Mastergradsoppg. 2010

CANCER-RELATED MIRNAS LIKE LET-7 AND MIR-21 ARE ALREADY DIFFERENTIALLY EXPRESSED IN BENIGN TUMOURS

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MASTER THESIS 60 CREDITS 2010

Acknowledgments

This study was performed at Akershus University Hospital, institute for clinical epidemiology and molecular biology (EpiGen), as part of the Master program at the Norwegian University of Life Sciences (UMB), The Department for Chemistry, Biotechnology and Food Science (IKBM).

I would like to thank my supervisor, Pr. Vessela Kristensen for her guidance and advice throughout the project, and for always being supportive and helpful. I would like to thank the entire breast cancer research group at EPIGEN for their support and thoughts in the preparation of this paper. Their advice has been very valuable to me.

Special thanks go to Post doc. Torben Lüders for his support, guidance and knowledge, and for helping me throughout the project with lab work and data analysis. I will always be grateful to you for helping me and always having time for me during my time at EPIGEN.

Thanks to my external supervisor, Dr. Espen Enerly at Norwegian Radium Hospital for giving me support and exchange of thoughts when needed.

I would like express my gratitude to my family and my husband for always encouraging me and giving me everlasting support.

Lørenskog, 15. Mai 2010

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Oppsummering

MikroRNA (miRNA) er små, ikke-kodende RNA som spiller en essensiell rolle i reguleringen av genuttrykk. Denne studien ble utført for å analysere miRNA uttrykk profiler i godartete svulster, ondartete svulster og normalt brystvev. Ved bruk av miRNA mikroarrayteknologi har vi demonstrert at godartete brystsvulster har større likhet med ondartete brystsvulster enn med friskt brystvev, utifra deres miRNA-profil. RNA ble isolert fra 20 biopsier av kvinner med godartete brystsvulster (fibroadenomer/fibroadenomatoser), 13 vevsprøver av ondartetede brystsvulster og 29 prøver av normalt brystvev fra friske kvinner, for å kunne utføre miRNA-mikroarrayanalyser. Ikke-veiledet, hierarkisk klyngeanalyse av uttrykksnivået til 342 miRNA viste 3 hovedgrupper som skiller de tre forskjellige histologiske vevstypene.

En tre-klasse SAM analyse identifiserte 81 miRNAer som var forskjellig uttrykt mellom godartetede brystsvulster, ondartede brystsvulster og normalt brystvev. Blant miRNAene som var mest forskjellig uttrykt er medlemmer av let-7 familien, miR-125b, miR-145, miR-155 og medlemmer av miR-200 familien (miR-200b, miR-200c, and miR-141), miR-205 og miR-21. Disse miRNAene har tidligere blitt påvist å være kreftfremkallende og promotere vekst i forskjellige typer kreft, inkludert brystkreft. Vi identifiserte 31 miRNA som er likt uttrykt i både godartetede og ondartetede brystsvulster og som er mest ulikt uttrykt sammenlignet med friskt vev. Blant de indentifisert miRNAene var miR-21 og let-7. MiR-21 har flere tumorsuppressorgener som mål mens let-7 har flere oncogener som mål, blant dem er genet *RAS* som er deregulert i mange typer kreft hos mennesker. Både miR-21 og let- 7 har kreftfremkallende potensiale, og deregulering av disse miRNA fører til deregulering av målgener som kan føre til kreft hos mennesker.

Utviklingen av klinisk validerte biomarkører for kreft har forblitt en vanskelig oppgave til tross for nye avanserte metoder innenfor feltet for kreft molekylær biologi. MiRNA har mange egenskaper som gjør den til en ideell biomarkør, spesielt på grunn den naturlige stabiliteten og fleksibiliteten. Funn av kreftrelaterte miRNA i godartete svulster indikerer at disse miRNAene kan være potensielle diagnostiske markører og sannsynlige faktorer involvert i tidlig utvikling av brystkