been identified and intensively studied; Luminal A, Luminal B, HER2-enriched (Human epidermal growth factor receptor 2), Basal-like and normal breast-like (6–8).

The gene expression in the luminal subtype correlates with the gene expression in the normal breast epithelia; this includes transcriptional factors such as GATA3 and FOXA1, which are important for luminal differentiation (9). The luminal subtype often express HER2 and, partly based on the expression of estrogen receptor (ER), progesterone receptor (PR) and HER2, the luminal subtype is divided in to two subclasses: Luminal A and Luminal B (10). The luminal A subtype is HER2 negative and is characterized by a high expression of ER, PR and a number of ER-targets and a overall low proliferation (11). Luminal B tumors also express high levels of ER and PR and are either HER2+ (Luminal-HER2 group) or HER2- accompanied by a high proliferative rate (12). The basal-like subtype has a gene expression resembling normal basal/myoepithelial cells of the breast. This subtype frequently lack the expression of hormone receptors and HER2 (6), show mutations in the tumor suppressor gene tumor protein p53 (*TP53)* (7), and has high expression of proliferation-related genes (6,7,13). Furthermore the initial gene expression profiling experiments demonstrated that the basal-like subtype (together with the HER2 overexpressing subtype) were associated with a particularly poor prognosis (7).

A significant fraction of the basal-like subtype is ER, PR and HER2 negative (i.e. triple negative breast cancer) (14). In fact, the majority of basal-like breast cancers are also triple-negative breast cancer and most triple-negative breast cancer (approximately 80%) are also basal-like breast cancer (15). However, not all basal-like tumors demonstrate a triple negative phenotype and conversely, not all triple-negative breast cancer have a basal-like gene expression profile (16,17). Triple-negative breast cancer is associated with a poor prognosis and a unique pattern of recurrence characterized by high rates of recurrence only in the period from 1 to 4 years after diagnosis (18,19).

The HER2 enriched subgroup has a low expression for ER, and other genes associated with ER (6). HER2 overexpression is usually caused by gene amplification and this seems to drive genomic instability along chromosome 17q leading to overexpression of other genes on the 17q amplicon, such as the growth factor receptor-bound protein 7 (GRB7) (20). This subtype is found in 10-20% of breast cancers and is associated with a poorer prognosis (21). A complication that has been relatively common for the HER2-enriched subtype is brain metastasis (22), and about 37% of patients with HER2-positive breast cancer relapse due to intracranial disease (23). Several factors of HER2-enriched subtype are likely to account for the increased risk of brain metastasis, and one study showed that there was an increased risk if the patient had received prior treatment with trastuzumab (24). The HER2-enriched subtype is an aggressive subtype and is associated with a higher risk of both local and regional relapse (23,25). In general, an activation of HER-2 tyrosine kinase receptor triggers a complex array of signaling pathways that regulates normal cell growth and promotes tumorigenesis; cell proliferation, survival, migration, differentiation and angiogenesis (26,27).

Normal-like breast cancer has a gene expression pattern characterized by high expression of genes that is characteristic for basal epithelial cells and adipose cells and a low expression of the genes that is characteristic for luminal epithelial cells (6). It has also been proposed that the normal-like subtype is mainly an artifact of having a high percentage of “contamination” in the tumor specimen. Another explanation

could be that the normal-like subtype is a group of slow growing basal-like tumors that lack expression of the proliferation genes or a potential new subtype that has been referred to as Claudin-low tumors (20,28). Molecular characterization of the Claudin-low subtype revealed that these tumors are significantly enriched in EMT (Epithelial–mesenchymal transition) and stem cell-like features while showing a low expression of luminal and proliferation-associated genes (20,29).

Although the current classification of human breast tumors has proven to be fundamental for prognosis and predictive evaluation, a number of important limitations remain. First, substantial variation in response to therapy and clinical outcome still exists, even for tumours that seem to share similar characteristics both clinically and pathologically. Second, this classification does not reflect the underlying biology or the molecular pathways driving the disease in the different subtypes. Recent studies has suggested that the integrated changes at the genomic level could give rise to a new classification system, based on the changes in CNA`s (Copy number aberration) (30), which is an acquired alteration of DNA that results in an abnormal number of copies of one or more segments of DNA (1kilobase or larger). Based on this it was found 10 new integrative clusters (intClust 1-10), where all of the ten subtypes are associated with different outcome and had distinct clinical features (31). In the future, breast cancer classification will most likely involve several levels of assessment integrating clinical information about the patient, histopathology information about the tumor and molecular data revealed by genomic, transcriptomic and proteomic profiling. At the genomic level, the next generation sequencing technology will contribute to delineate the complete genomic background including structural rearrangements, somatic mutations, variations in copy number and epigenetic changes. This will undeniably be essential to further elucidate the mechanisms driving each subtype and lead to fundamental improvements in our approach to the classification of breast cancer subtypes, the biological characterization, and the management of breast cancer (32).

1.4 Breast cancer cell lines

Breast cancer cell lines is the most widely used model system to investigate how proliferation, apoptosis and migration becomes deregulated in breast cancer (33). Breast cancer cell lines are easily propagated, relatively tractable to genetic manipulation and, under well-defined experimental conditions, they generally yield reproducible and quantifiable results (34). Human cells are thought to have more relevance to human disease compared to other models like mice and rats, and there is a debate about whether the same genetic alterations transforms both mouse and human epithelial cells (35,36).

Three different subtypes of cancer cell lines are identified based on gene expression; Luminal, Basal A and Basal B (37). The luminal subtype contains only ER positive cell lines, and is characterized by enriched expression of ER and good prognosis signatures (37). The basal A subtype only contains ER negative cell lines, resembles basal-like tumours and is associated with the erythroblast transformation specific pathway (ETS-pathway) and the breast cancer 1, early onset genes (BRCA1) signatures (37). The basal-B subtype is characterized by markers that is associated with more aggressive tumours including mesenchymal and stem/progenitor-cell characteristics and up regulation of the epidermal growth factor (EGF) (37) .

The majority of the luminal cell lines is similar to the luminal-A or luminal-B tumours, the basal-A show similarities with the basal-like tumors and the basal-B cell line is compatible with the basal-like or the HER2-enriched tumors (37).
The discrepancy of subtypes between primary tumours and cell lines might be due to the fact that the cell line expression profile are not “contaminated” with normal epithelial or stromal cells so that the cluster resolve more clearly in the cell lines. The difference could also be a result of the absence of stromal or physiological interactions and/or signalling in cell culture (38). However, it has been shown that the differences between the genome aberration patterns for the basal-like and luminal clusters in the cell lines do not reflect the differences in these subtypes in primary

tumours, suggesting that the cell lines may be derived from subpopulations of tumour cells that are selected because they grow well in culture.

Still, comparison between cell lines and primary tumors have shown that cell lines mirror both the genomic heterogeneity and the recurrent genome copy number abnormalities found in primary tumours (39). Thus, breast cancer cell lines seem to represent an appropriate model system to investigate the functional consequence of gene deregulation and identification of molecular features of breast cancer as well as the discovery of biomarkers and new breast cancer genes.

1.5 Non-protein coding RNA molecules

Since the complete human genome sequence is available, the need and necessity to understand it and use that information to learn about the biology of human disease is tremendous (40). Earlier, research has generally been focused on protein-coding genes, but in recent years it has been well acknowledged that the non-protein coding genes in the human genome are essential for cancer biology. As much as 90% of the human genome is actively transcribed and the protein-coding genes account for less than 2% of the genome sequence (40,41). Hence, a huge amount of non-protein coding RNAs (npcRNAs) are transcribed from the genome. Most of these npcRNAs fulfil crucial functions, such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) essential for mRNA translation, small nuclear RNAs (snRNAs) involved in splicing and small nucleolar RNAs (snoRNAs) involved in rRNA modification. But in addition to these well-known "housekeeping" npcRNAs, many regulatory npcRNAs have been discovered and characterized. These regulatory npcRNAs are generally grouped into two major classes based on transcript size; long ncRNAs (lncRNAs) and small ncRNAs (42–44).

1.6 Small regulatory RNA molecules

Over a long period of time the area of RNA molecular biology has been thoroughly transformed compared to other areas of biology, and one of the most significant discoveries has been the detection of the small, approximately 20-30 nucleotides (nt) noncoding RNAs that are involved in the regulating of genes and genomes. The regulation can happen at several of the central levels of genomic function; Chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability and translation.

The effects of small RNAs are generally inhibitory and the equivalent regulatory mechanism is therefore mutually incorporated under the heading of RNA silencing (45). Based on their function, small regulatory RNA are divided into three different classes; small interacting RNA (siRNA), piwi-interacting RNA (piRNA) and micro RNA (miRNA); all existing only in eukaryotes (45).

SiRNAs are thought to be primarily exogenous in origin, deriving directly from a virus, transposon, or transgene trigger. It is excised from long, fully complementary double-stranded RNAs (dsRNAs) (46), implemented in RNA-Induced-Silencing-Complex and regulates gene expression by direct base-pairing with mRNA inducing mRNA cleavage (45).

PiRNAs are typically 24–32 nt long RNA molecules that are generated by a Dicer-independent mechanism. They have been implicated in germ cell development, stem cell self-renewal, and retrotransposon silencing (47).

MiRNAs are evolutionary conserved, small (18–25 nt), single-stranded RNA molecules involved in specific regulation of gene expression in eukaryotes (48). It is predicted that the miRNA genes comprise 1–2% of the human genome and that miRNAs control the activity of about 50% of all protein-coding genes (49,50)
They were first discovered in the nematode *Caenorhabditis elegans* (51) and regulate gene expression post-transcriptionally primarily by binding to complementary sequences in the 3′ untranslated region (UTR) of messenger RNAs (mRNAs). This

interaction results in either degrading or blocking translation of target mRNAs (or a coupled process involving both degradation and inhibition of translation) leading to reduced protein expression of the targeted gene (52,53). The number of miRNAs in the human genome is currently estimated to be 1600 precursor- and 2042 mature miRNAs (miRBase, release 19, august 2012) (54).

1.7 The biogenesis of miRNA

The biogenesis of miRNAs in mammals (illustrated in Fig 1.1) can be summarized in two main processing steps, starting in the nucleus and ending in the cytoplasm. In the nucleus primary miRNA (pri-miRNA) is mainly transcribed by RNA polymerase II (Pol II) (55), however some are transcribed by RNA polymerase III (Pol III) (56). These transcripts can be thousands of nucleotides long and are characterized by hairpin structures (pri-microRNAs). The pri-microRNAs are further processed in the nucleus by the RNAse III enzyme Drosha. Drosha binds to the DiGeorge syndrome critical region gene 8 protein (DGCR8), where Drosha functions as the catalytic component while DGCR8 recognizes the pri-miRNA and stabilizes the interaction with Drosha (55). The resulting pre-miRNAs are 70- to 100 nt long hairpin precursors with 3´overhangs at the base of the stem-loop (55).
In addition, there is an alternative miRNA biogenesis pathway, the so-called miRtron pathway, which is not dependent on Drosha-mediated cleavage. This is because miRtrons (miRNAs localized within introns of protein-encoding or non–protein-encoding genes) are directly processed by the cells splicing machinery to generate pre-miRNAs (57–59).

Exportin 5 (Exp5) and its cofactor Ran-guanosine triphosphate (Ran-GTP), recognizes the pre-miRNAs, with a high affinity for the hairpin structure and the 3'overhang, and mediates the translocation from the nucleus to the cytoplasm. In the cytoplasm, the pre-miRNA interacts with another RNase III enzyme, called Dicer (55). Dicer is a multi-domain protein containing a RNAhelicase/ATPase, Domani of unknown function-283- (DUF283-) and Piwi-Argonaute-Zwille-Domains (PAZ-domains), two neighboring RNase III-like catalytic domains (RIIIDs) and a double

stranded RNA binding domain (dsRBD) (60). During this second cropping process (dicing) Dicer is associated to TRBP (TAR RNA-binding protein) or the protein activator of the interferon-induced protein kinase (PACT) (also known as PRKRA) and Argonaute (Ago1-4) (55).
The PAZ domain in Dicer recognizes the 3´overhang and cleaves the pre-miRNA (mediated by RIIIDs) approximately two helical turns away from the base of the stem-loop (60). The result is a miRNA duplex (about 22 nt long), which includes the mature miRNA guide and the complementary passenger strand (miR-3p/miR-5p).

Dicer, TRBP and PACT participate in the assortment of mature miRNA strands and the transfer of the mature miRNA into the RNA-Induced-Silencing-Complex (RISC). RISC preferentially includes the mature single-stranded miRNA molecule and Ago2 proteins, that act as guiding molecules to deliver the complex to target mRNA (61).

The micro RNA-induced silencing complex (miRISC) can now, dependent on the miRNA sequence, regulate the expression of target mRNAs. Binding to target mRNAs usually happens through partial complementarity and lead to mRNA degradation or inhibition of translation, depending on the sequence complementarity between the small RNA and the target mRNA (62).

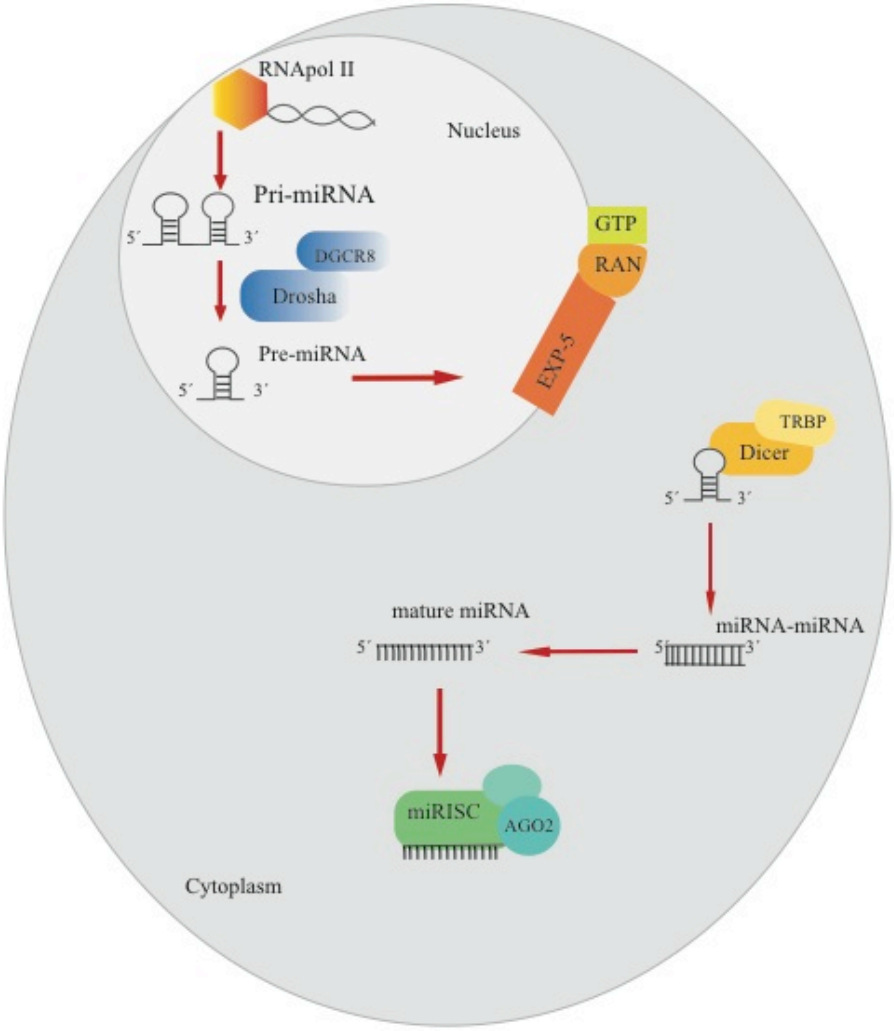


Figure 1.1. The biogenesis of miRNA; Pri-miRNA is transcribed by RNA polymerase II (55) in the nucleus. Drosha then further processes the transcript, yielding a hairpin precursor that is approximately 60-70 nt in length. Pre-miRNA is then exported into the cytoplasm by Exportin-5 (57–59). The pre-miRNA is then further processed into 19-25 nt miRNA duplex structures by the RNase III protein Dicer. Generally, the less stable of the two strands in the duplex is incorporated into RISC where the miRNA and its mRNA target interact (62).

1.8 The MiRNA genes

The majority of the miRNA genes are are embedded within introns or exons of protein coding genes and share the same transcriptional control of the host gene (63). (63). These intronic miRNA genes are oriented in the same direction as the host gene and are, with a few exceptions, transcribed from the same promoter and processed from the introns of host gene transcripts.

Mirtron is a notable exception where the intron is the exact sequence of the pre-miRNA with splice sites on either side, and is spliced out of the host transcripts into the direct substrate of Dicer (59).

Intergenic miRNA genes are, on the other hand, found in genomic regions distinct from known transcription units. These miRNAs genes can be either monocistronic with their own promoters, or polycistronic, where several miRNAs genes share a promoter and are transcribed as cluster of primary transcripts (64).

Exonic miRNAs are quite rare and often overlap an exon and an intron of a noncoding gene. These miRNAs are generally transcribed by their host gene promoter and their expression often excludes host gene function (63).

Somewhere between 36% and 47% of miRNAs are found in genomic clusters (65,66). Genomic miRNA gene clusters can be both intronic and intergenic and are defined as groups of miRNA genes with arrangement and expression pattern implying transcription as a multi-cistronic primary transcript. The miRNAs within a genomic cluster are often, though not always, related to each other; and related miRNAs are sometimes but not always clustered (67).

1.9 The mechanism of miRNA mediated gene silencing

The exact mechanism for how miRNAs is regulating its target genes is not yet fully understood, however several controversies surround the topic (45,68–70). Initially it was suggested that in animals, miRNAs repress their targets mainly at the level of translation, causing little or no influence on mRNA levels. In contrast, plant miRNAs were thought to promote mainly target cleavage and degradation and these differences were assumed to stem from the fact that the degree of base pairing complementarity between miRNAs and their targets is much less extensive in animals than in plants. Nevertheless, it is now clear that miRNAs can induce mRNA degradation in animals, but whether target silencing occurs predominantly at the level of translation or by mRNA degradation has been debated exceedingly, with lines of evidence supporting both views (45). Moreover, translational repression has been suggested to occur in four different ways: inhibition of translation initiation; inhibition of translation elongation; co-translational protein degradation; and premature termination of translation (45,68–70).

In 2010, Bartel and colleagues investigated the contribution of translational repression to silencing of miRNA targets using ribosome profiling and found that miRNAs cause a decrease in cellular mRNA levels that can explain most of the reduction (84%) in protein production (71). They also found that the mRNA fraction that was not degraded was translated less efficiently, concluding that regardless of whether mRNA destabilization occurs before or after a translational block, it still provides the main contribution to the reduction in protein levels. Indeed, studies have demonstrated that miRNAs trigger deadenylation and decapping even when the mRNA target is not translated, and this indicates that mRNA degeneration is not a consequence of a primary effect of miRNAs on translation, but an independent mechanism by which miRNAs silence gene expression (72).

When the miRNA is part of the RISC complex, it can recognize the binding sequence on the target mRNA and bind with a Watson and Crick base pairing (73). The degree of complementarity between the miRNA and its target RNA seems to influence the mechanism of regulation (45). The mechanism by which miRNA sequence complementarity conveys functional binding to mRNA targets has been thoroughly studied, providing rules for miRNA target prediction algorithms. Numerous biochemical and structural findings has shown that the 5′ region of a miRNA from nucleotides 2 to 8 (known as the 'seed' region) is of particular importance in targeting (74).

The seed region is the most evolutionarily conserved region of miRNAs (75), and it is most frequently complementary to target sites in 3′-UTRs of its target mRNA (76). Despite the importance of the seed region, there are still examples that miRNA also can be functional even if the binding degree is low and there is little complementarity in the seed sequence (77). Binding of miRISC to the target mRNA 3´UTR sequence is thought to induce deadenylation and decay of target (78,79). MRNAs repressed by deadenylation (or at the translation initiation stage) co-localizes with Ago proteins and miRNAs to cytoplasmic foci known as p-bodies. These p-bodies are discreet cytoplasmic domains that contain most enzymes required for mRNA degradation and it seems as translation repression by RISC delivers mRNAs to p-bodies, either as a cause or as a consequence of inhibiting protein synthesis (78,80,81).

An important question that remains unanswered is whether translation is inhibited before deadenylation, or whether there is an initial event that renders the mRNA target more accessible to the decay enzymes and at the same time interferes with translation. Bringing together the accumulated data in the field, it seems that miRNA mediated silencing of mRNA begins with the recognition of the target by miRISC including the AGO proteins. The AGO proteins then interacts with the GW182 proteins, which consists of multiple glycine(G)-tryptophan(W) repeats and has a molecular mass of 182 kilo Dalton (kD) (82). The GW182 proteins have been shown to be essential for miRNA-mediated gene silencing in animal cells (81,83–85) The GW182 in turn, interacts with the cytoplasmic poly(A) binding protein (PABPC). The

assembly of this complex on the mRNA finally triggers deadenylation, resulting in a rapid degradation by the major 5′-to-3′ exonuclease, exoribonuclease 1 (XRN1) (79,86,87)

1.10 Circulating miRNA

MiRNAs were identified in cell-free circulation in 2008 (88,89). This opened up the exciting prospect of utilizing circulating miRNAs as non-invasive diagnostic markers for cancers and other diseases. Circulating miRNAs have many of the crucial features of good biomarkers: First, they are extremely stable in the circulation and are resistant to RNase digestion, extreme pH, boiling, extended storage, and multiple freeze-thaw cycles. Second, the majority of miRNAs are conserved in closely related animals, such as human and mouse. Many are also conserved more broadly among the animal lineages. Third, several studies have shown that among detectable miRNAs, specific signatures exist in the miRNA profiles that distinguish healthy from diseased individuals. Finally, miRNAs levels in body fluids can easily be determined by various methods (90–95). The stability of circulating miRNAs in blood begs the question of what mechanism that protects circulating miRNAs from degradation and different opinions exist regarding this issue. In 2010 Wang et al. reported that miRNAs are associated with different RNA-binding proteins, most importantly nucleophosmin 1 (NPM1), which may play a role in protecting miRNAs from degradation (96).

It has also been shown that miRNA associates with AGO 2, leading to the hypothesis that circulating miRNAs may be by-products of dead/dying cells that remain stably bound to AGO 2 in the extracellular environment (97). However, several studies have revealed that miRNAs are localized and protected either in microvesicles (up to 1 μm) or in small membrane vesicles of endocytic origin called exosomes (50–100 nm) (98,99).

MiRNA in exosomes are functionally active and can be delivered to recipient cells where they exert gene silencing through the same mechanism as cellular miRNAs (100). Moreover, a study by Pigati and colleagues showed that

miRNAs is selectively released into blood, milk and ductal fluids, and that this selectivity correlated with malignancy (101). It therefore seems likely that profiling of secretory miRNAs could represent a valuable cancer diagnostic and prognostic tool in near future.

1.11 Exosomes

Exosomes were originally described in 1983, however the attention towards these vesicles has increased dramatically in the last years, after the finding that they contain mRNAs and microRNAs (99). Exosomes are small, secreted vesicles with a diameter between 30 and 100 nanometers (nm) that form inside intracellular multivesicular compartments and are released upon fusion of these compartments with the plasma membrane. They are formed when cell membrane proteins transfer to early endosomes by inward budding. Intraluminal vesicles are then generated through invagination of endosome membranes, generating intracellular multivesicular bodies (MVBs). The molecules that are inside the intraluminal vesicles can either be sorted for degradation by fusion of the MVBs with lysosomes, or secreted when the MVBs fuse with the plasma membrane and release the exosomes (76,80). The mechanism behind the process that controls the development of MVBs into exosomes is not yet clear but the endosomal-sorting complex required for transport (ESCRTs) is suggested to play a key role in the development of the lysosomes, while the development of the exosomes may be ubiquitin dependent (102). The microvesicles are rich in proteins located in internalization-prone membrane domains and molecules engaged in fission, scission, and vesicular transport (103–106) exosomes also harbor selected mRNA and miRNA (99). The recruitment of miRNA into the exosomes is facilitated by a coupling of the RNA-induced silencing complex, to different components of the sorting complex; where GW bodies (containing GW182) that is sorted into MVB, promotes continuous assembly/disassembly of membrane-associated miRISCs (107,108). Once they are shed, the exosomes can interact with surrounding cells and release their internal content (e.g., RNA) into the cytosol of recipient cells, facilitating the horizontal delivery of bioactive molecules. Several studies have shown that exosome binding/uptake can severely alter target cells, as demonstrated for immunosuppression, T cell activation, and transformation to a

malignant phenotype (109–112) These findings support that at least some exported miRNAs are used for cell-to-cell communication, however the mechanisms that determines how miRNAs are specifically targeted for secretion, recognized for uptake, and what information can be transmitted via this process requires further investigation.

A number of different profiling platforms are currently used for detection of miRNAs in body fluids. Relative quantification by stem-loop RT-PCR is a powerful method for the analysis of serum miRNAs, and this method is commonly used for the sensitive detection of low abundance circulating miRNAs (113). Microarray is another method that is commonly used for detection of miRNAs, however this generally requires more starting material than qRT-PCR and (as with qRT-PCR) requires prior knowledge of the miRNA sequences for analysis (90).

Deep sequencing appears to be a very promising technique for identifying novel miRNA biomarkers since this technology allows the concomitant quantification of miRNAs, identification of isomiRs, and detection of novel miRNA sequences (114,115). Although these methods open exciting avenues for non-invasive quantification of miRNAs, there has been concerns about reproducibility and variability between the profiling platforms (116,117) and more work seems necessary to establish standardized and normalized protocols.

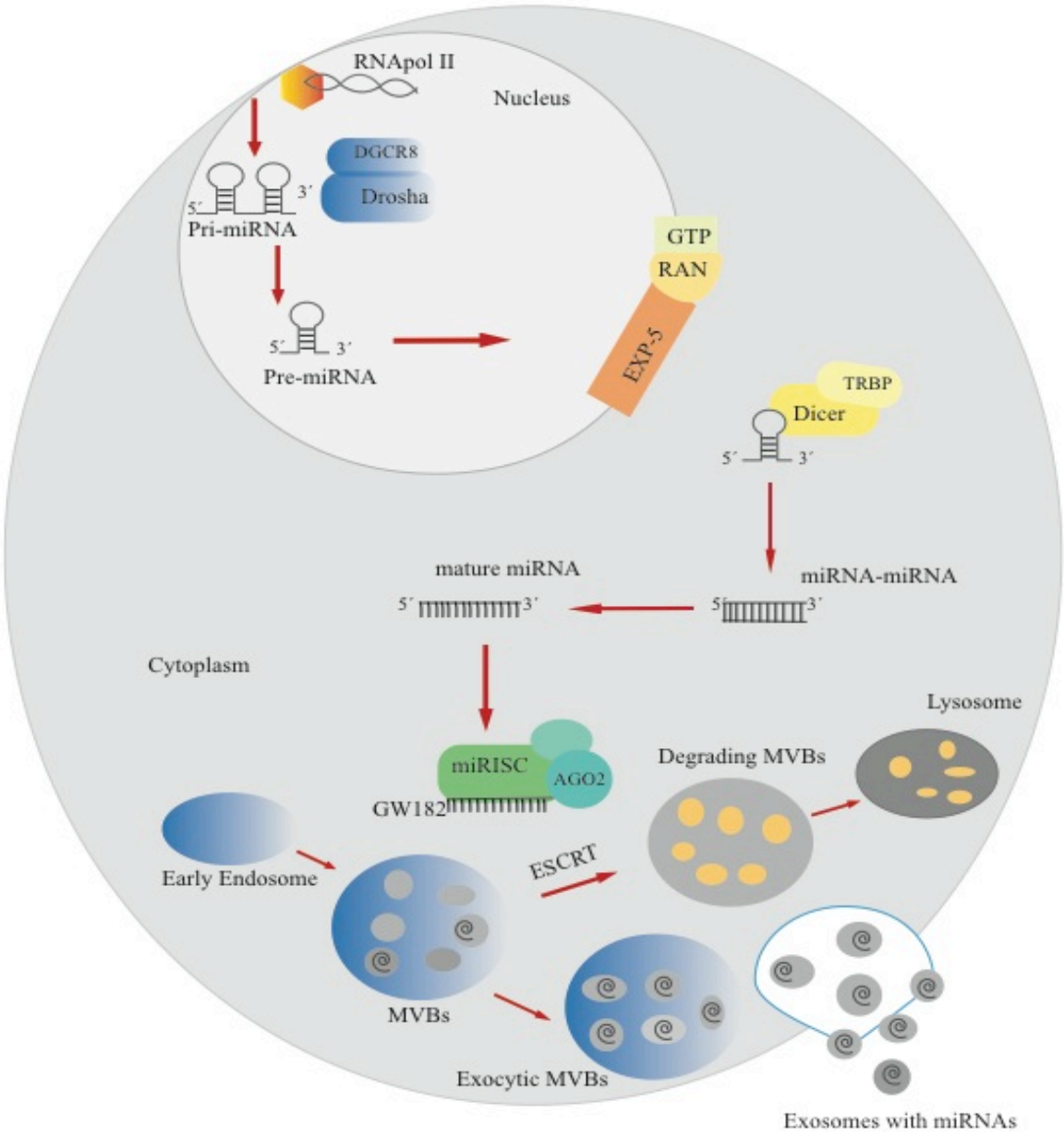


Figure 1.2. In the nucleus, miRNAs are transcribed from DNA. A precursor hairpin miRNA (pre-miRNA) is formed after cleavage by the RNase III enzyme Drosha. After transport into the cytoplasm, the pre-miRNA is further cleaved into 19- to 23-nucleotide miRNA duplexes and one strand of the miRNA duplex is loaded into RISC where it guides the RISC to specific mRNA targets and prevents the translation of the mRNA into protein. In the cytoplasm, the miRISC can also be incorporated into exosomes which originate from the endosome and are released from cells when multivesicular bodies (MVB) fuse with the plasma membrane.

1.12 MiRNA and breast cancer

In the early 1990s a region of chromosome 13 that was frequently deleted in chronic lymphocytic leukemia (CLL) was identified, and it was assumed that the region contained tumor suppressor genes.

In 2002, researcher Carlo Croce, finally found the genes for two microRNAs in the deleted region; miR-15 and miR-16. Croce and his postdoc George Calin, then showed that both genes were either absent or had reduced activity in two-thirds of CLL patients, proposing that the miRNAs were tumor suppressors. Later they confirmed this supposition, showing that miR-15 and miR-16 induce apoptosis by targeting the key survival protein Bcl-2, which is overexpressed in CLL (118).
Since then, miRNA-expression profiling of human tumors have identified specific signatures associated with diagnosis, tumor stage, tumor progression, prognosis and treatment response (119).

MiRNAs contribute to oncogenesis both as tumor suppressors and oncogenes (Oncomirs) and the genomic abnormalities that influence the activity of miRNAs, are the same as those affecting protein-coding genes; chromosomal rearrangements, genomic amplifications or deletions and mutations (120,121).

In one of the first miRNA profiling studies of solid breast tumors, 13 miRNAs that discriminated tumors from normal tissues with an accuracy of 100% were identified (122). Some of the most significant miRNAs are found to be differentially expressed in this initial study, have later been shown to have an important role in the biology of breast cancer.

MiR-21 is often overexpressed in breast cancer and exerts its oncogenic role by mediating cell survival and proliferation by directly targeting the tumor suppressor genes PTEN, PDCD4 and TPM1. Elevated levels of miR-21 is also associated with advanced clinical stage, lymph node metastasis and poor prognosis (123–126).

MiR-155 is one of the most multifunctional miRNAs whose overexpression has been found to be associated with different types of cancer including breast cancer. MiR-155 targets the tumor suppressor gene, suppressor of cytokine signaling 1 gene (SOCS1) (127,128) in breast cancer (128), and promotes TGF-beta-induced EMT and cell migration and invasion by targeting RhoA (129). It is associated with resistance towards chemotherapy, cell survival and cell migration in breast cancer and overexpression of miR-155 correlates positively with cell proliferation, tumor growth and the development of metastasis (128,130).

The majority of miRNA function is based on repression of their target genes, which means that a miRNA will be tumor suppressive if its target gene is an oncogene (131). Tumor suppressor miRNAs are often down regulated or lost in cancer, and several miRNAs are reported to be tumor suppressors.

The miR-125a and miR-125b isoforms have both been found to be significantly down regulated in breast cancer patients and miR-125a functions as a tumor suppressor by repressing the translation of the RNA binding protein Human Antigene R (*HuR*), which is up regulated in several different cancers, through a target site in the 3' UTR (132). The miR-125a and miR-125b have also been shown to suppress HER2 mRNA and protein levels, resulting in reduced cell growth, motility and invasiveness (133).

MiR-34a is also a tumor suppressor and the expression of this miRNA is associated with the reduced risk of metastasis in breast cancer and a lower risk of relapse of the disease and death from breast cancer (134). Similar to miR-34a, miR-31 is also associated with the ability to inhibit development of metastasis in breast cancer patients, and the expression of this miRNA is negatively correlated with the presence of metastasis and the probability for relapse independent of other biological markers. Alone, miR-31 inhibits several steps of metastasis, including local invasion, initial survival at a distant site, and metastatic colonization. This miRNA repress a number of metastasis-promoting genes, including RhoA, and it has been demonstrated that ectopic miR-31 expression alone inhibit metastasis in otherwise aggressive breast cancer cells while inhibition of miR-31 allowed otherwise-nonaggressive breast cancer cells to metastasize (135).

Overall, as summarized in Fig. 1.3, many miRNAs have been identified as biomarkers and/or characterized as essential regulators of breast cancer development, including cancer initiation, metastasis, and therapy resistance. Future advancement in individual miRNA profiling technology for cancer patients will certainly facilitate personalized cancer medicine which in turn must be based on comprehensive miRNA functional studies.

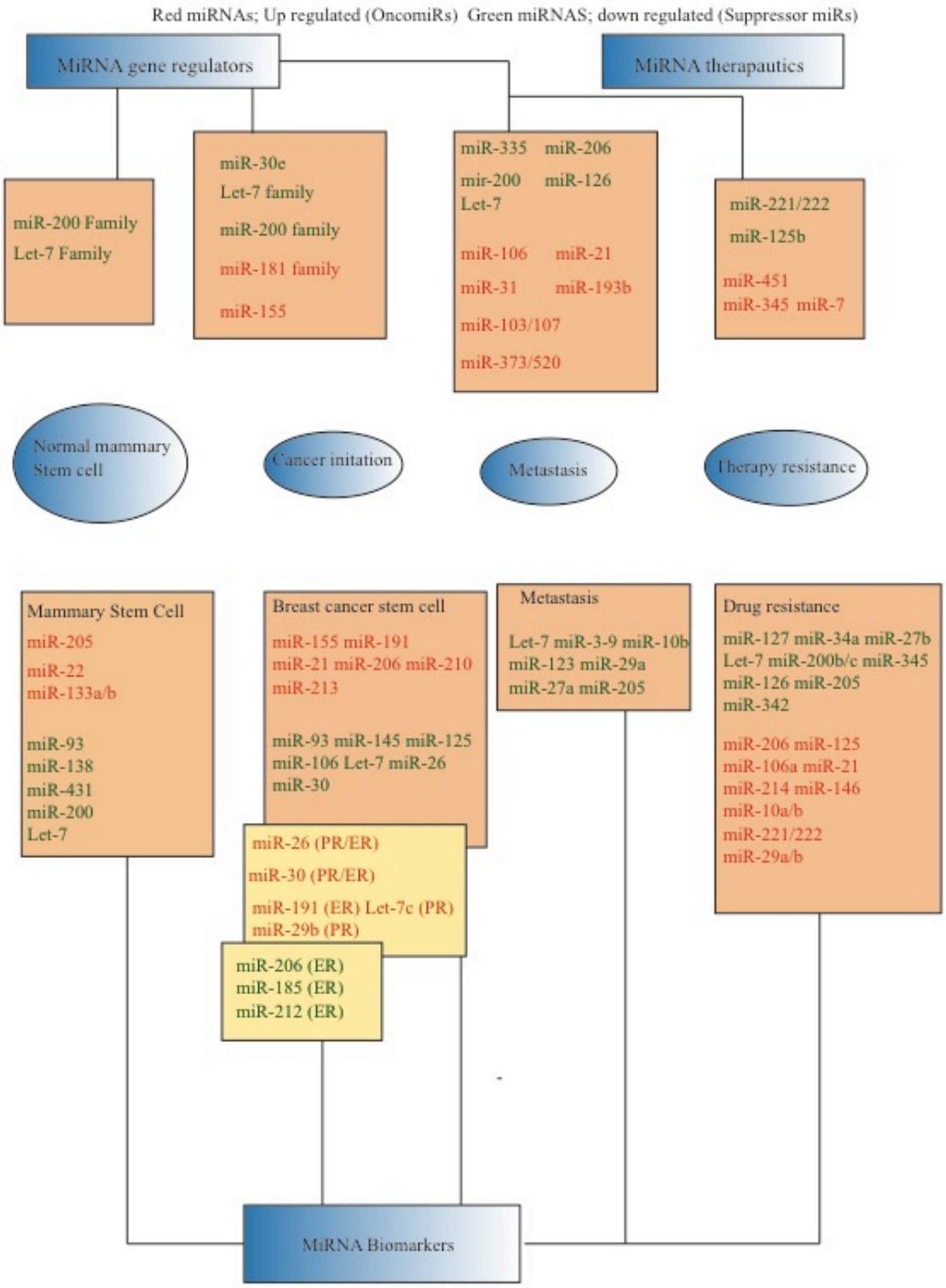


Figure 1.3. A summary of miRNA reported to act as regulators and biomarkers in the development of the normal mammary gland, breast cancer initiation, metastasis, and therapy resistance. The top panels show miRNA regulators (suppressor-miRNAs in green and oncomirs in red), and the bottom panels list miRNA biomarkers (up-regulated miRNAs in red and down-regulated miRNAs in green). Modified from (136).

2 Materials and methods

2.1 Cell lines

The cell lines that were analyzed in this thesis were: MA11, HCC 1569, MDA-MB 231, AU 565, HCC 1428, HCC 1187, DU 4475, MCF-7, Hs578T, MCF-7(N), Hs578Bst, MCF-10A, HCC 1187BL and SKBR3 (summarized in figure 2.1), They were all obtained from American Type Culture Collection (ATCC/LGC Standards).

The MA11 cell line originates from a 65 year old female. It was originally an invasive lobular carcinoma that had metastasized to the bone marrow (137).

HCC 1569 is derived from a 70-year-old black female with a germline mutation in the FHIT gene. The patient received prior chemotherapy and had no family history of breast cancer. The tumor was classified as TNM stage IV, grade 3, metaplastic carcinoma with 4 out of 18 lymph node metastasis. HCC 1569 is classified as a basal-A subtype and express HER2 (37,39).

HCC1187 was initiated from a 41 year old White female with a TNM stage IIA, grade 3, primary invasive ductal carcinoma. HCC 1187 is negative for HER2, PR and ER (triple negative), express mutated TP53 and is classified as a basal-A subtype (37,39). HCC1187BL is a B lymphoblastoid cell line initiated by Epstein-Barr virus (EBV) transformation of peripheral blood lymphocytes obtained from the same patient as HCC1187 (138,139).

DU 4475 is also a triple negative cell line that expresses TP53 and is classified as a basal-A subtype. It was retrieved from a 62 year old female(140).

The AU565 cell line is derived from a pleural effusion of a 43 year old white female with breast carcinoma. AU565 overexpress HER2, and is classified as a luminal subtype (37,39).

SKBR3 is established from pleural effusion from the same patient as AU565 (140).
HCC 1428 derived from a 49 year old white female and is classified as a luminal subtype. This cell line expresses a low level of TP53 and is negative for ER and PR (140).

MCF-7 is derived from a 69 year old white female and is classified as a luminal breast cancer cell line. It expresses ER and PR and a low levels of TP53 (39,140). The cell line referred to as MFC-7(N) is the same as the MCF-7 cell line.

MDA-MB231 is derived from a pleural effusion of a 51 year old white female. It is triple negative and classified as a basal-B subtype cell line (37,39).

Hs578T is derived from a 74 year white female. The cell line is classified as a basal-B subtype and is triple negative (37,39,141).
Hs578Bst is derived from the same patient as Hs578T, but is established from normal tissue peripheral to the tumor and is myoepithelial in origin (140).

MCF 10A is derived from a 36 year old white female and is a non-tumorigenic epithelial cell line that display characteristics of luminal ductal cells (142).

The cell lines were grown and sub-cultivated according to the guidelines from ATCC for the different cell lines (141). The different cell lines required different types of growth medium (see figure 2.1). Dulbecco´s Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI) and Dulbecco´s Modified Eagle's Medium/Ham's F-12 50/50 were purchased from Sigma Aldrich.

Cell line	*Subtype*	*ER*	*PR*	*Her2*	*P53*	*Age of Patient*	*Growth medium*	*Other conditions*
MA11	Basal-B	+	-			65	RPMI-1640/ 10% FBS	
HCC 1569	Basal-A	-	-	+		70	RPMI-1640/ 10% FBS	
MDA-MB231	Basal-B	-	-	-		49	DMEM/ 10% FBS	
AU565	Luminal	-	-	+		43	RPMI-1640/ 10% FBS	
SK-BR-3	Luminal	-	-	+		43	DMEM/ 10% FBS	
HCC 1428	Luminal	+	+	-	-	49	RPMI-1640/ 10% FBS	
HCC 1187	Basal-A	-	-	-	+	41	RPMI-1640/ 10% FBS	
HCC1187 BL	Basal-A						RPMI-1640/ 10% FBS	
DU4475	Basal-A					70	RPMI-1640/ 10% FBS	
Hs578T	Basal-B	-	-			74	DMEM/ 10% FBS	Insulin 0.01 mg/ml
Hs578Bst	Basal Normal					74	Hybri-Care Medium/ 10% FBS	EGF 20 ng/ml,
MCF-7	Luminal	+	+	-		69	DMEM/ 10% FBS	insulin, 0.01 mg/ml
MCF-7 (New)	Luminal	+	+	-		69	DMEM/ 10% FBS	insulin, 0.01 mg/ml
MCF 10A	Basal Normal					36	DMEM/Ham's F-12/ 5% horse serum	EGF 20 ng/ml, cholera toxin 100 ng/ml, insulin, 0.01 mg/ml hydrocortisone 500 ng/ml

Figure 2.1. The cell lines that were used in the thesis and their condition requirements.

2.2 Isolation of total-RNA from cell lines

The cell lines that was used to isolate total RNA was MCF-7(N), MA11, HCC 1428 and DU 4475. This was done according to the protocol provided by RNA and molecular pathology (RAMP) research group the University of Tromsø.

The growth media was removed and approximately 5-10 X 10^6 cells were washed once with PBS (Phosphate buffered saline). Then, trypsin/EDTA solution was added and the cells were resuspended in 8 ml growth media after detachment. The cells were pelleted for 3 minutes at 1000 rotations per minute (rpm) before the growth media was removed. The pellet was resuspended in 10ml PBS and the cells were centrifuged for another3 minutes at 1000 rpm.

1. Cell lysation: The cells were lysed by adding 1 ml Trizol-reagent (Sigma Aldrich) to the cell pellet and by repetitive pipetting to completely dissolve the pellet. The Trizol-reagent is a one-phase solution that contains Phenol and Guanidin Isothiocyanate that deactivates RNases, lysates the cell and stabilizes the RNA. The solution was left on ice for 5 minutes to permit the complete dissociation of nucleoprotein complexes and transferred to a 1,5 ml LoBind Eppendorf tube.
2. Phase separation: 0.2 volumes of chloroform were added and the tubes were left on ice for 20 minutes with gentle vortexing every second minute. The mixture was centrifuged for 30 minutes at 9000 rpm. Following centrifugation, the mixture separates into lower phenol-chloroform phase, an interphase, and a upper aqueous phase. RNA remains exclusively in the aqueous phase. The lower organic phase was removed followed by another centrifugation for 5 minutes at 9000 rpm. The upper aqueous phase was then carefully transferred to a new 1,5 ml LoBind Eppendorf tube without disturbing the interphase or the rest of the organic phase.
3. RNA precipitation: The volume of the aqueous phase was measured and 1 volume of isopropanol was added. The sample was mixed well and incubated for 2 hours at 4^0C and centrifuged for 30 minutes at 15000 g-forces (g) before removing the supernatant completely.

4. RNA wash: 1 ml ice cold 80% Ethanol was added, followed by a new centrifugation at 15000 g for 5 minutes. The supernatant was removed, and the pellet was dried in a workbench for 15-30 minutes.
5. Redissolving RNA: The dried pellet was dissolved in 10 micro litres (µl) of nuclease free water.
6. The quality and quantity of the total RNA was assessed with Qubit fluorometer 2.0 and Agilent 2100 Bioanalyzer.

After isolation of total RNA, the sample was enriched for miRNA. The pureLink miRNA isolation kit (Invitrogen/Life Technologies) was used for this purpose.

2.3 Total Exosome isolation from cell culture medium

We isolated exosomes from the following cell lines: AU565, DU4475, HCC1187, HCC1428, HCC1569, Hs578T, MA11, MCF-7(N) and MDA-MB231.
This was done using the Total Exosome Isolation reagent (from cell culture media) according to the protocol (Invitrogen/Life Technologies), publication number MAN0006949 (143).

2.4 Total Exosome RNA and protein Isolation

This was done using the Total Exosome RNA and Protein isolation Kit according to the protocol (Invitrogen/Life Technologies), publication number MAN0006962 (144), The protocol was modified at the organic extraction step where an extra centrifugation was added (Isolate RNA, step 6 and 7). The lower organic phase was removed after the first centrifugation step followed by another centrifugation with the subsequent transferral upper aqueous phase to a new 1,5 ml LoBind Eppendorf tube.

2.5 Quibit® Fluorometer

Qubit® Fluorometer utilizes specifically designed fluorometric technology using Molecular Probes® dyes to quantitate RNA, DNA or proteins. The fluorescent dyes emit signals only when bound to specific target molecules. In this thesis Qubit® Fluorometer was used to detect and quantify the isolated total RNA and miRNA from the cell lines and the exosomes. Measurements was done according to the protocols from Invitrogen/ Life Technologies (145).

2.6 Agilent 2100 Bioanalyzer

Agilent 2100 Bioanalyzer is a system that can be used to analyse the size and quality of proteins, cells, DNA and RNA. Through the use of microfluidic technology, a minimum of 1 μl of nucleic acid sample is required for separation in micro-channels that are filled with a gel and a fluorescent dye. When an electrical voltage is applied to the microchip, the sample migrates through micro channels etched in the chip surface. As the sample moves, RNA, DNA, proteins or cell fragments of different sizes separate according to their mass. Intercalating dye within the gel allows the migrating RNA, or DNA, proteins or cell fragments to be detected. A Computer connected to the instrument controls records the fragment sizes and concentrations. Different microchips are used to analyse different samples, and for total RNA, RIN value is calculated. The RIN (RNA Integrity Number) value is an algorithm based on the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products, to determine sample integrity. The RIN algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact. In this thesis, three different chips were used. For the analysis of total RNA, RNA 6000 nano chip was used. For the analysis of the miRNAs after miRNA enrichment, a Small RNA chip was used. To analyse the quality and quantity of the cDNA from the library preparation, a DNA High Sensitivity chip was used. All the procedures was done according to protocol from Agilent technologies (146). The pictures from the analysis are available in the Appendix (see section 6).

2.7 Sequencing

In this thesis, the Applied Biosystems SOLiD™ **(**Sequencing by Oligonucleotide Ligation and Detection) 5500 XL deep sequencing technology, a next-generation sequencing technology, was used to investigate the miRNA expression in breast cancer cell lines and exosomes derived from breast cancer cell lines. The SOLiD™ sequencing technique, and all other is next-generation sequencing technologies, is based on the Sanger sequencing that is still the most commonly used sequencing method. It was first described in 1977 (147). It is based on detection of nucleotide-specific end-marked DNA fragments of different sizes, which is separated by gel electrophoresis. However, this method is both time-consuming and expensive, at least when large amounts of DNA sequence information is needed.

New research has led to development of new and more efficient methods, so called next generation sequencing technologies. The significant strength of the NGS is their ability to produce massive amounts of data, and this improvement has made NGS one of the preferred technologies for RNA deep sequencing, and has been applied in analysis of miRNAs. These sequencing technologies does not require prior knowledge about the RNA that is sequenced, and therefore allows any organism or target to be easily studied (148).

2.8 Preparation of the cDNA library

The preparation of the cDNA library was done according to the protocol form Applied Biosystems SOLiD™ 5500 XL systems – small RNA library preparation from Invitrogen (145).

The small RNA fraction that is enriched from the total RNA, is first hybridized and ligated to an adaptor mix that contains a set of oligonucleotides with a single stranded degenerated sequence at one end and specific sequence at the other end; the P1 adaptor and the internal adaptor which is required for SOLiD sequencing. The small RNA is then reverse transcribed to yield cDNA, which in turn is purified and size selected using acrylamide gel electrophoresis. The gel was made manually, and was a 10% Acrylamide gel. It contained 25 ml 40% acrylamide, 10 ml 10 X TBE, 30 ml water and 12 g Urea. This was sterile filtered through a 22 micrometer (μm) filter. The gel was run for exactly 45 minutes before the cDNA fragments between 60 to 80 nt were cut out from the gel.
The cDNA fragments was then subjected to a polymerase chain reaction (PCR) amplification step, where a common 5´primer and a barcoded 3´PCR primer are added. The barcoded primers enable simultaneous sequencing of different samples in a single run.

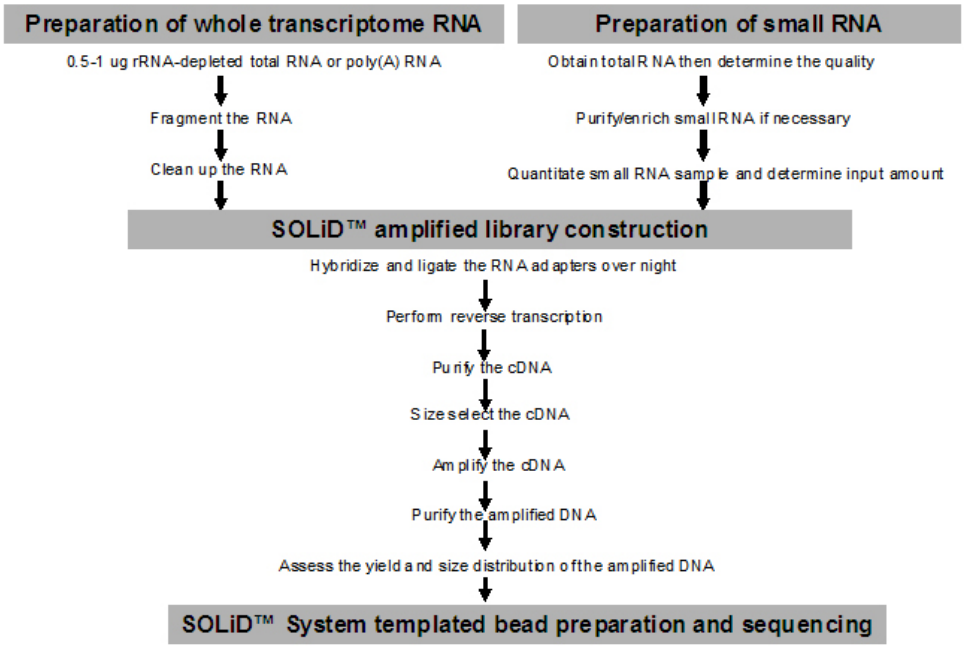


Figure 2.2. The SOLiD cDNA library preparation modified from (145).

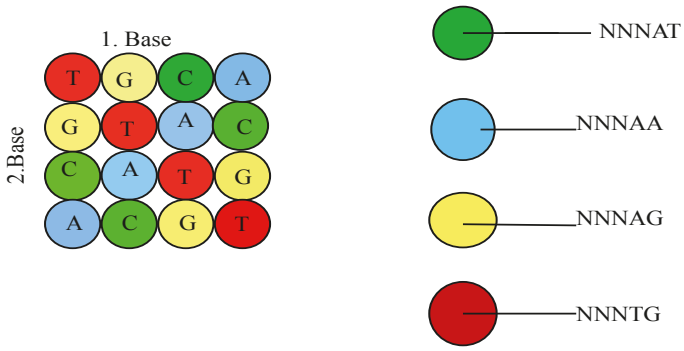
2.9 Sequencing by Oligo Ligation and Detection (SOLiD): SOLiD™ 5500 Xl Sequencing

The SOLiD™ 5500 XL system differs from other NGS techniques by their sequence by ligation technology and two base encoding probes. This technique can sequence up to 300 gigabases (GB) in one run and has an accuracy greater than 99.99% with the use of exact cell chemistry (ECC) module (149).

The cDNA library (section 2.5) is amplified and analysed for yield and size distribution before proceeding to emulsion PCR (ePCR). The ePCR step is an amplification of single templates in micro-reactors created by several droplets of an aqueous phase that is distributed in an oil phase. The water droplets contain all the components required for the PCR. The small RNA library, now a cDNA library, is clonally amplified onto SOLiD P1 DNA beads, which are small magnetic beads with P1 adaptors attached to them, in the ePCR reaction (Figure 24b). The protocols that were used here was EZ bead™ Emulsifier and EZ bead™ amplifier from Applied Biosystems/Life Technologies (145).

In the ePCR reaction approximately 30´000 copies of a single template are amplified onto each magnetic P1 bead. The templates are denatured, and a bead enrichment step is preformed to separate beads with extended templates from the ones who do not have any templates attached to them. This is done according to the protocol EZ bead™ enricher protocol from Applied Biosystems/Life Technologies (145). In the enrichment step polystyrene beads with P2 adaptors attached are used to capture the template-amplified beads. The enriched P2 beads are separated with a glycerol gradient where they are situated in the top phase. The enrichment P2 beads are washed away after enrichment and the 3´end of the template beads are modified by addition of a (Deoxyuridine-Triphosphate) dUTP in order to chemically cross link template beads onto an amino-coated glass slide. To make sure that the magnetic beads are separated it is preformed several declumping sonication steps, ant the beads that do not cross-link to the amino glass slide are washed away during the sequencing initiation.

SOLiD uses a DNA ligase instead of a DNA polymerase to elongate the template sequence. A set of four fluorescently labelled di-base probes compete for ligation to the sequencing primer. The specificity of these probes is achieved by interrogating every first and second base in each ligation reaction. After ligation imaging will record the fluorescent signal, and the probe is cleaved between nucleotide 5 and 6 releasing the fluorescent label. When the fluorescent label is released, a new round of hybridization and ligation will be initiated. Numerous cycles of ligation, detection and cleavage are performed, and the desired read length determines the number of cycles. After a series of ligation cycles, the extension product is chemically removed and the template is reset with a primer complementary to the n-1 position for a second round of ligation cycles. A total of five rounds of primer reset are completed for each sequence tag. Through this process, each base is detected in two independent ligation reactions by two different primers (148,150–152).



2.3. Color code combination for ligation probes used during SOLiD sequencing.

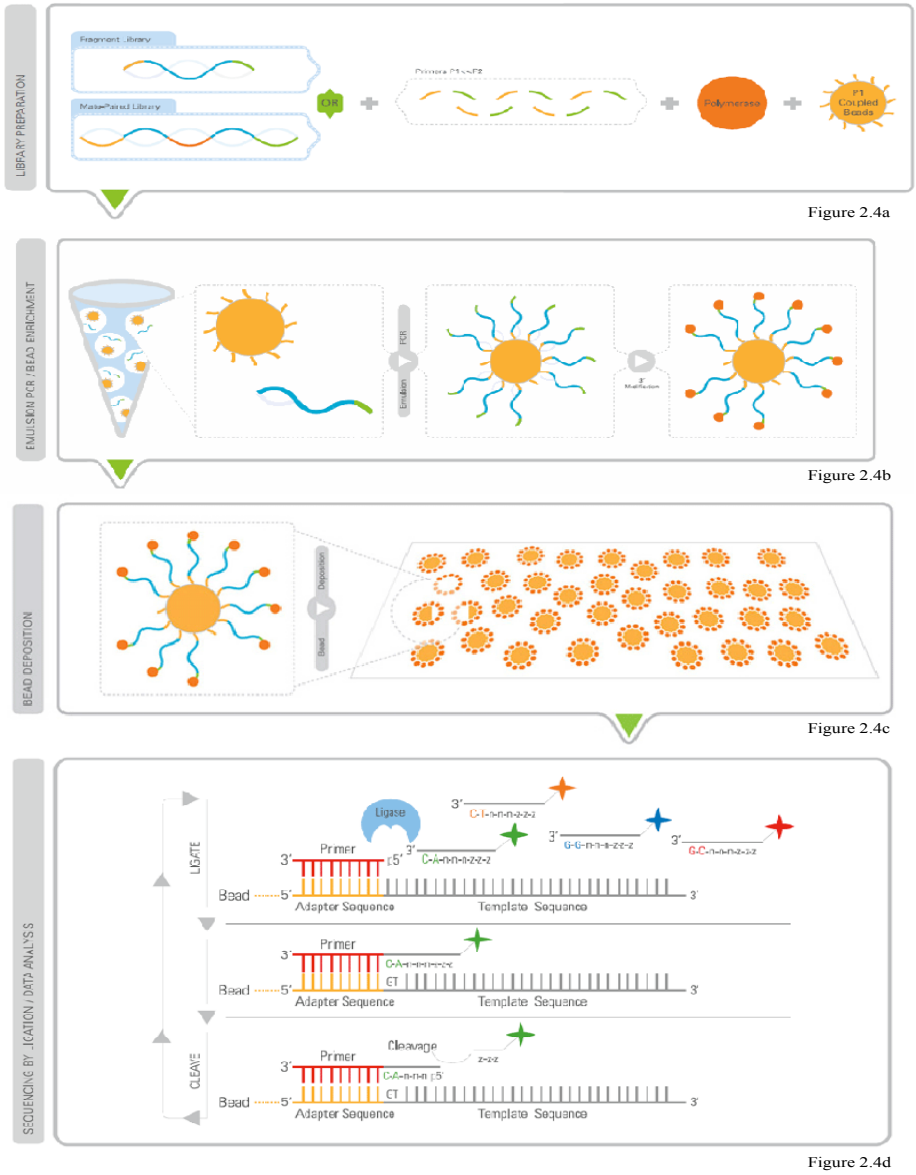


Figure 2.4. Illustration of the library preparation in figure 2.4a, the emulsion PCR in figure 2.4b, the bead deposition on the amino-coated glass slide in figure 2.4c and the sequencing by ligation in figure 2.4d, modified from (145). Color code combination for ligation probes (also see figure 2.3) used during SOLiD sequencing is done by ligation instead of template extension by DNA polymerase, and the ligation probes are specific for the sequence of two nucleotides. This allows the sequence to be presented in color space. Ligation probes consists of fluorescently labelled 8-mer oligonucleotides with degenerated 5´ends, between nucleotides 3 and 8. A specific combination of the first and second base and a set of fluorescent labels are attached to the ligation probe according to the combination of the first two bases. After the ligation imaging records the fluorescent signal and the probe is cleaved between the two nucleotides, 5 and 6, which will release the fluorescent label. A new round of hybridization and ligation follows this; this will ultimately allow each base to be sequenced twice, as seen in 2.4d.

2.10 Bioinformatics and CLC workbench

The bioinformatics is used to store, analyse and interpret biological results. There are several bioinformatics platforms on the market, but in this thesis the results from the sequencing was analysed with CLC Genomics workbench from CLC bio. The CLC Genomics workbench is used to visualise and analyse the results from the NGS. The CLC program was used to trim the adaptors and count the annotated sequences listed in miRBase, version 19 (54). After the analysis in the CLC Genomics workbench, the data was imported into Microsoft Excel for further analysis.

3 Results

MiRNAs are small RNA molecules that regulate gene expression post-transcriptionally. They play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. Hence, altered miRNA expression contributes to the development and progression of human disease, including cancer.

In this thesis, we used a strategy of small RNA profiling by Applied Biosystem's next-generation sequencing system (SOLiD) to analyze the different genome-wide miRNA expression profiles in breast cancer cell lines and exosomes originating from breast cancer cell lines. Our hypothesis is that some key miRNAs may be detectable in both cell lines and exosomes, and such miRNAs could be useful for early breast cancer detection. We sequenced miRNA from 11 breast cancer cell lines belonging to three different subtypes, and the exosomal miRNA from 9 of the corresponding cell lines. This was done to investigate the potential differences in the miRNA expression patterns, both between the different subtypes of breast cancer cell lines and the exosomes originating from the cell lines. We aimed to identify miRNAs with a consistent high expression among all the cell lines and/or exosomes since such miRNAs could have a potential as biomarkers. We also did a comparison of the miRNA expression pattern between the exosomes and the cell lines in order to investigate whether exosomal secretion of miRNA is a passive or selective process.

3.1 SOLiD 5500™ XL sequencing of different subtypes of breast cancer cell lines.

To analyse the miRNA expression patterns in the different subtypes of cell lines, we used a strategy for small RNA SOLiD 5500XL sequencing (see section 2.9). The results from the sequencing of the cell lines are shown in table 3.1. The sequencing gave 467,364,513 total reads and 66167 miRNA hits in miRbase (19 edition released August) for the14 cell lines.

Cell line	Reads	Trimmed reads	Annotated reads	Annotated Small RNAs with miRBase
SKBR3	42908208	23615840	12586253	8083
HCC1428	7748833	1771838	638910	1803
AU565	44308276	32530626	8740399	7110
MCF-7(N)	8524635	2371884	1366267	1817
MCF-7	16527986	13788631	4402003	4394
DU4475	9943490	1533581	739558	1898
HCC1187	53889797	26685734	7386244	6078
HCC1569	83201686	28398422	8000141	6290
Hs578T	41229727	27168436	7956951	6821
MA11	7606681	1498020	614730	1543
MDA-MB231	25790716	17559387	4887027	4401
Hs578Bst	63850618	46364738	15403682	7762
HCC1187BL	46999070	22185439	4548176	4472
MCF-10A	14834790	10576212	3102074	3695
TOTAL	467364513	256048788	80372415	66167

Figure 3.1. Number of total reads and the number of annotated exact mature miRNA detected in the SOLID 5500XL sequencing.

3.2 The miRNA expression does not follow the classification of the breast cancer cell lines

In order to examine whether the miRNA expression in the different cell lines correlated, we subjected the data to a Pearson's Correlation analysis (figure 3.2). Pearson's Correlation is a measure of the correlation, or dependence between two variables and is presented as a value between -1 and +1. The Pearson correlation is +1 in the case of a perfect positive correlation, −1 in the case of a perfect negative relationship (anticorrelation), and a value between −1 and 1 in all other cases, representing the degree of linear dependence between the variables. The closer Pearson's Correlation is to 0, zero the less correlation (closer to uncorrelated) and the closer the coefficient is to either −1 or 1, the stronger the correlation between the variables. Since it is a linear correlation plot it is easily affected by values that astray from the majority of the values. This could mean a significant bias in the interpretation of the results and must always be considered.

The highest correlation was seen between the AU565 and SKBR3 cell lines, with a correlation value of 0,95. This is not unexpected since these cell lines originate from the same patient (luminal subtype). SKBR3 stood out as the cell line with the highest correlation rate with the majority of the cell lines. Also, Hs578Bst and Hs578T showed a high correlation (0,92). Similarly to AU565 and SKBR3, these cell lines originates from the same patient, however Hs578Bst is a normal breast epithelial cell line while Hs578T is a Basal-B breast cancer subtype.

Hs578Bst had an all over low correlation with the other cell lines, most likely due to the fact that this is a non-cancerous cell line. Hs578T correlated least with the MA11 cell line (0,3) even though and MA11 and Hs578T are both Basal-B subtypes. MA11 had the best correlation with HCC 1569 (Basal-A subtype) with a value of 0,84. Furthermore, HCC 1569 correlated well with MDA-MB231 (Basal-B subtype), showing a correlation value of 0,89. MDA-MB231 also showed a high degree of correlation (0,87) with MA11, which is also a Basal-B cell line.

DU4475 had the highest correlation with and HCC 1187 (both the Basal-A subtype). HCC1187 BL is a normal lymphoblastoid cell line originating from the same patient

as HCC 1187, however this cell line correlated most with HCC 1569 (0,75) and the least with Hs578Bst (0,39) which is also a normal cell line.
HCC 1428 (luminal subtype) showed the highest degree of correlation (0,83) with MA11 (Basal-B subtype) and least correlation with Hs578Bst (0,45). The fact that cancer cell lines show a low correlation with normal cell lines is expected since cancer cell lines generally displays a higher degree of genetic alterations that ultimately affect miRNA expression. Indeed Hs578Bst shows the highest degree of correlation (0,68) with MCF-10A, which also is a non-cancerous cell line derived from breast epithelia. MCF-7 and MCF-7(N) is the same cell line, however MCF-7(N) was subjected to miRNA profiling shortly after purchase from ATCC, while MCF-7 has been sub cultured and frozen several times. The relatively low correlation (0,8) between MCF-7(N) and MCF-7 could be caused by a potential cross-contamination from another cell line in MCF-7, or more likely long-term sub culturing has placed selective pressure on this cell line, leading to an enrichment of a subpopulation and/or an acquirement of additional genetic changes that has altered the original functional characteristics of this cell line. Taken together, the miRNA expression varies greatly between the different breast cancer cell lines and the correlation between these cell lines cannot be attributed to their molecular subtypes.

We also did a cluster analysis (figure 3.3) of the cell lines where they are grouped together in clusters based on similarity in miRNA expression. The cell lines that are clustered displays a higher degree of correlation in their miRNA expression compared to the miRNA expression in the cell lines in other clusters. As shown in figure 3.2, the results generally mirror the Pearson's correlation analysis. Both AU565 and SKBR3 and Hs578T and Hs578Bst still clusters, respectively. Interestingly MCF-7(N) and MCF-7 show a higher degree of correlation in the cluster analysis compared to the Pearson's Correlation analysis. As with the Pearson's Correlation analysis, MDA-MB231 and HCC 1569 groups together, including the MCF10A cell line which also had a high degree of correlation with MDA-MB231 and HCC 1569 (0,87 and 0,84, respectively). Furthermore, both HCC 1187 and DU 4475 clusters (0,8 in the Pearson's correlation analysis) and MA11 and HCC 1428 (0,83 in the Pearson's Correlation analysis) clusters, confirming the results found in the Pearson's Correlation analysis.

	AU565	HCC1428	MCF-7(N)	MCF-7	SKBR3	DU4475	HCC1187	HCC1569	Hs578T	MA11	MDA-MB231	HCC1187BL	Hs578Bst	MCF-10A
AU565	1	0,8	0,65	0,78	0,95	0,7	0,76	0,58	0,68	0,77	0,78	0,63	0,65	0,78
HCC1428		1	0,75	0,79	0,81	0,72	0,73	0,76	0,51	0,83	0,74	0,67	0,45	0,72
MCF-7(N)			1	0,8	0,71	0,62	0,63	0,59	0,47	0,68	0,61	0,7	0,36	0,63
MCF-7				1	0,8	0,71	0,74	0,65	0,55	0,7	0,7	0,62	0,46	0,74
SKBR3					1	0,77	0,79	0,81	0,69	0,81	0,78	0,69	0,61	0,81
DU4475						1	0,8	0,68	0,51	0,76	0,72	0,68	0,43	0,74
HCC1187							1	0,7	0,55	0,74	0,71	0,67	0,48	0,78
HCC1569								1	0,68	0,84	0,89	0,75	0,67	0,84
Hs578T									1	0,3	0,69	0,45	0,92	0,71
MA11										1	0,83	0,7	0,57	0,81
MDA-MB231											1	0,73	0,69	0,87
HCC1187BL												1	0,39	0,75
Hs578Bst													1	0,68
MCF-10A														1

Figure 3.2. Pearson´s correlation between the breast cancer cell lines. They are grouped together with their subtypes. Ligt; Luminal, Medium; Basal-A, Dark; Basal-B and Hvite; Normal cell line.

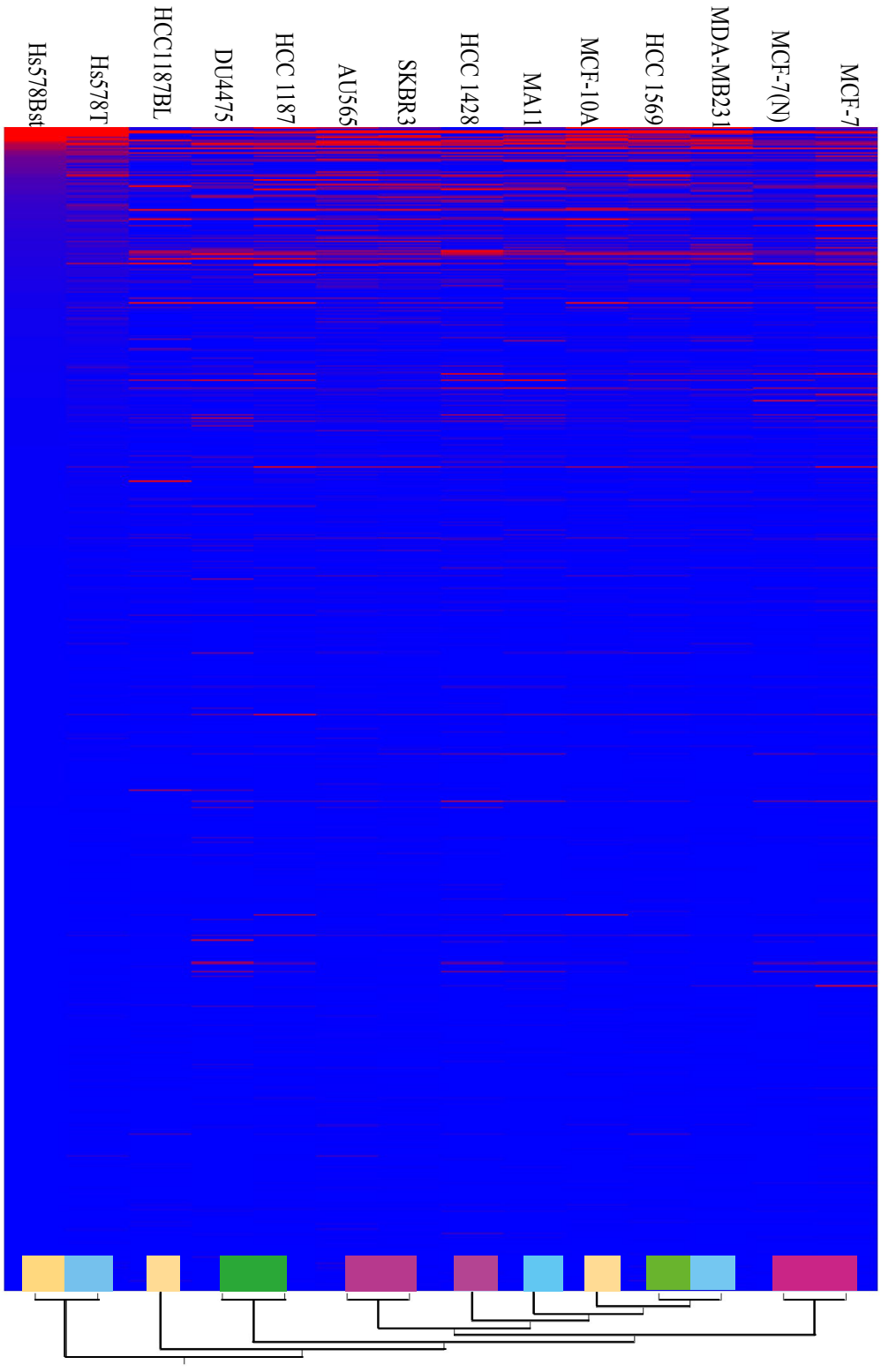


Figure 3.3. Cluster analysis of the cell lines. This is done to investigate if some are more similar than others. Pink; Luminal subtype, Blue; Basal-B, Green; Basal-A and Nude; Normal cell line.

3.3 A comparison of the miRNA expression pattern in breast cancer cell lines and normal cell lines

Although the miRNA expression varies greatly between the different breast cancer cell lines and cannot be used to confirm their molecular subtypes, some of the more abundant miRNAs in the breast cancer cell could potentially be important for breast cancer development and/or be possible candidates for biomarkers. We therefore aimed to investigate the identity of the most abundant miRNAs in the breast cancer cell lines and compare these to the expression of the same miRNAs in the three normal cell lines. The results presented in fig 3.4 shows the 20 most abundant miRNAs in the breast cancer cell lines compared to the same miRNAs in the normal cell lines. The individual miRNA expression was also taken into consideration, as miRNA highly abundant in just a few of the cell lines will potentially affect the average miRNA expression and give a false impression of a high general abundance. When assessing the miRNA expression in the breast cancer cell lines and the normal cell lines, miR-103a-2//miR-103a-1 showed the highest expression (fig. 3.4). This miRNA is a mature 3´prime miRNA and it has earlier been called miR-103 but is now known as miR-103a (153). MiR-103a-2//miR-103a-1 represents the two different loci in the chromosome that they originate from. Although this miRNA has a higher abundance in the breast cancer cell lines compared to the normal cell lines, it shows an overall high expression, being the second most abundant miRNA in the normal cell lines. Evaluating individual miRNA expression in the breast cancer cell lines confirms an overall high abundance of miR-103a-2//miR-103a-1. MiR-103/107 (miR-103 is homologous to miR-107) has previously been shown to attenuate miRNA biosynthesis by targeting Dicer. In breast cancer, high levels of miR-103/107 are associated with metastasis and poor outcome (154).

MiR-29b-1//29b-2 (29b) is the second most abundant miRNA in the breast cancer cell lines. Interestingly, this miRNA is associated with both tumor metastasis in breast cancer patients and the repression of tumor metastasis (155,156). However this miRNA shows a higher expression in the normal cell lines than the breast cancer cell lines. In cancer cells the expression of miR-29b expression is thought to be regulated by the transcription factor GATA3, while miR-29b inhibits the expression of genes involved in suppression of angiogenesis and metastasis (156). MiR-29b belongs, together with miR-29a, and miR-29c, to the miR-29 cluster. As seen in fig. 3.4, all members of this cluster are represented among the 20 most abundant miRNAs in the breast cancer cell lines, and both miR-29a, and miR-29c is more abundant in the breast cancer cell lines compared to the normal cell lines. MiR-29c downregulates the expression of the tumor suppressor Phosphatase and tensin (PTEN) protein directly by targeting the PTEN 3′-UTR while miR-29a has previously been reported to be abundant in invasive breast cancer cells compared to non-tumourigenic cell types and is coupled to increased stabilization and subsequent over-expression of the oncogenic Human antigen R (*HuR*) protein (157).

Three miRNAs are significantly up regulated in the normal cell lines compared to the breast cancer cell lines; miR-125b, miR-20a and miR-145. Although an up-regulation of miR-125b have been shown to confer chemotherapy resistance in breast cancer (158), miR-125b has also been reported to be down regulated in several types of cancer, including bladder cancer, thyroid anaplastic carcinomas, squamous cell carcinoma of the tongue, ovarian cancer and breast cancer (159). MiR-20a is a member of the miR-17/20 cluster, and this cluster is known to often undergo loss of heterozygosity in several different cancers, including breast cancer (160). MiR-145 has been shown to inhibit the EMT in breast cancer cells by blocking the expression of octamer-transcription factor 4 (Oct-4), and the transcription factors, Zinc finger protein 1 (SNAIl), Zinc binding E-box-binding protein homeobox 1 and 2 (ZEB1 and ZEB2) (161). MiR-20a-5´ and miR-19a-3´ are members of the well-known oncogenic miR-17-92 cluster, a polycistronic miRNA cluster encoding miR-17-5´, miR-17-3´, miR-18a, miR-19a, miR-20a, miR-19b and miR-92-1. Overexpression of the miR-17-92 cluster have been observed in several different cancers (162), however our results

show that neither of these miRNAs shows a higher expression in the breast cancer cell lines compared to the normal cell lines (Fig. 3.4).

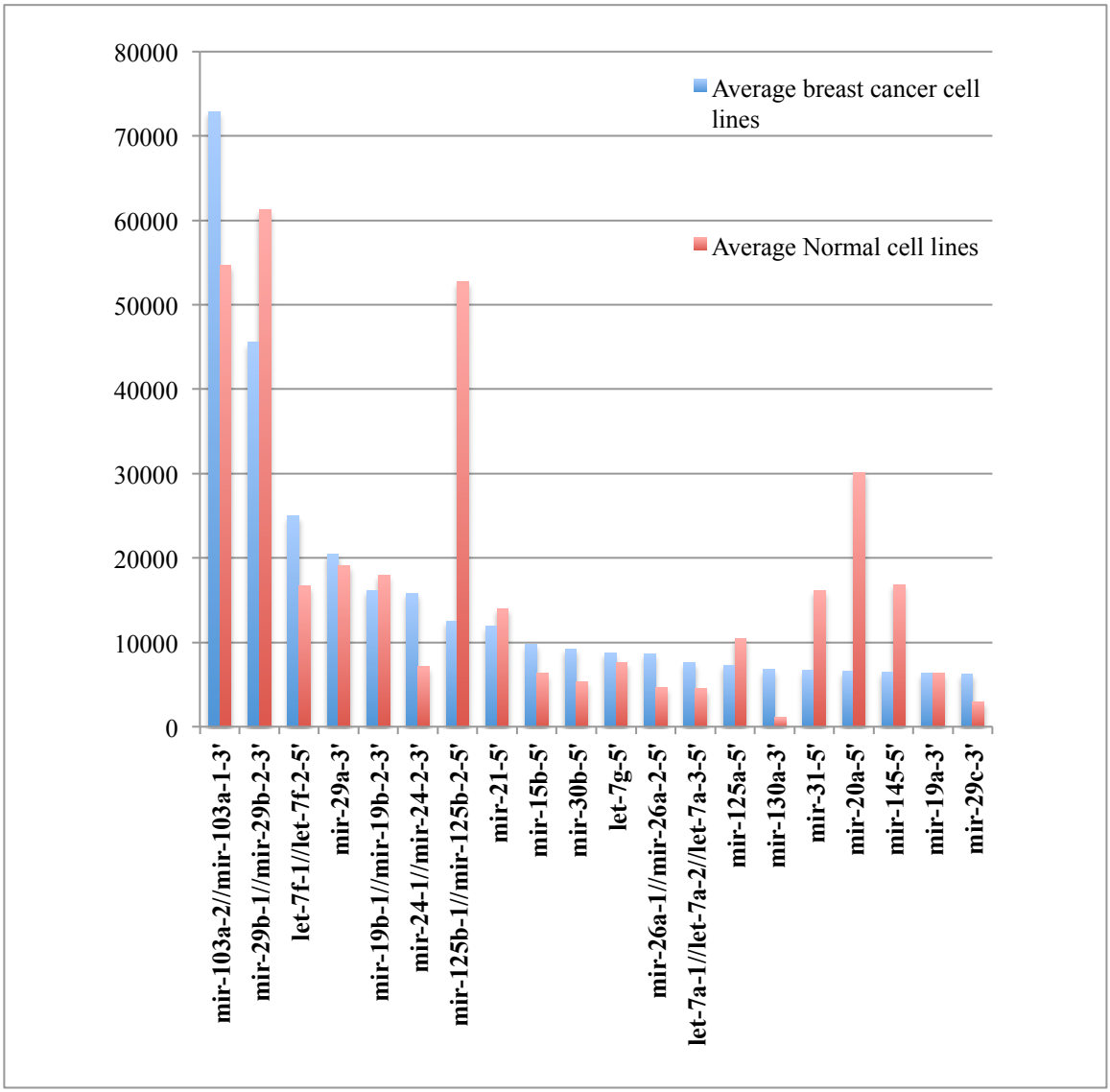


Figure 3.4. A comparison between the 20 most abundant miRNAs in the breast cancer cell lines compared to the same miRNAs in the normal cell lines.

We also did an analysis displaying the highest fold change values when comparing the miRNA expression in the breast cancer cell lines and the normal cell lines (Fig. 3.5). Two relatively highly expressed miRNAs that were not detected in the normal cell lines, miR-489-3 and miR-875-5´ (had the value 0), were given the value 0,1 reads per million to include them in the analysis.

MiR-489 has been shown to inhibit cell growth in head and neck cancer cell lines, but there are currently no reports on neither of these miRNA in relation to breast cancer. miR-141 has a role in potentiating the transition from luminal cancer cells to Cancer Stem Cells (CSCs) (163) and miR-375 has been reported to be a key driver of proliferation in ERα-positive breast cancer cell lines (164).

In the normal cell lines miR-155-3´ and miR-142-5´ had the highest fold change compared to the breast cancer cell line. As mentioned in the Introduction (1.12), miR-155 correlates positively with cell proliferation, tumor growth and the development of metastasis in breast cancer. The expression of miR-142 has not previously been reported as significant in relation to breast cancer.

In summary these results show that there is no significant difference in the expression pattern of miRNA with known functions as oncomirs or tumor suppressors in breast cancer when comparing our selection of breast cancer cell lines and normal cell lines.

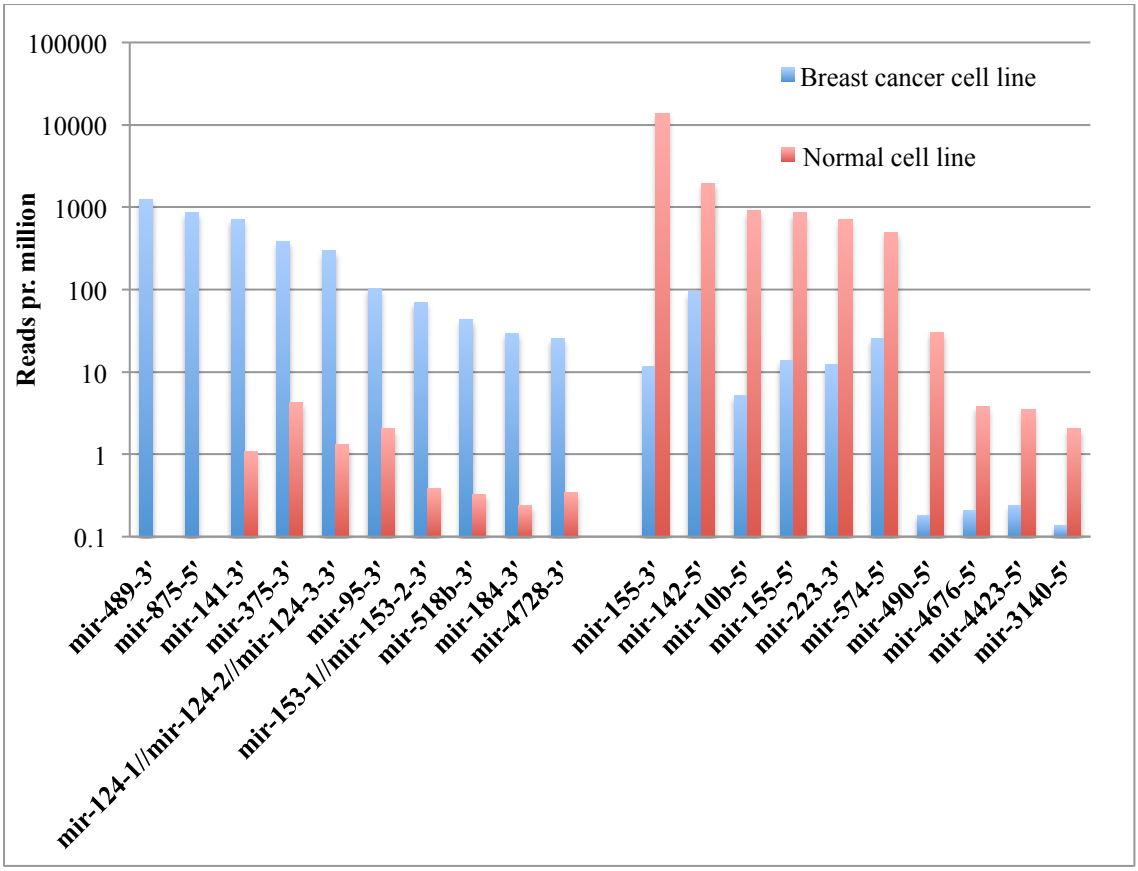


Figure 3.5. Top fold change values comparing the miRNA expression in the breast cancer cell lines compared to the normal cell lines in a logarithmic scale in reads per million. The miRNAs that was not detected in the normal cell lines (and therefore had a value 0) was given the value 0,1 in order to include them in the fold change analysis.

3.4 The miRNA expression pattern in exosomes from breast cancer cell lines

Exosomes from breast cancer cells contain intact and functional miRNAs that potentially can alter the cellular environment to favor tumor growth. MiRNA profiling could increase our understanding of the role of exosomes in breast cancer progression and may lead to discovery of useful biomarkers.

In order to investigate the miRNA expression pattern in exosomes derived from breast cancer cell lines, exosomes were isolated from the breast cancer cell lines: MDA-MB231, AU565, MA11, HCC 1187, HCC 1569, HCC1428, DU4475, MCF-7(N) and Hs578T. Following exosome isolation, the exosomal miRNA was purified and the concentration was increased to meet the demand for preparation of the cDNA library prior to sequencing by SOLiD 5500XL.

It was also made attempts to isolate exosomes and purify miRNA from the normal cell lines; however we were not able to retrieve a sufficient amount of material. Whether this is caused by a low amount secreted exosomes or a low amount of exosomal miRNAs is not known, nevertheless the difference between the breast cancer cell lines and the normal cell lines in this matter is certainly an interesting observation.

The results presented in fig 3.6 shows the 25 most abundant miRNAs from the exosomes secreted by the 9 selected breast cancer cell lines. When evaluating the miRNA expression in the exosomes from the breast cancer cells, miR-24-1//miR-24-2 showed the highest expression (fig. 3.6). Assessing individual exosomal miRNA expression confirms an overall high abundance of miR-24-1//miR-24-2. Indeed five out of nine cell lines had the miR-24-1//miR-24-2 as their highest expressed exosomal miRNA, while it was represented among the top 15 expressed exosomal miRNA in the remaining four cell lines. It has been reported that ectopic expression of miR-24 can promote breast cancer cell invasion and migration. *In vivo* experiments has also indicated that miR-24 expression could enhance tumor growth, invasion to local

tissues, metastasis to lung tissues, and decreased overall survival in a murine system (165).

MiR-103a-2//103a-1 is the second most abundant miRNA in the exosomes, and is represented amongst the four most abundant miRNAs in the exosomes from all the breast cancer cell lines. As mentioned in section 3.3, this miRNA showed the overall highest expression (fig. 3.4) in the breast cancer cell lines, and has previously been shown to be associated with breast cancer (166).

Several miRNAs that are abundant in the cell lines are also highly expressed in the exosomes, like miR-29a and miR-19b that are also mentioned in section 3.3. Some of the 25 miRNAs with the highest average count in the exosomes were not highly expressed in the breast cancer cell lines; Expression of miR-17 was low in breast cancer cell lines, but abundant in the exosomes. MiR-17 has been shown to downregulate AIB1 (amplified in breast cancer 1) and insulin-like growth factor 1 (IGF1), and has a clear role as a tumor suppressor in breast cancer cells (167).

Similarly, expression of miR-34a was low in breast cancer cell lines, but abundant in the exosomes. MiR-34a is a direct transcriptional target of p53 and expression of this miRNA promotes apoptosis and leads to global alterations in gene expression. Among the targets are transcripts that control the cell-cycle, apoptosis, DNA repair, and angiogenesis and miR-34a is a crucial component of the p53 tumor suppressor network (168).

When assessing the exosomal miRNA expression pattern, it is clear that both miRNAs with oncogenic and tumor suppressive roles are among the most abundant. Based on these results, it is difficult to draw a conclusion whether miRNAs are selectively secreted into the exosomes, or if specific criteria for which miRNAs that are selected for secretion exist.

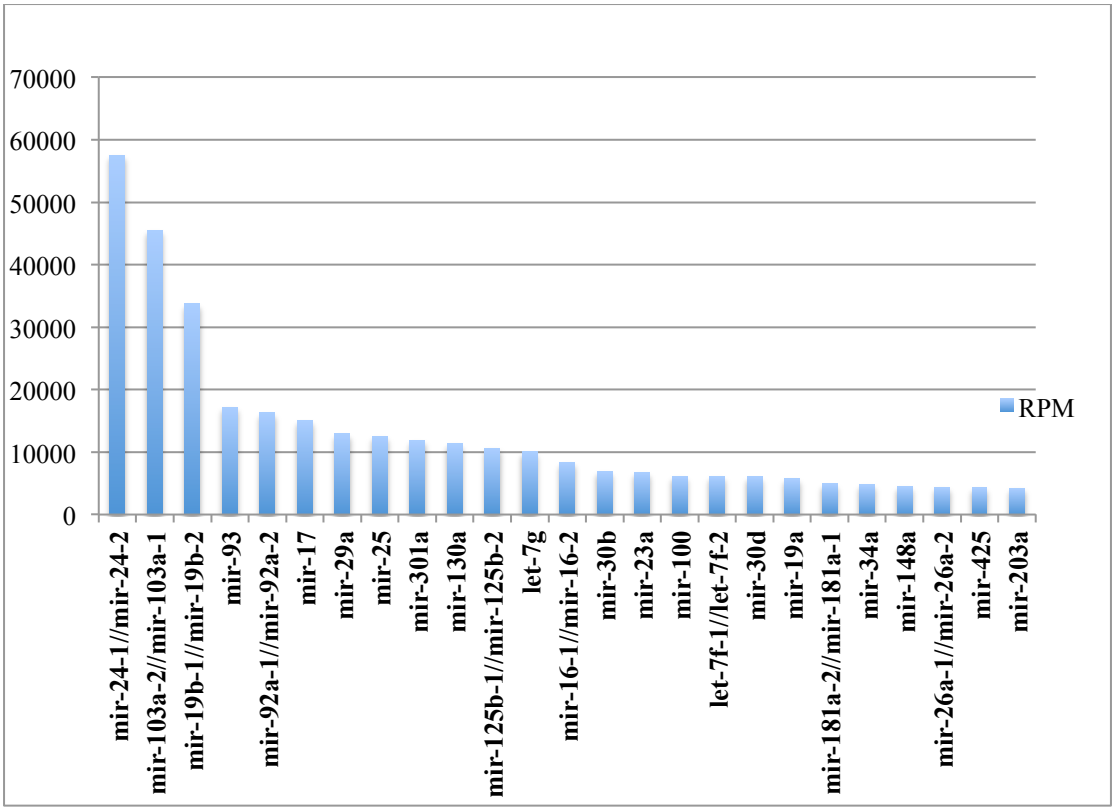


Figure 3.6. The 25 miRNAs with the highest average count in the exosomes, derived from nine breast cancer cell lines presented in Reads per million.

3.5 The miRNA expression in the breast cancer cell lines partly follows the expression in the exosomes

Exploring the full spectrum miRNA expression signatures in breast cancer cells and exosomes, and comparing these signatures, will potentially provide the starting point for the generation of a miRNA map of breast cancer derived exosomes that possibly can be used as biomarkers. However, little research has been completed that investigates miRNA expression profiles in breast cancer cells versus those in exosomes. In this study, we profiled miRNA expression in breast cancer cells and their corresponding exosomes, and compared this expression.

In order to investigate the correlation between the miRNA expression patterns in the breast cancer cell lines and the exosomes from these cell lines, we subjected the data to a Pearson's Correlation analysis (Table 3.7). The Pearson's Correlation analysis shows that the AU565 cell line has the highest correlation between the miRNA expression pattern in the cells and the exosomes (0,86). Second is HCC 1187 with a correlation value of 0,84. In general, these correlation values are comparable to the values from the analysis comparing the miRNA expression in the cell lines (Table 3.2), and overall fig.3.7 show a high degree of correlation between the breast cancer cell lines and the exosomes.

Cell line	R-Value	Exosomes
AU565	0,86	AU565
HCC1428	0,79	HCC1428
MCF-7(N)	0,78	MCF-7(N)
DU4475	0,74	DU4475
HCC1187	0,84	HCC1187
HCC1569	0,81	HCC1569
MDA-MB2311	0,81	MDA-MB231
MA11	0,81	MA11
Hs578T	0,82	Hs578T

Figure 3.7. Showing Pearson's correlation analysis showing the correlation of miRNA expression between the breast cancer cell lines and their corresponding exosomes. They are grouped according to subtype. Dark; Luminal, Medium; Basal-A and light; Basal-B.

To further explore the correlation between miRNA expression in the breast cancer cell lines and the exosomes, the data is presented in a scatterplot diagram (Fig. 3.8). The scatterplot confirms the results from the Pearson's Correlation analysis, showing that most of the breast cancer cell lines group together with their respective exosomes. These results suggest that a correlation of miRNA profiles between cells and cell-derived exosomes exist, showing that the exosomal miRNome at least to some degree represents the miRNA signatures within their originating cells.

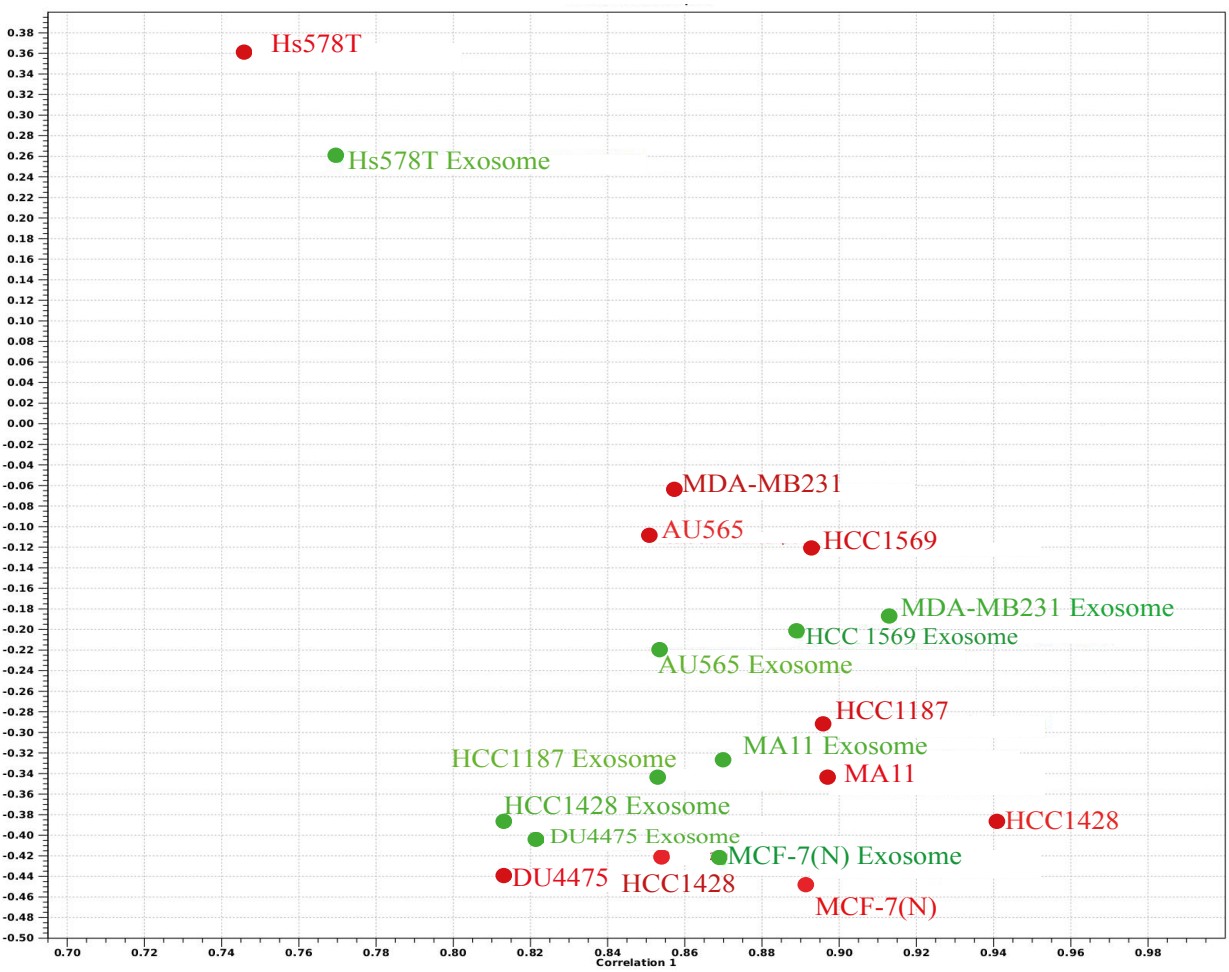


Figure 3.8. A scatterplot analysis, showing the correlation between the miRNA expression pattern in the breast cancer cell lines (red) and the expression in the exosomes (green).

We also chose to present the results as a cluster analysis (Fig.3.9). As shown in Fig.3.9, only two cell lines, Hs578T and DU4475, shows the highest correlation with the exosomes from the breast cancer cell lines respectively. Interestingly, in this cluster analysis the exosomal miRNA expression pattern for six out of nine cell lines (HCC 565, HCC 1569 HCC 1187, HCC 1428, MA11 and MCF-7(N)) shows a higher degree of correlation than with the miRNA expression pattern of the cell lines in which the exosomes originated from.

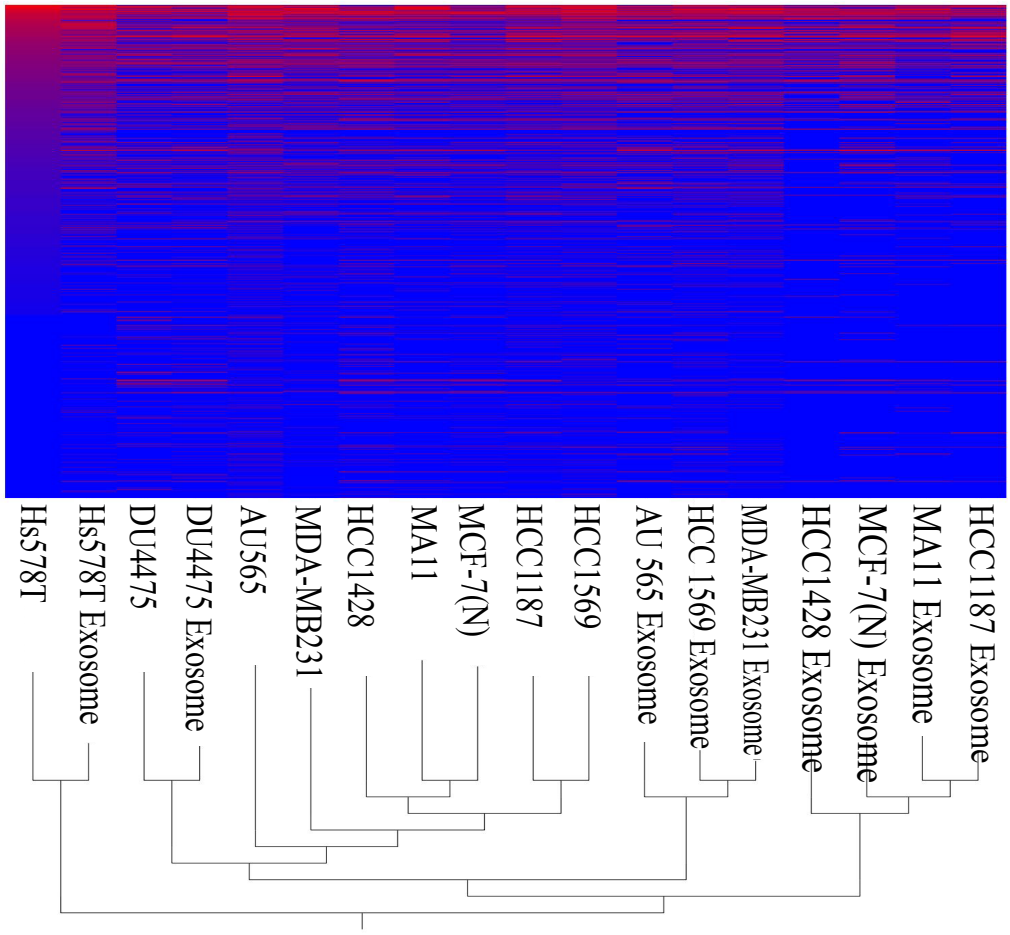


Figure 3.9. A cluster analysis comparing the miRNA expression pattern of the breast cancer cell lines and the cell-derived exosomes.

Figure 3.10 shows the 25 most abundant miRNAs in the exosomes compared to the same miRNAs from the breast cancer cell lines. As seen in this figure, the intracellular and exosomal miRNA expression pattern clearly differs, with some miRNAs (like miR-103a miR-29a and miR-125b) having an overall high abundance and some that show a strikingly differential expression. MiR-93 shows a significantly higher expression in the exosomes compared to the breast cancer cell lines (Fig.3.10). This miRNA is thought to be an oncomir, and utilizes its oncogenic potential by down-regulating the protection that the antioxidant gene, Nuclear Factor erythroid 2-related factor 2 (NRF2) naturally gives against mutations. NRF2 is an antioxidant responsive transcription factor that is suggested to play an important role in the antioxidant-mediated preclusion of oxidative stress (169). As mentioned in section 3.3, miR-34a is abundant in the exosomes, but not in the breast cancer cell lines. Studies have shown that miR-34a/c expression is significantly decreased in metastatic breast cancer cells and human primary breast tumors with lymph node metastases and one of its functions is to regulate breast cancer migration and invasion through targeting of the Fra-1 oncogene (170).

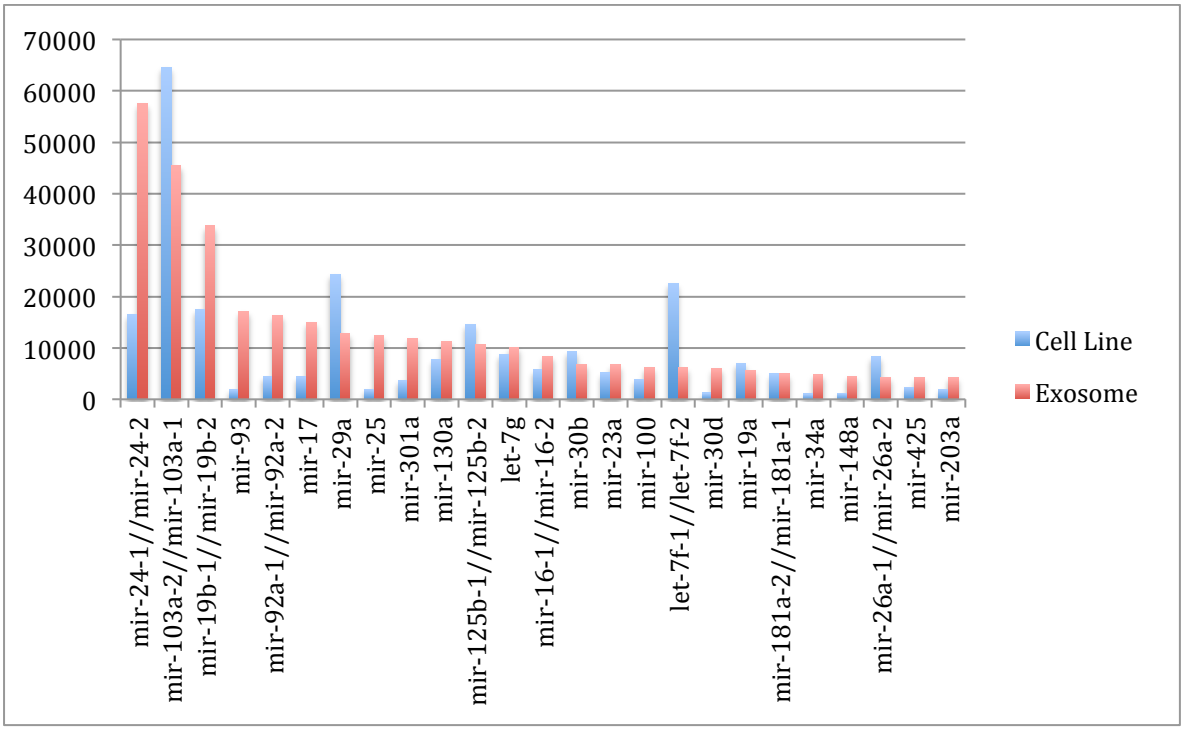


Figure 3.10. The 25 most abundant miRNAs in the exosomes, compared to the same miRNAs in the breast cancer cell lines shown in Reads Per Million. It is evident that the intracellular and exosomal miRNA miRNA expression pattern clearly differs.

Having observed that the miRNA expression pattern in the the breast cancer cell lines and the exosomes were noticeably dissimilar, we wanted to identify the miRNAs with the maximum difference in expression. Figure 3.11 A-H shows the top 20 fold change values for all the breast cancer cell lines and their corresponding exosomes. As seen in this figure, some miRNAs like miR-31, miR-29b, miR-21 are almost consistently amongst the ones that show the highest fold change when comparing the miRNA expression in the cell lines and the exosomes. When comparing the miRNA expression in exosomes to the cell lines, miR-521, miR-93, miR-97, miR-92 and let-7d displays a high fold change in several of the exosomes, as seen in fig 3.11

Figure 3.12 shows a comparison of the miRNA expression in all the nine breast cancer cell lines and the corresponding exosomes presented in fold change values. The fold change value is positive if the expression is higher in the breast cancer cell line and negative if the expression is higher in the exosomes. As seen in this figure, a significant number of miRNAs are differentially expressed between the cell lines and the exosomes. From Figure 3.12, we selected 11 miRNA and presented the results in Figure 3.13. As seen in this figure, miR-29b, miR-31 and miR-96 are significantly more abundant in the breast cancer cell lines than in the exosomes, while miR-93, let-7d and miR-521 are more abundant in the exosomes compared to the cell lines.

Overall these results suggest a partially selective secretion of miRNA into exosomes. Although the intracellular and exosomal miRNA expression pattern show a relatively high correlation, a significant number of miRNAs are differentially expressed comparing the breast cancer cell lines and their corresponding exosomes. It seems as some miRNAs are actively retained in the breast cancer cell lines, while others are secreted into the exosomes. These results demonstrate that exosomes released by breast cancer cell lines have distinct miRNA signatures. Such signatures can potentially be utilized for diagnosis and as biomarkers for early breast cancer detection.

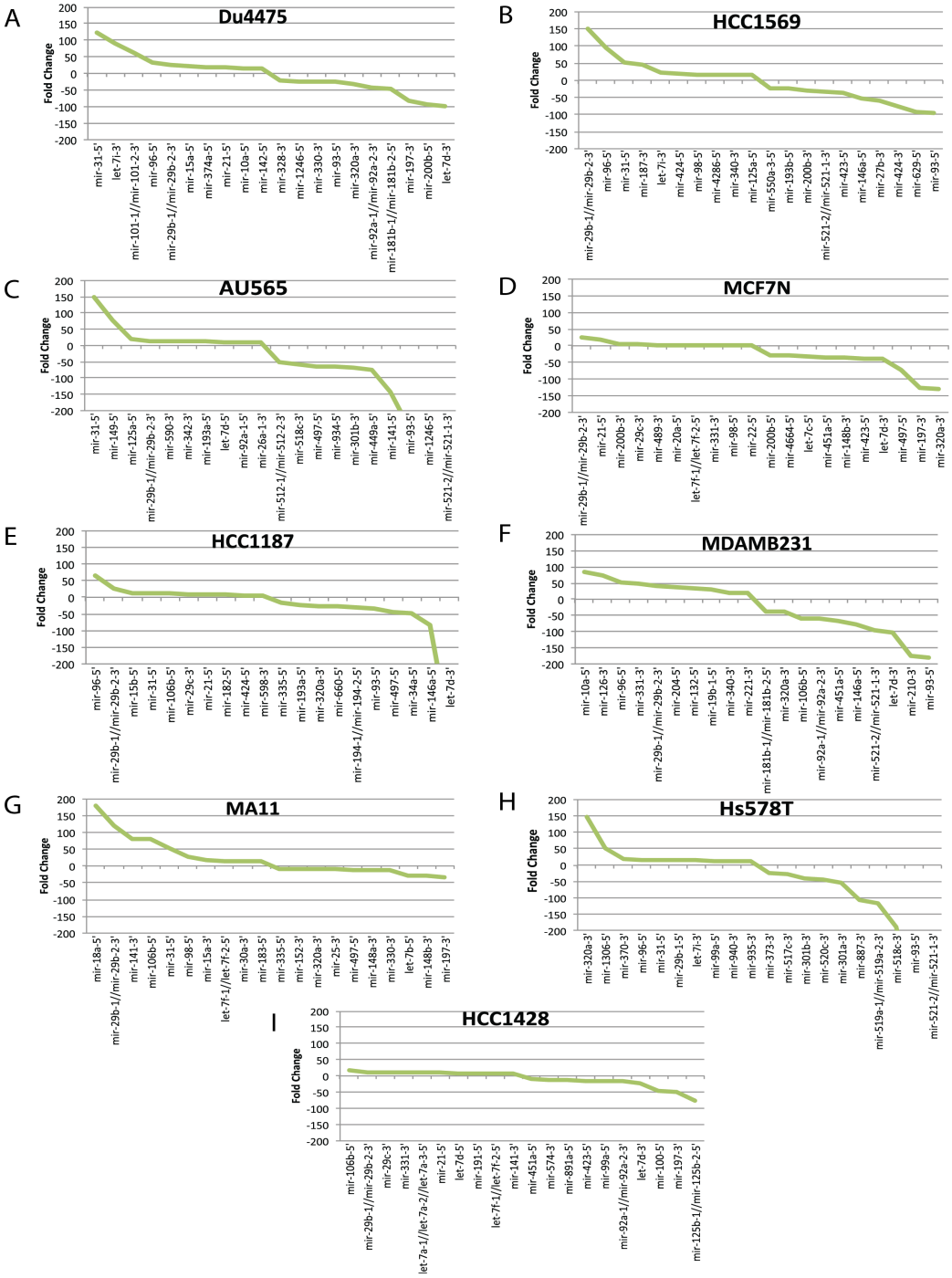


Figure 3.11. The top 20 miRNA expression fold change values for all the breast cancer cell lines and their corresponding exosomes. A. DU 4475, B. HCC 1569, C. AU565, D. MCF-7(N), E. HCC 1187, F. MDA-MB 231, G. MA11, H. Hs578T and I. HCC 1428. A positive value indicates that the miRNA expression is higher in the cell line compared to the exosomes and a negative value indicates that the miRNA expression is higher in the exosomes compared to the cell line.

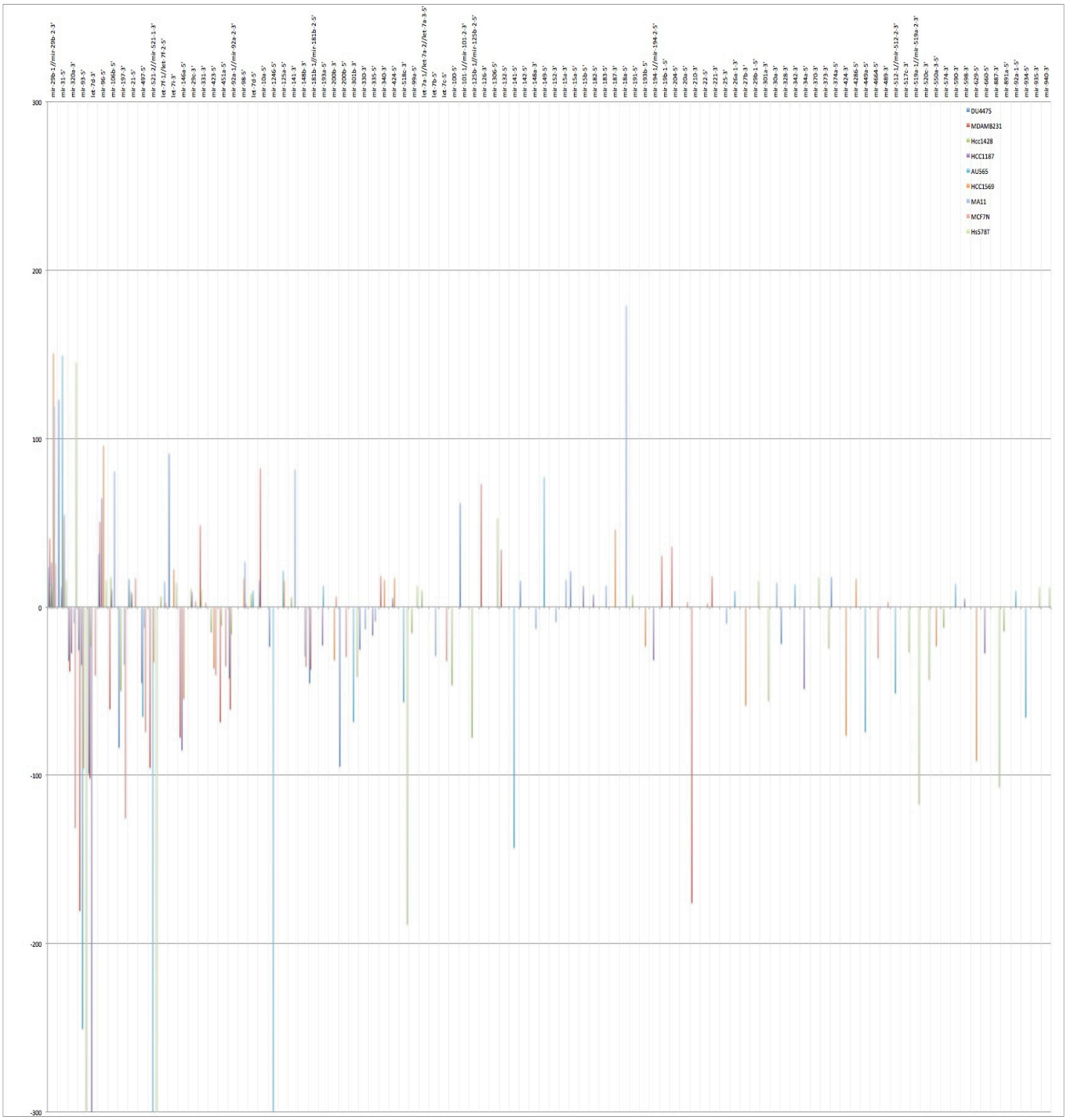


Figure 3.12. A comparison of the most differentially expressed miRNAs expression in all the nine breast cancer cell lines and the corresponding exosomes presented in fold change values. Positive values indicate a higher intracellular expression while negative values indicates a higher exosomal expression.

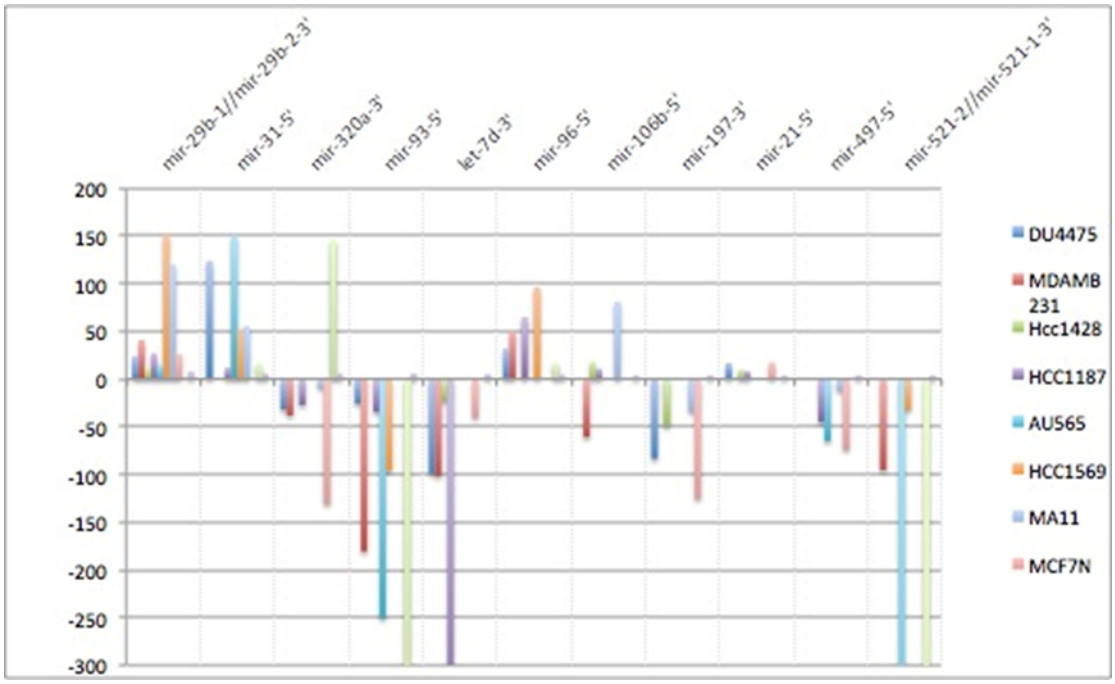


Figure 3.13. Selected miRNAs differentially expressed between the cell lines and the exosomes. Results presents as fold change values.

4 Discussion

Breast cancer is the leading cause of cancer deaths among woman worldwide, and one in ten women will experience the disease during their lifetime. Breast cancer accounted for 23% of the total new cancer incidences and 14% of the total cancer deaths in 2008, and in 2010, 2839 women and 13 men were diagnosed with the disease in Norway. One way to potentially improve long-term cancer survival statistics is earlier detection. That includes appropriate initiation of earlier treatment in high-risk patients, since it is well known that the survival rate in stage I or II is noticeably better than for stage III or IV. The critical question is: How do we improve earlier detection? There are several ways to achieve this including: 1. Improved imaging techniques such as mammography and ultrasound although both have their challenges and limitations. 2. Better, prognostication by the identification of new, accurate prognostic and predictive factors. 3. Discovery and characterization of biomarkers. To date, there are no highly sensitive and specific minimally invasive biomarkers for detection of breast cancer at an early stage. An ideal biomarker is stable and sensitive enough to be able to detect breast cancer at an early stage, and nevertheless it should be cost-efficient (93,171–173). The presence of circulating microRNAs (miRNAs) in blood components (including serum and plasma) has been repeatedly observed in cancer patients as well as healthy controls (90–95). Because of the significance of miRNA in carcinogenesis and their remarkable stability in body fluids, profiling of circulating miRNAs has been used in a number of studies to identify novel minimally invasive miRNA biomarkers (171,174,175). Breast cancer is a very heterogeneous disease and the diversity within the tumors as well as among the individuals that is affected with cancer, determines the rate of disease progression and the effectiveness of the therapy (5). This heterogeneity will also most likely be reflected in the miRNA expression pattern since different molecular pathways are involved in different subtypes of breast cancer. However, the ideal breast cancer biomarker is consistently present in body fluids independent on the molecular subtypes, emphasizing the need to search for miRNA that are universally abundant in body fluids of breast cancer patients.

In an initial attempt to characterize the miRNA expression pattern in breast cancer cell lines, we used the SOLiD 5500 XL next generation sequencing technology to sequence the miRNome in 11 breast cancer cell lines belonging to three different molecular subtypes and 3 normal cell lines (see Fig 1.1). As shown in the results section (3.1), we chose to annotate the sequences against miRbase 19. This means that only exact mature miRNAs are included in the analysis. However, the RNA fraction that was not annotated includes isomirs, potential new and uncharacterized miRNAs and other RNA species with unknown function. This leaves the possibility that members of this RNA fraction may have important roles in regulation of gene expression in breast cancer and could possibly also have potential as biomarkers.
Evaluating the miRNA expression pattern in breast cancer cell lines revealed considerable differences, both among the breast cancer cell lines and between the breast cancer cell lines and the normal cell lines. Nevertheless, we identified some miRNAs that were highly expressed in all the breast cancer cell lines. They had an overall high abundance of miR-103a-2//miR-103a-1 and this miRNA has previously been shown to inhibit miRNA biosynthesis by targeting Dicer, and is associated with metastasis and poor outcome in breast cancer (154). MiR-24 also showed a high general expression compared to the normal cell lines, and this miRNA has previously been reported to have higher expression in breast carcinoma samples than in breast benign tissues. It has also been shown that ectopic expression of miR-24 promotes breast cancer cell invasion and migration (165). MiRNA with a consistent high expression among all the breast cancer cell lines may be candidate biomarkers. However the potential as biomarkers is highly dependent on the presence of these in body fluids. Extracellular miRNAs exist in different forms and several studies have shown that miRNAs can be localized in exosomes (98,99).

The results shown in section 3.4 confirmed the expression of a large number of different miRNAs in the exosomes derived from the breast cancer cell lines. The exosomal miRNome was annotated against miRbase 19 and only exact mature miRNAs were included in the analyses. As with the breast cancer cell lines, this leaves the possibility that members of the sequenced exosomal RNA fraction not analyzed in this thesis might have important roles in regulation of gene expression in breast cancer, and may possibly also have potential as biomarkers. Some the most

prominent miRNAs in the exosomes were miR-24, MiR-103a-2//103a-1 and miR-19b. These miRNAs were also highly expressed in the breast cancer cell lines, accentuating their potential as possible biomarkers. However some of the most abundant exosomal miRNAs were not highly expressed in the breast cancer cell lines. Both miR-17 and MiR-34a showed a low expression in the cell lines, but were abundant in the exosomes.

Comparing the miRNA expression between the breast cancer cell lines and the exosomes, showed an allover good correlation (See Fig 3.7, Fig 3.8 and Fig 3.9), nevertheless a significant number of miRNAs were differentially expressed in the exosomes and the cell lines. Examples of such miRNAs were miR-17 and miR-34a. Interestingly, both of these miRNAs have been shown to act as tumor supressors in breast cancer. The discrepancy in miRNA expression between the exosomes and the cell lines, suggests at least a partially selective secretion of miRNA into exosomes. Selective packaging of miRNAs into exosomes have also been reported in other studies, supporting our findings that the miRNA profiles in exosomes do not reflect the miRNA profiles observed in the parental cells (99,176–178).

The observation that miRNAs are at least partly selectively secreted into exosomes, raises the question of whether exosomally-derived miRNAs are specific mediators of cancer cell function. When assessing the exosomal miRNA expression pattern in this study, both miRNAs with oncogenic and tumor suppressive roles were found among the most abundant. Moreover, the expression of miRNAs with oncogenic roles seems slightly higher in the breast cancer cell line compared to the exosomes. As shown in fig 3.4, miR-21 is highly expressed in the breast cancer cell lines, but not present amongst the most abundant miRNAs in the exosomes (Fig 3.10). Based on our results it is not clear whether the breast cancer cells selectively secrete the miRNAs that would preclude the oncogenic potential to the cell, or if the breast cancer cells secrete miRNAs into their microenvironment in order to signal the surrounding tissue and further induce malignancies. Indeed, studies have demonstrated that tumor-derived exosomes are pivotal in promoting tumor metastasis via a proinflammatory cytokine-driven expansion of myeloid-derived suppressor cells (179).

Although several studies have shown that miRNAs are localized in exosomes (50–100 nm) (98,99), others have reported that extracellular miRNAs are predominantly exosome free and are associated with Ago proteins (97). In order to investigate if miRNAs are associated with Ago2 and released into the cell media by breast cancer cell lines, we attempted to immunoprecipitate Ago2 both from protein extracts and from cell-free media. Unfortunately, we were unable to detect sufficient amount of the Ago2 protein to proceed with the isolation and identification of miRNAs from immunoprecipitated Ago protein complexes. Whether this was caused by insufficient starting material, or by or low levels of expressed Ago2 in our cell lines is not known.

To our knowledge, there have been no previous reports using SOLiD 5500 XL next generation sequencing technology to compare the miRNome in breast cancer cell lines and their corresponding exosomes. As a future prospect and extension of the study it would be of great interest to include more breast cancer cell lines and normal cell lines. As mentioned in section 3.4, we made some initial attempts to isolate exosomes from the normal cell lines, however we were unable to isolate a detectable amount of RNA. This could maybe imply that the secretion of exosomes is mainly restricted to the breast cancer cell lines and therefore associated with malignancies.
The fact that the miRNA profiles in exosomes did not reflect the miRNA profiles observed in the parental cell lines point towards a selective secretion of miRNA into exosomes. If in fact the same applies for breast cancer cells in patients, it would be of considerable interest to search for expression of the miRNAs found to be abundant in the exosomes used in this study. In support of this, several reports have described circulating miRNAs that are specifically elevated in the patients with tongue (95,171,174,180–182)and colorectal cancer (183,184)These findings suggest that blood-based miRNAs could emerge as new sources of biomarker for breast cancer diagnosis.

Very recently, Dvinge.H Et.al (30) described the miRNA expression profiles of 1,302 breast cancer tumors, and found a global decrease in miRNA expression in tumours compared to adjacent normal tissue, accompanied by a gradual decline with increasing tumour grade both in tumors and in breast cancer cell lines. They also reported that although several individual miRNAs that were expressed as classical oncogenes or tumour-suppressor genes, the profiling revealed that miRNAs behave

more consistently as fine-tuners/modulators of gene expression at the whole-genome level.

In the future, miRNAs in circulation system might function as promising biomarkers in early stage breast cancer detection. However, this concept still needs extensive investigation to validate a potential panel of miRNA specific for breast cancer.
It is also likely that circulating miRNA expression profiles will be used to differentiate between those breast cancer patients who are most likely to respond positively to neoadjuvant and adjuvant chemotherapy, and indeed serve as an overall prognostic factor and stratify patients into risk categories which would further guide their management. Similarly, it is also of great interest to identify a panel of circulating miRNA markers which could monitor the patient's response to therapiy. Ideally a suitable panel of miRNA biomarkers would show significant changes in miRNA expression level in good-responders while little or no change would be observed in non-responders.

Undoubtedly, the future will bring more details, which will contribute to solve the puzzle of miRNA detection in different body fluid; and this will hopefully bring a new era to the era to the field of diagnostic biomarkers in human cancers.

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6 Appendix

6.1 The highest expression in the exosomes

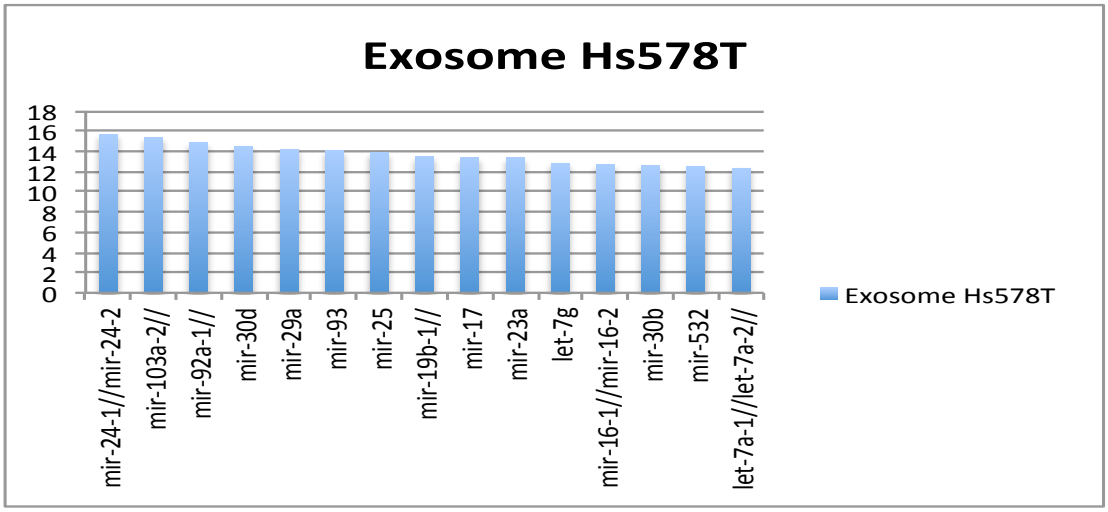

Figure 6.1. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line Hs578T.

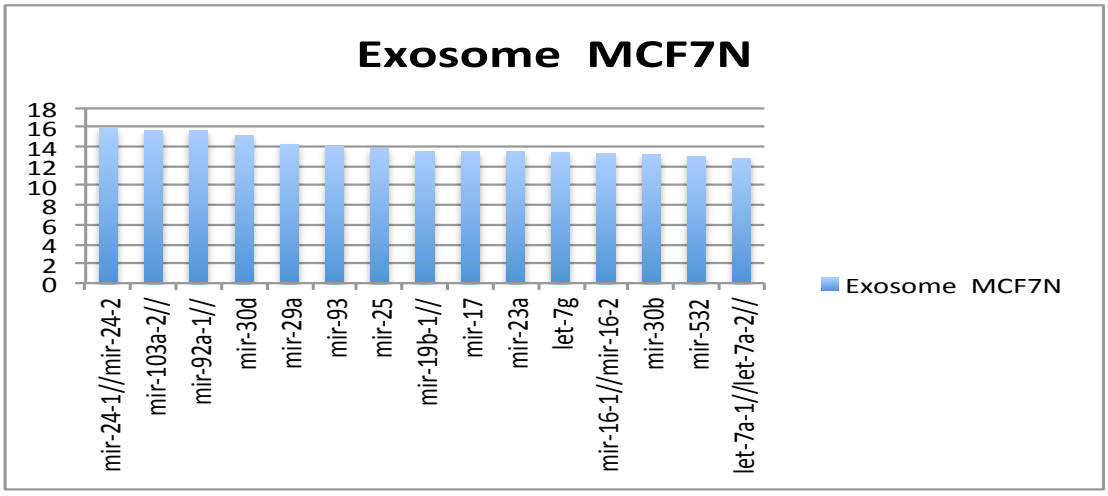

Figure 6.2. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line MCF-7(N).

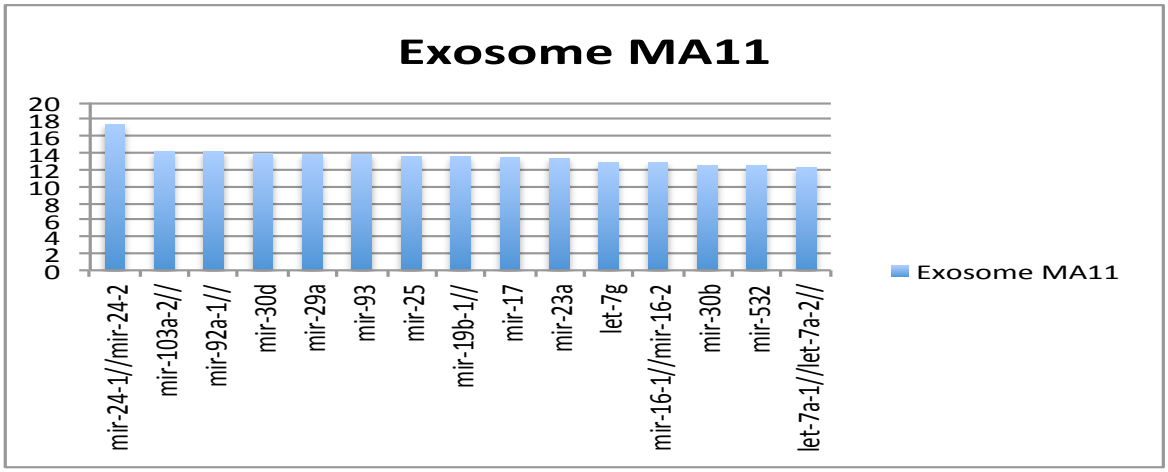

Figure 6.3. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line MA11.

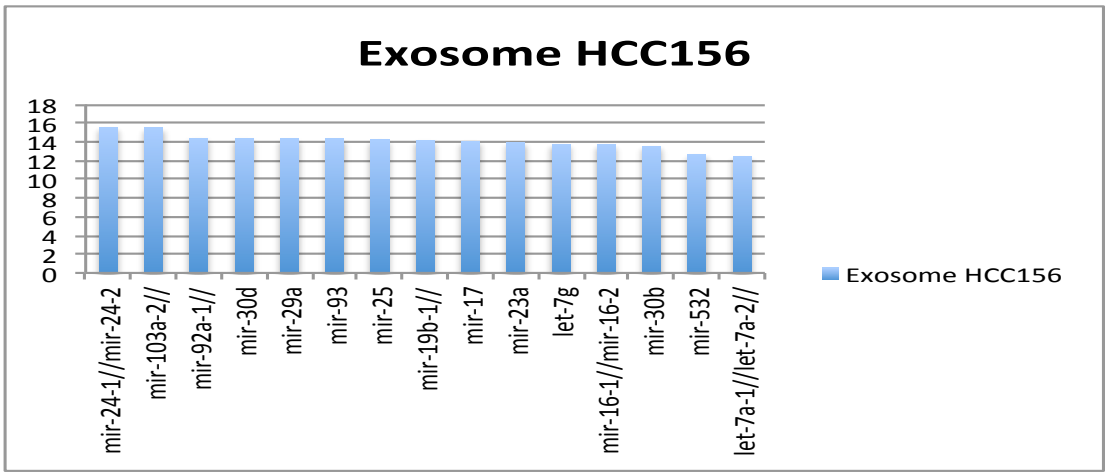

Figure 6.4. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line HCC 1569.

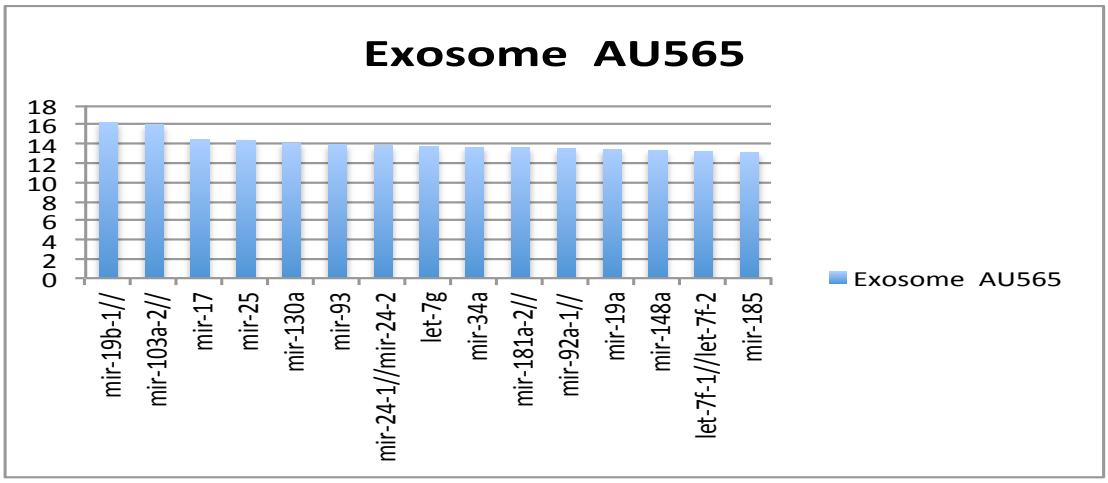

Figure 6.5. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line AU565.

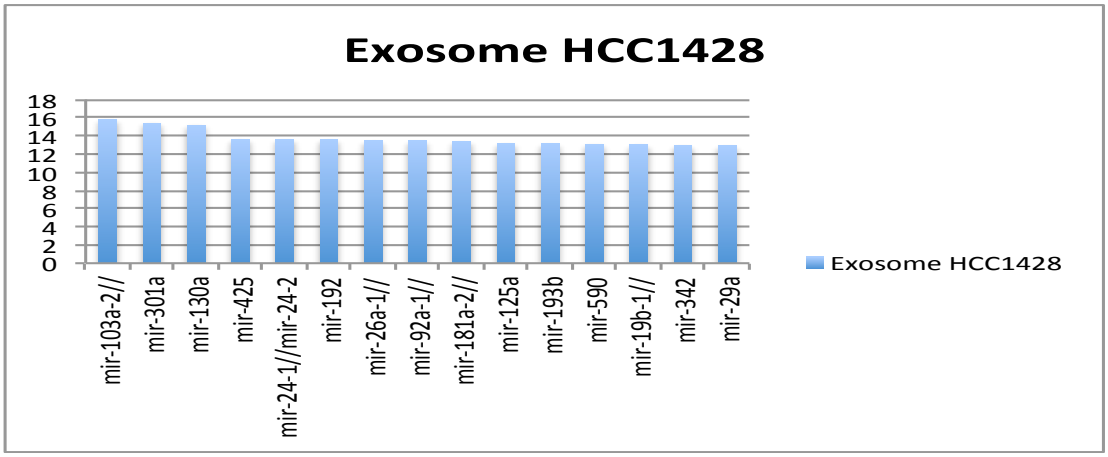

Figure 6.6. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line HCC 1428.

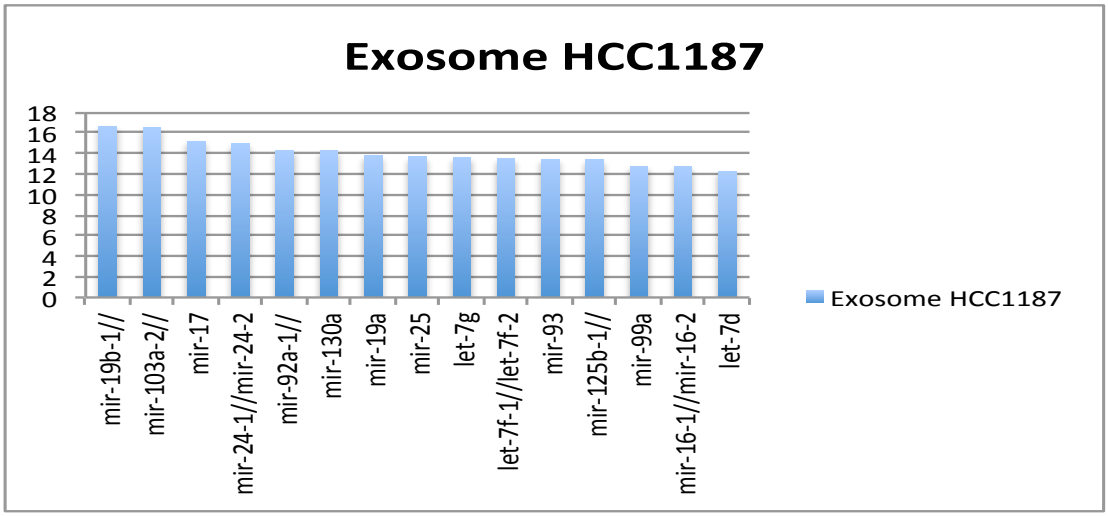

Figure 6.7. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line HCC 1187.

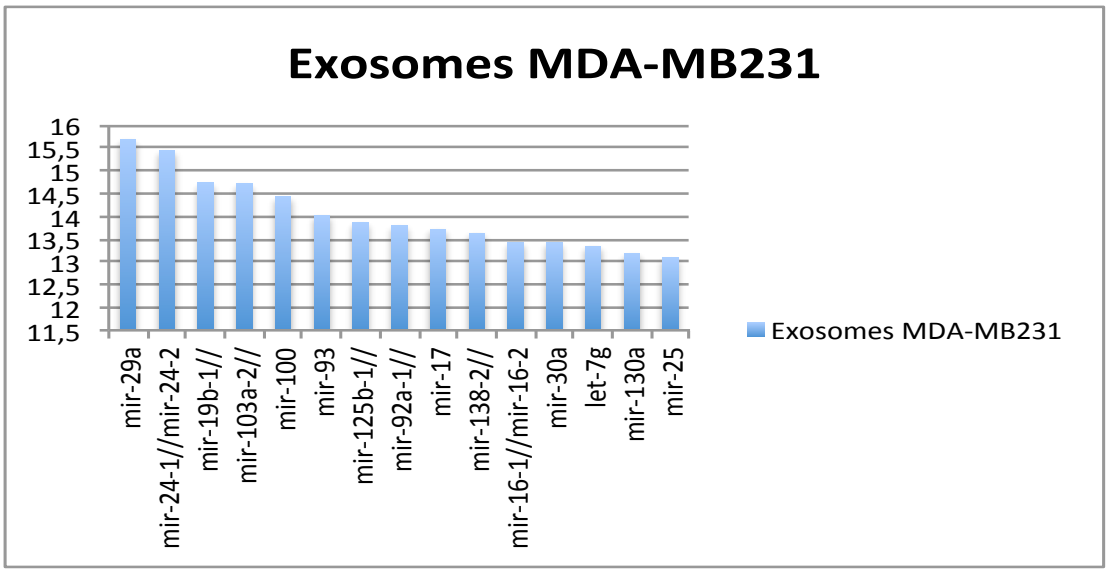

Figure 6.8. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line MDA-MB231.

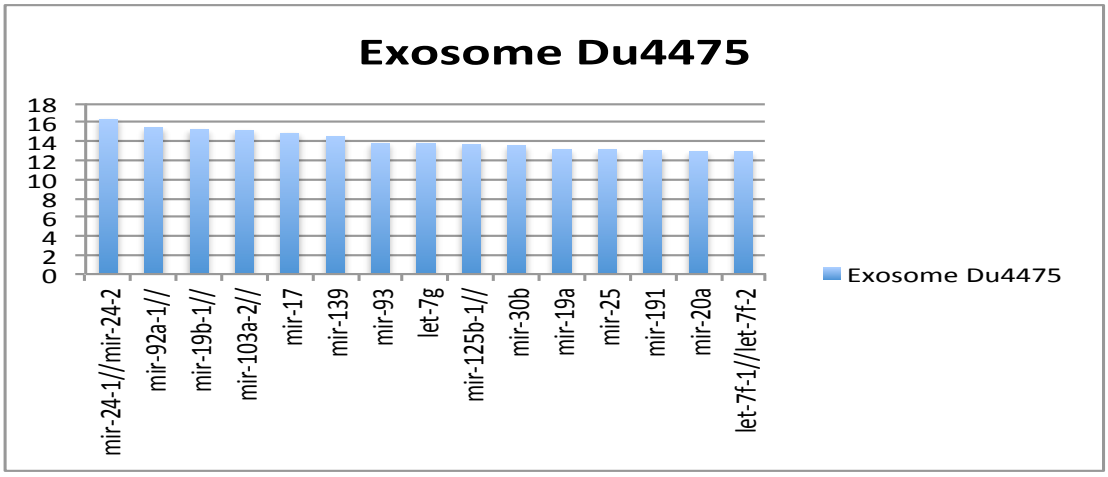

Figure 6.9. The fifteen most expressed miRNAs in the exosomes isolated from t
The breast cancer cell line DU 4475

6.2 The highest count of miRNA in the breast cancer cell lines

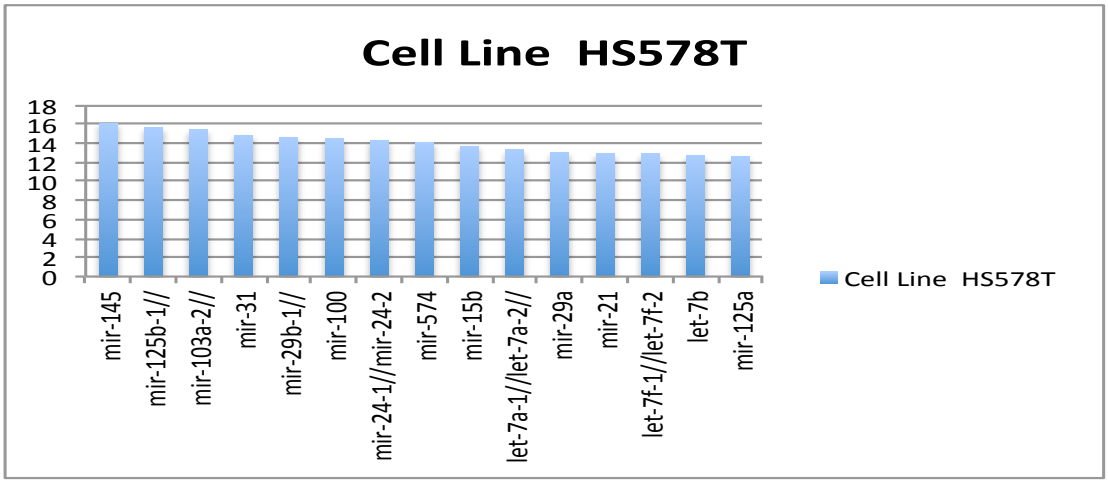

Figure 6.10. The fifteen most expressed miRNAs in the breast cancer cell
line Hs578T.

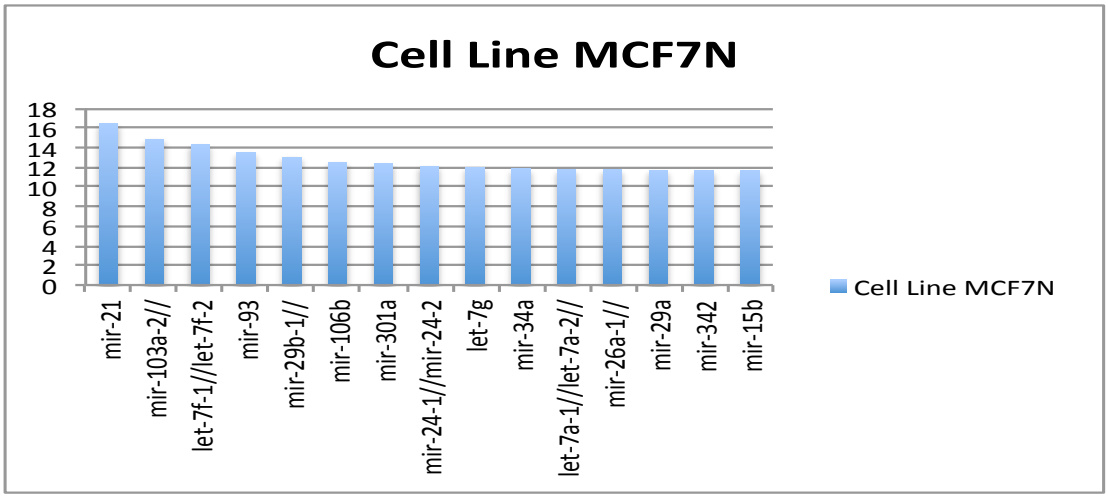

Figure 6.11. The fifteen most expressed miRNAs in the breast cancer cell
line MCF-7(N).

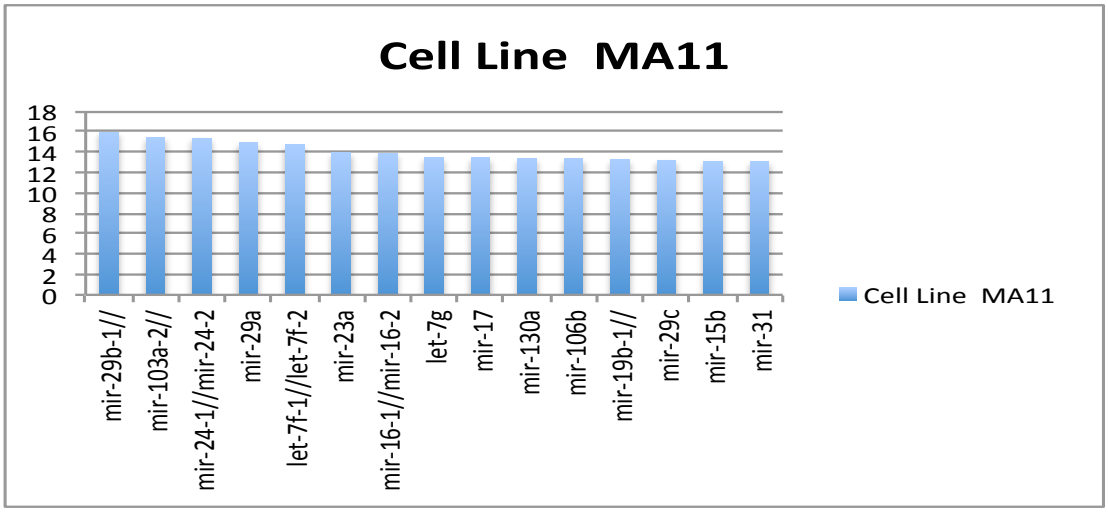

Figure 6.12. The fifteen most expressed miRNAs in the breast cancer cell line MA11.

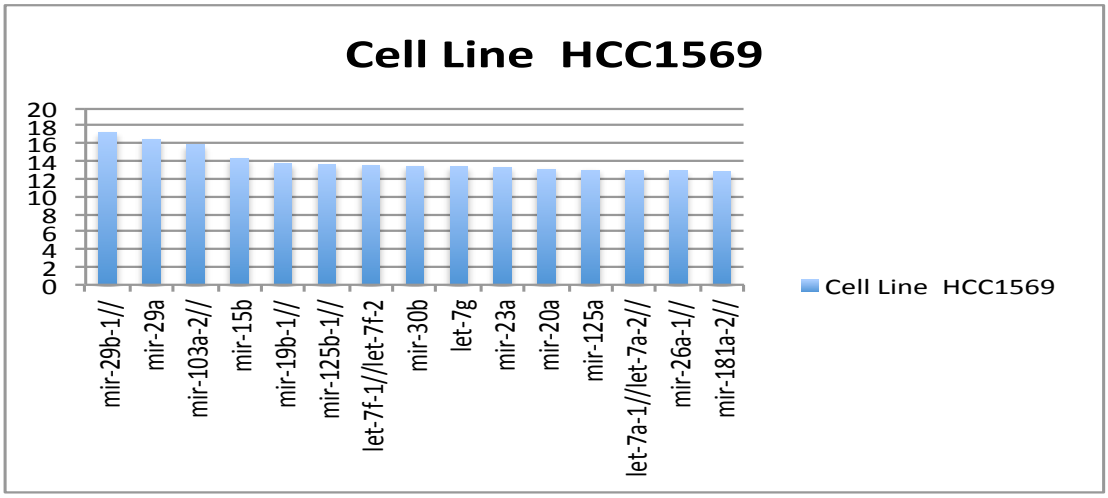

Figure 6.13. The fifteen most expressed miRNAs in the breast cancer cell line HCC 1569.

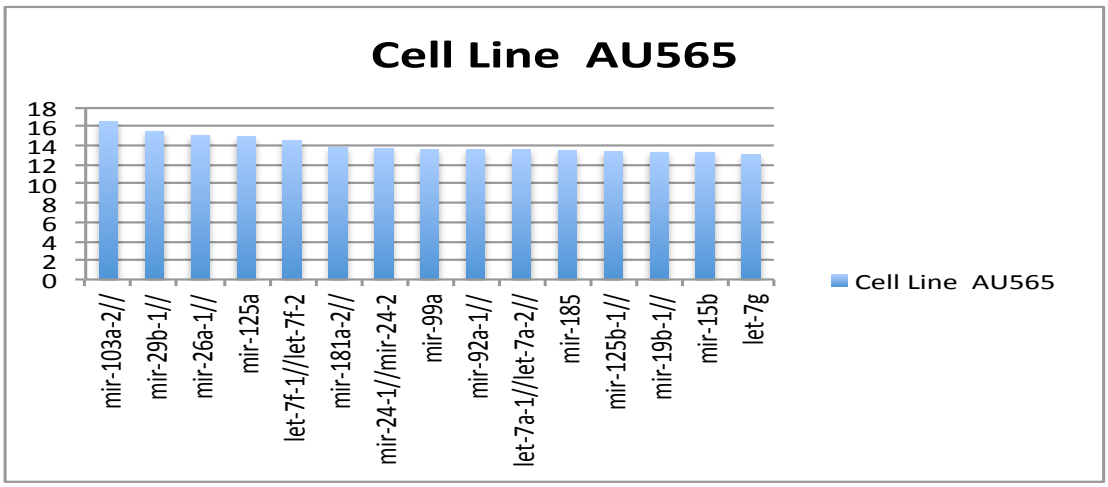

Figure 6.14. The fifteen most expressed miRNAs in the breast cancer cell line AU565.

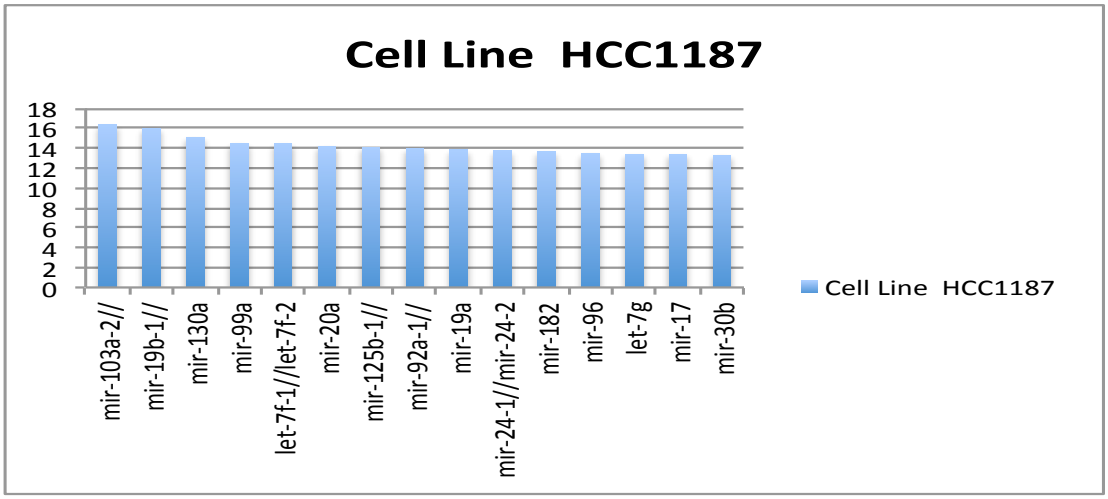

Figure 6.15. The fifteen most expressed miRNAs in the breast cancer cell line HCC 1187.

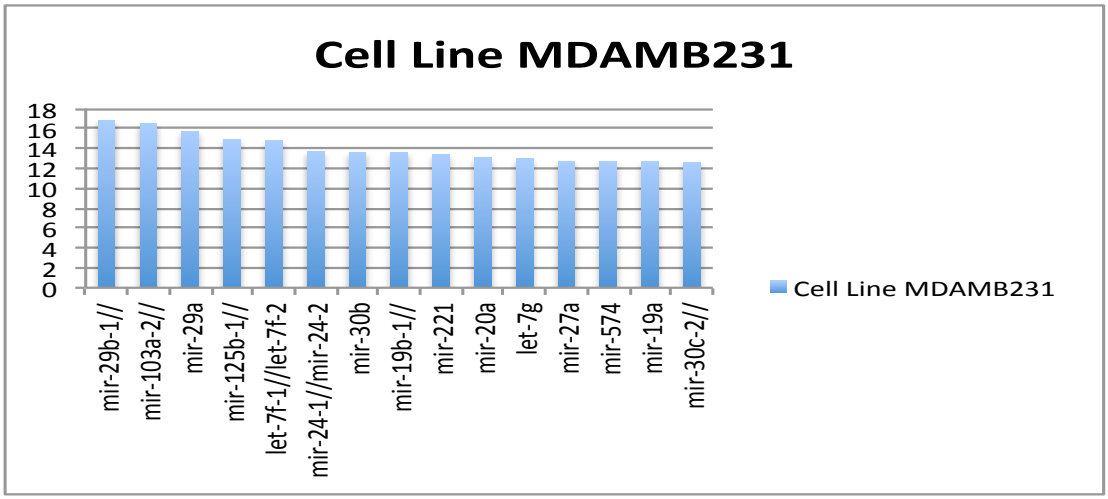

Figure 6.16. The fifteen most expressed miRNAs in the breast cancer cell line MDA-MB 231.

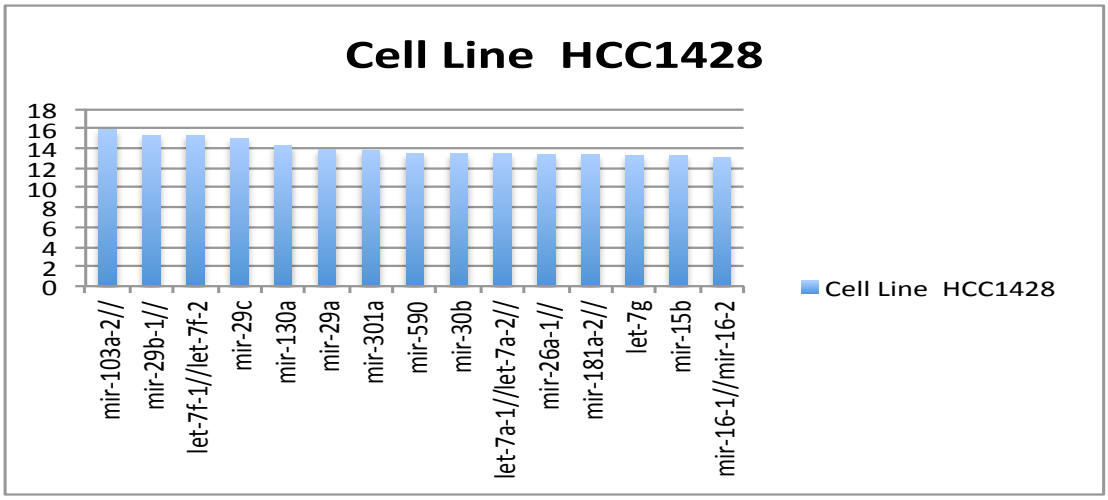

Figure 6.17. The fifteen most expressed miRNAs in the breast cancer cell line HCC 1428.

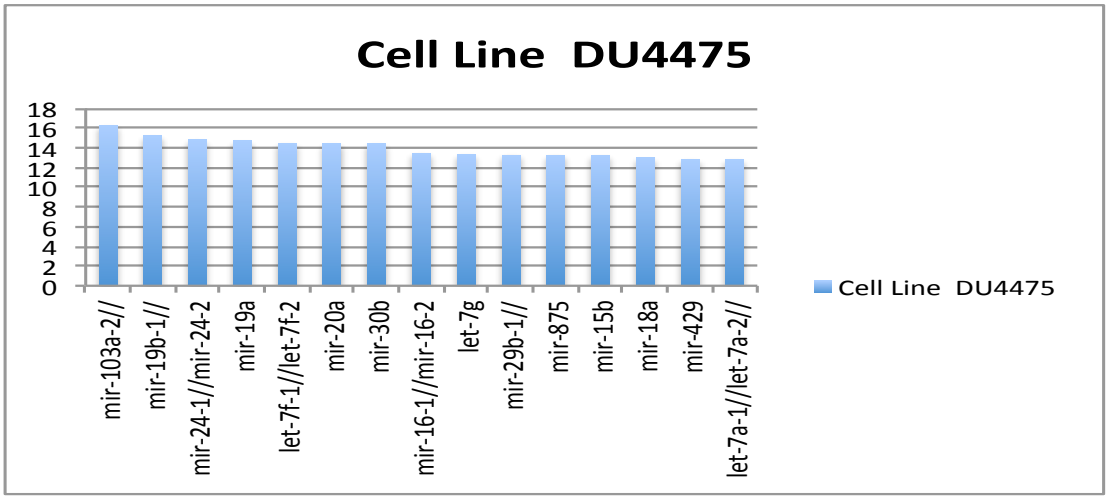

Figure 6.18. The fifteen most expressed miRNAs in the breast cancer cell line DU4475.

6.3 Scatter plot of the exosomes and the breast cancer cell lines

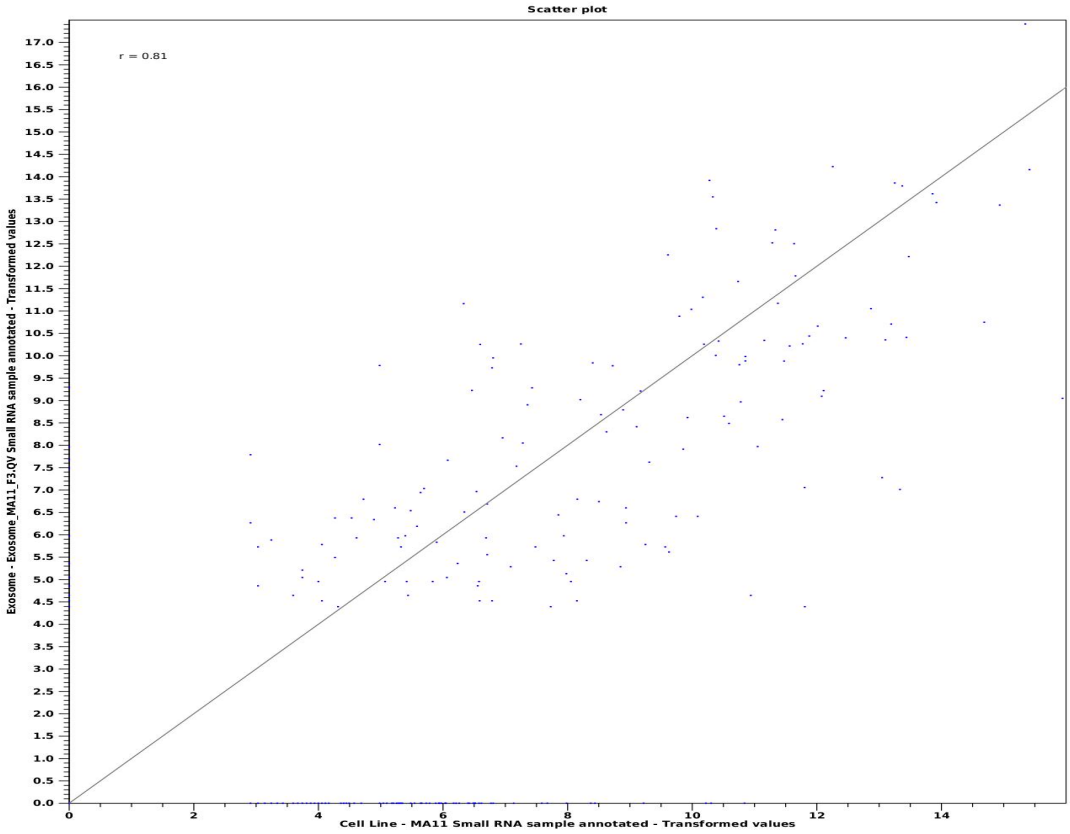

Figure 6.19. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line MA11.

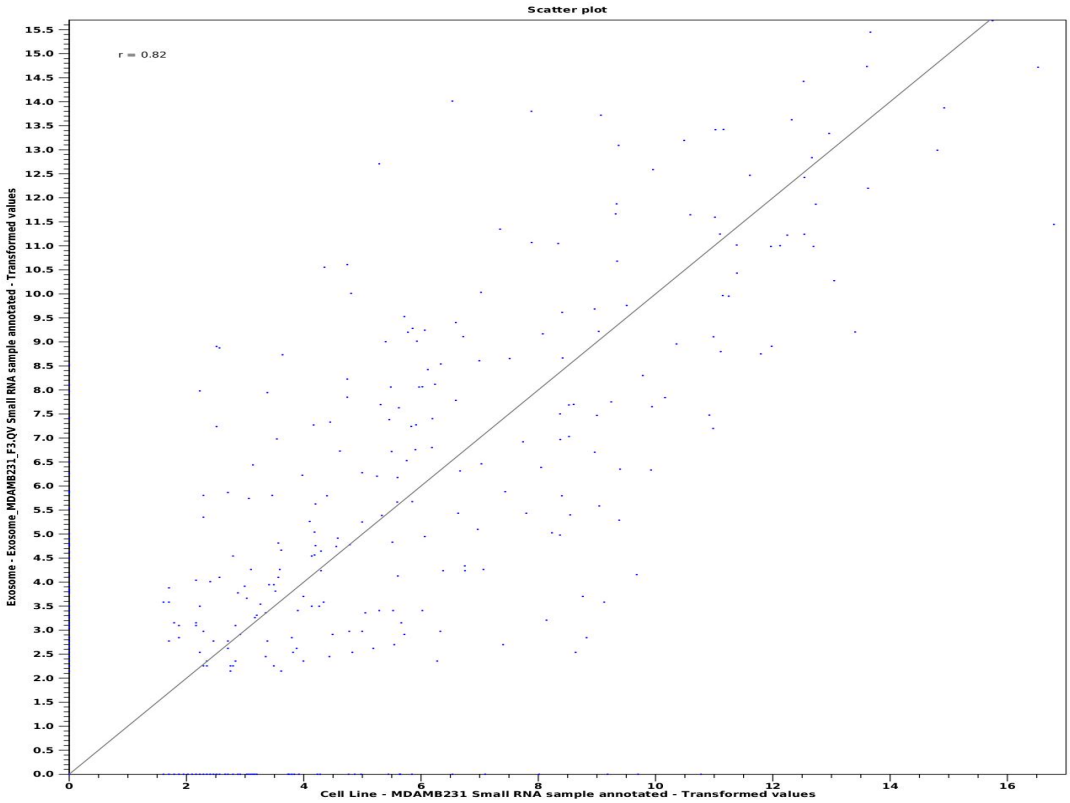

Figure 6.20. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line MDA-MB 231.

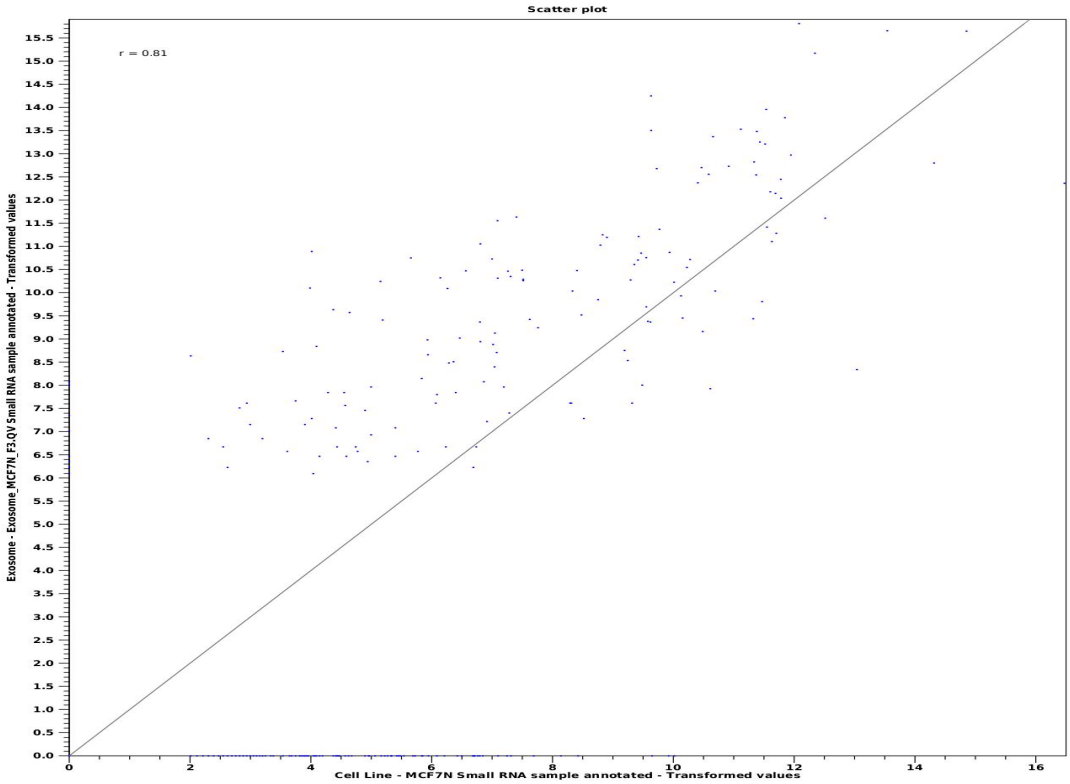

Figure 6.21. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line MCF-7(N).

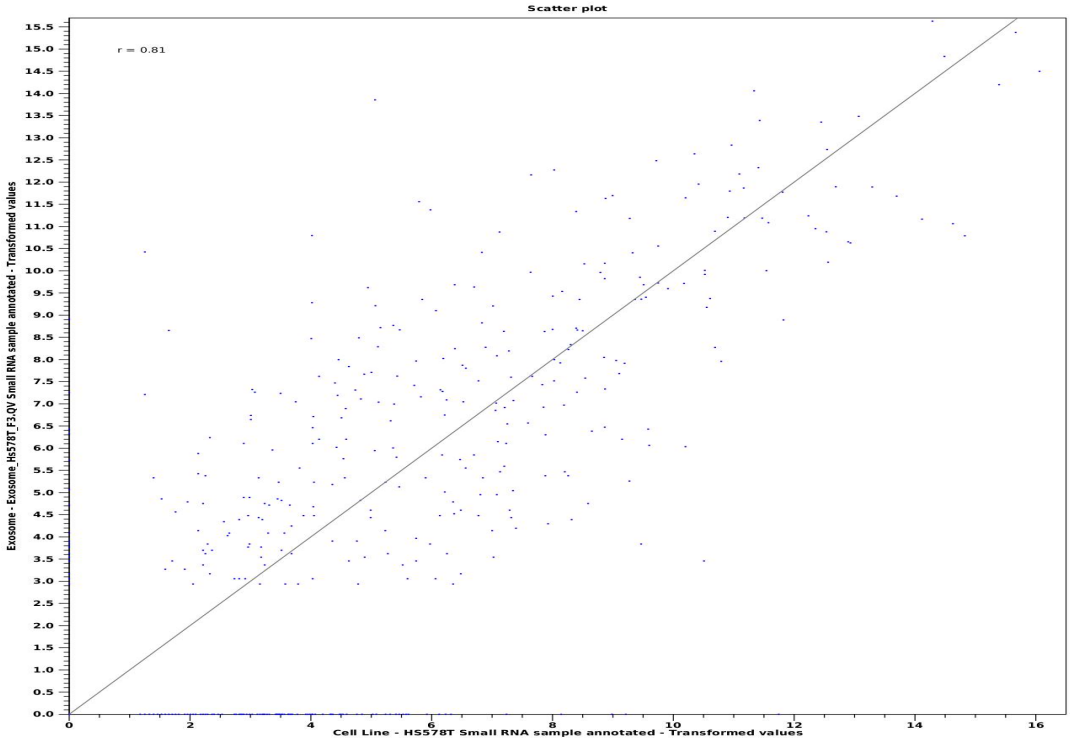

Figure 6.22. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line Hs578T.

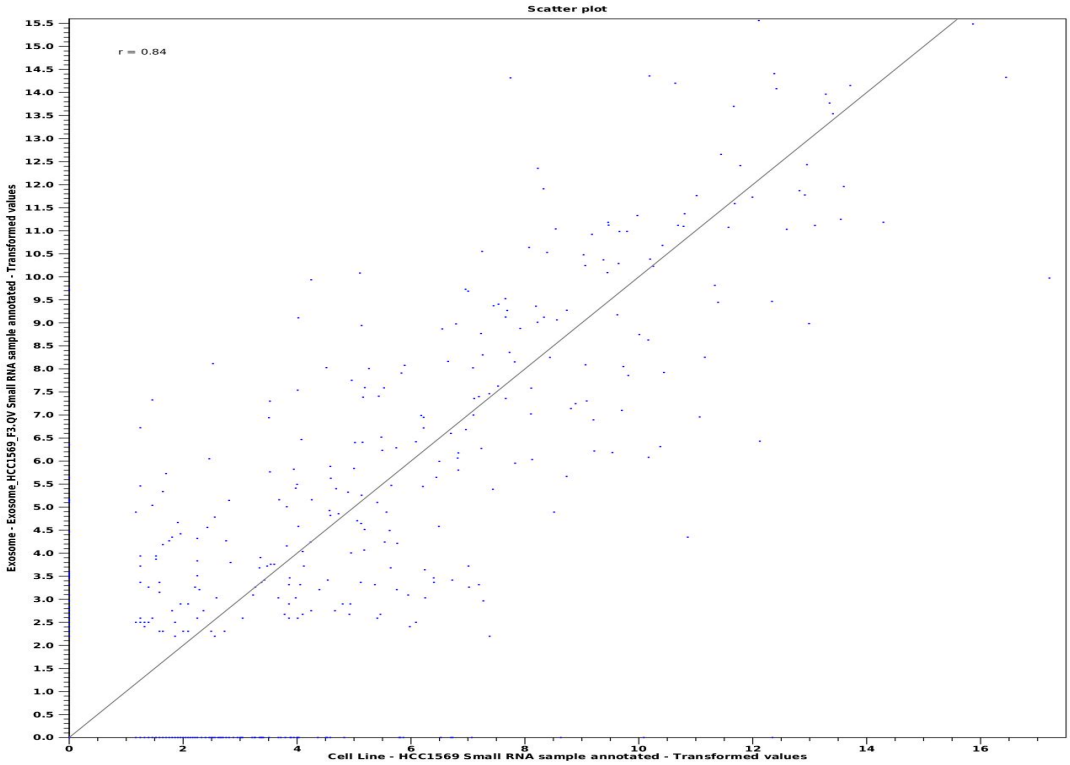

Figure 6.23. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line HCC 1569.

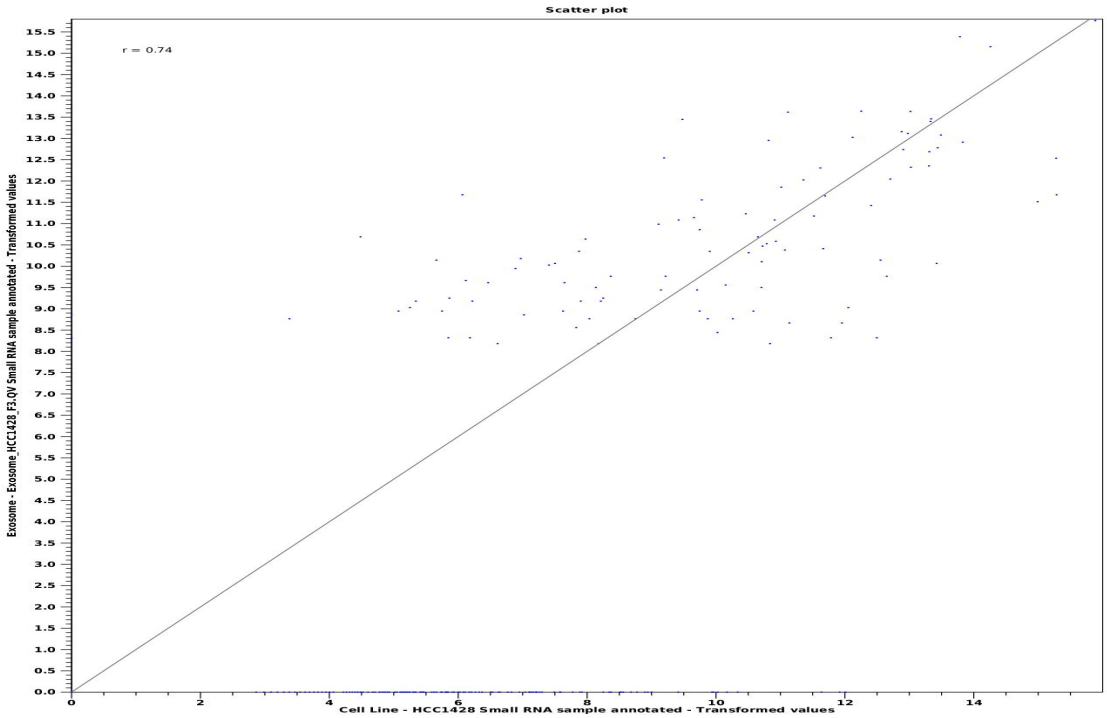

Figure 6.24. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line HCC 1428.

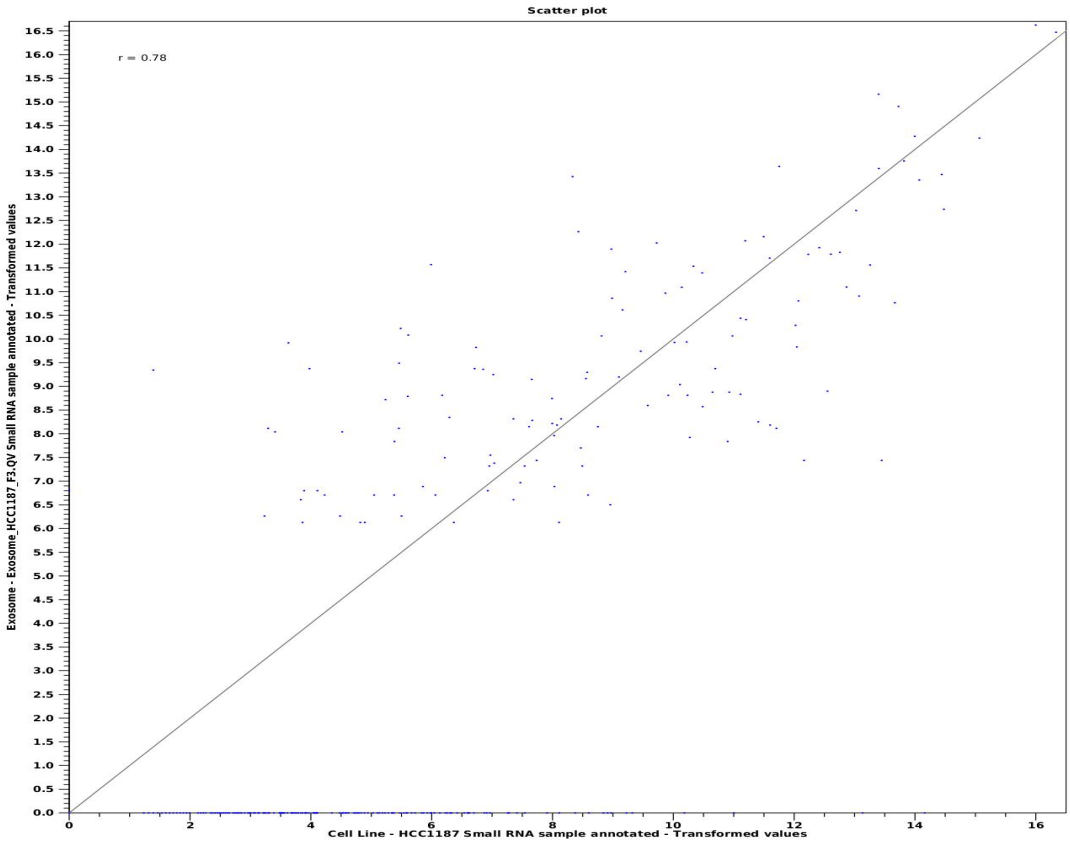

Figure 6.25. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line HCC 1187.

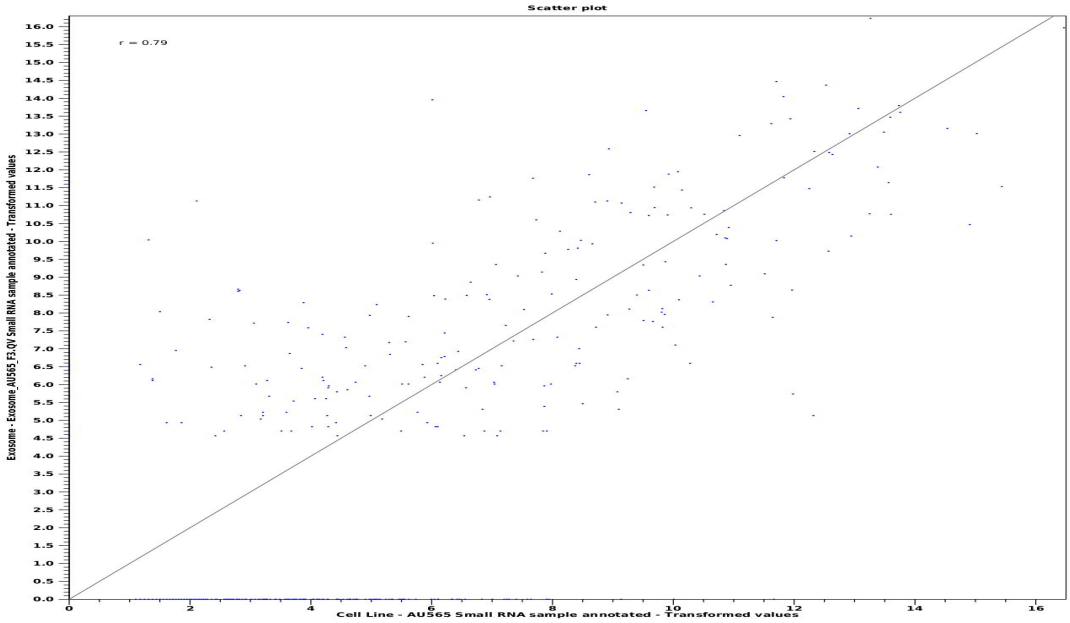

Figure 6.26. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line AU 565.

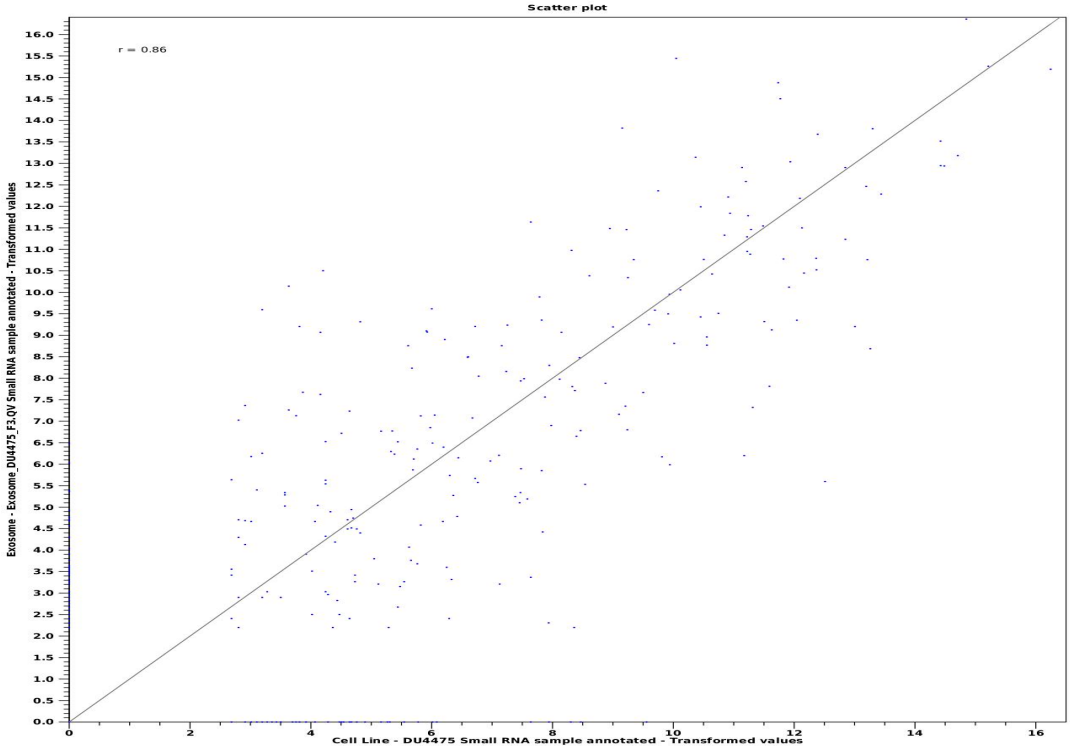

Figure 6.27. Scatter plot of the correlation between the miRNA expression in the exosomes and the miRNA expression in the breast cancer cell line DU 4475.

6.4 Diagrams of the of the small RNA enrichment from the total RNA analysed with Agilent 2100.

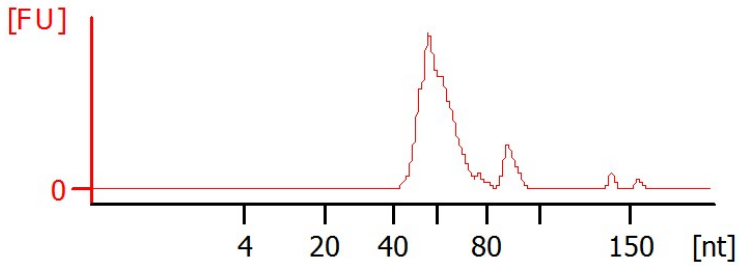

Figure 6.28. Small RNA enrichment from the total RNA isolation from the breast cancer cell line DU4475. This was done with a RNA 6000 nano chip.

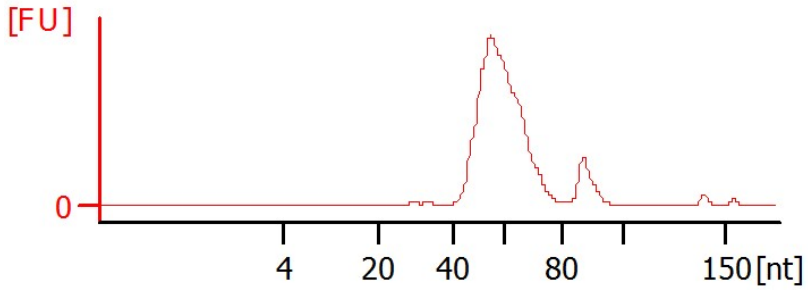

Figure 6.29. Small RNA enrichment from the total RNA isolation from the breast cancer cell line MCF-7(N), it was used a RNA 6000 nano chip.

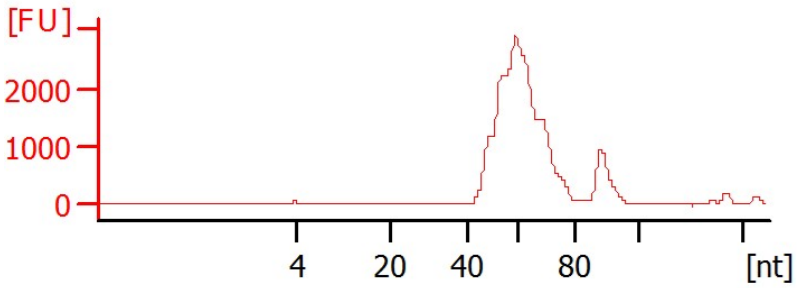

Figure 6.30. Small RNA enrichment from the total RNA isolation from the breast cancer cell line MA11, it was used a RNA 6000 nano chip.

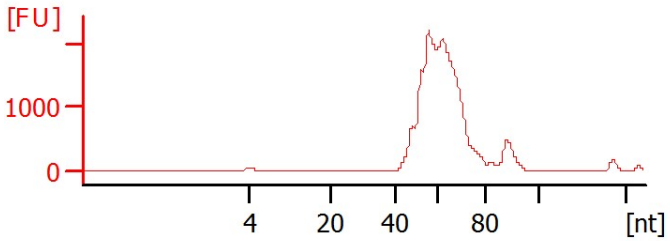

Figure 6.31. Small RNA enrichment from the total RNA isolation from the breast cancer cell line HCC1428, it was used a RNA 6000 nano cip.

6.5 Diagrams of the cDNA library of the breast cancer cell lines analysed with Agilent 2100

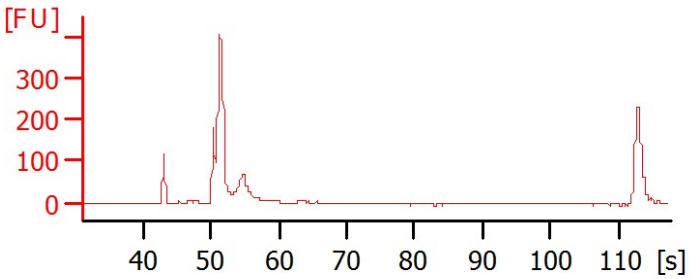

Figure 6.32. The cDNA library from the breast cancer cell line DU 4475, it was used a DNA high sensitivity chip.

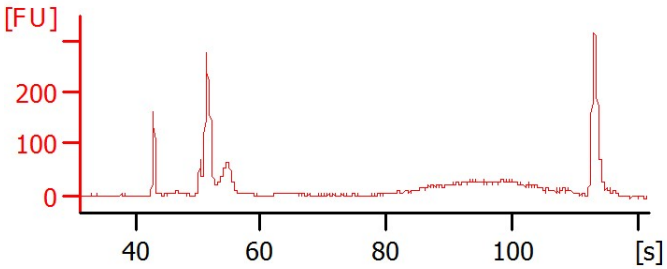

Figure 6.33. The cDNA library from the breast cancer cell line HCC 1428, it was used a DNA high sensitivity chip.

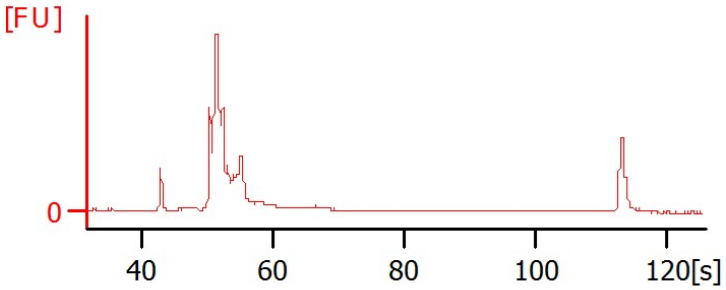

Figure 6.34. The cDNA library from the breast cancer cell line MCF-7(N), it was used a DNA high sensitivity chip.

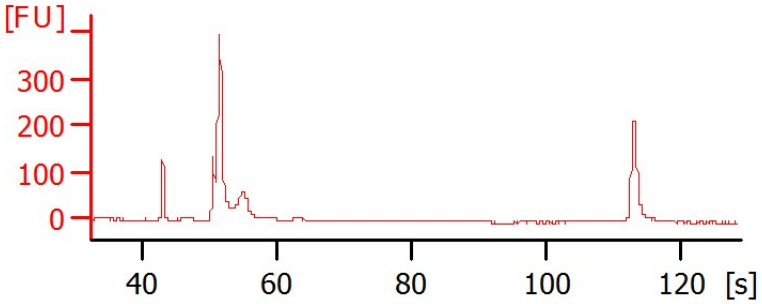

Figure 6.35. The cDNA library from the breast cancer cell line MA11, it was used a DNA high sensitivity chip.

6.6 Diagrams of the cDNA library of the exosomes isolated from the breast cancer cell lines analysed with Agilent 2100

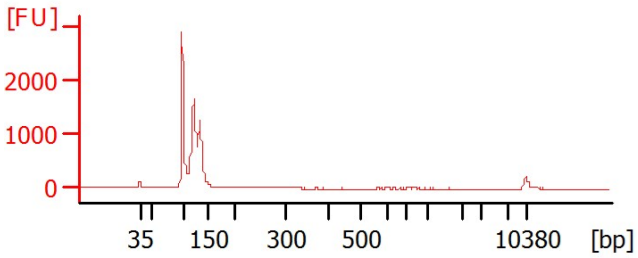

Figure 6.36. The cDNA library to the exosomes from the breast cancer cell line MCF-7N, it was used a DNA high sensitivity chip.

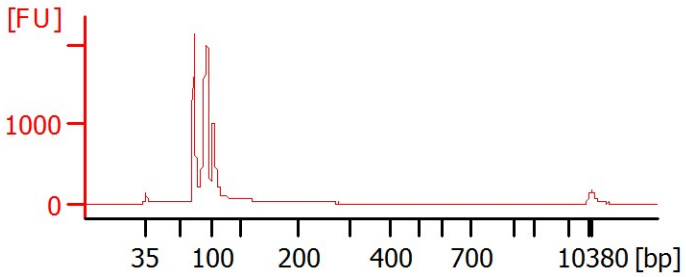

Figure 6.37. The cDNA library to the exosomes isolated from the breast cancer cell line Hs578T, it was used a DNA high sensitivity chip.

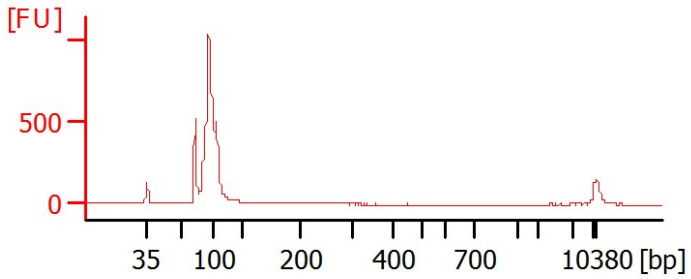

Figure 6.38. The cDNA library to the exosomes isolated from the breast cancer cell line MA11, it was used a DNA high sensitivity chip.

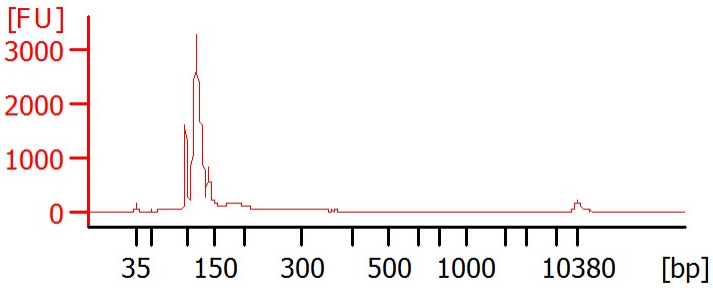

Figure 6.39. The cDNA library to the exosomes isolated from the breast cancer cell line HCC 1428, it was used a DNA high sensitivity chip.

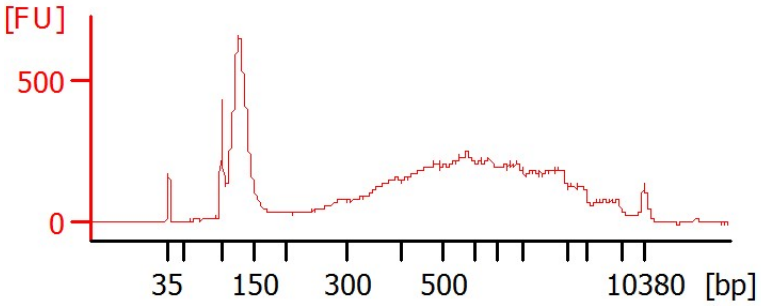

Figure 6.40. The cDNA library to the exosomes isolated from the breast cancer cell line HCC 1187, it was used a DNA high sensitivity chip.

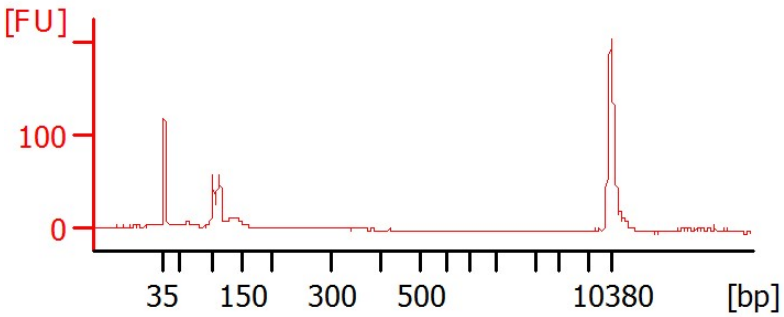

Figure 6.41. The cDNA library to the exosomes isolated from the breast cancer cell line AU565, it was used a DNA high sensitivity chip.

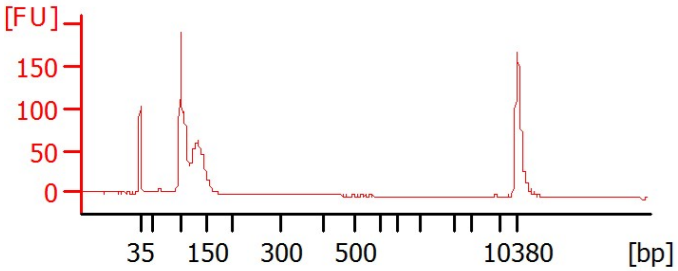

Figure 6.42. The cDNA library to the exosomes isolated from the breast cancer cell line MDA-MB 231, it was used a DNA high sensitivity chip.

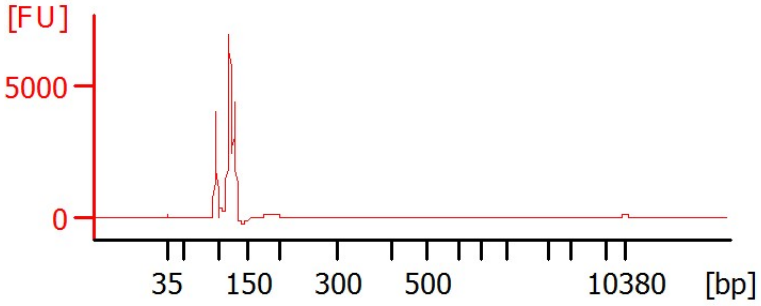

Figure 6.43. The cDNA library to the exosomes isolated from the breast cancer cell line DU 4475, it was used a DNA high sensitivity chip.

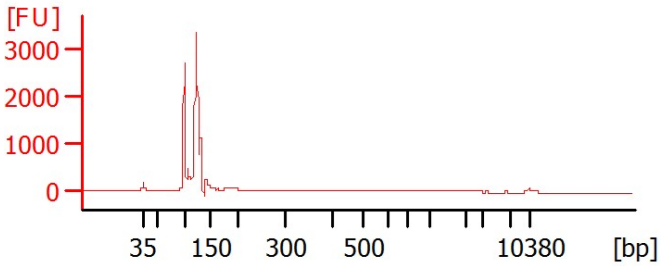

Figure 6.44. The cDNA library to the exosomes isolated from the breast cancer cell line HCC 1569, it was used a DNA high sensitivity chip.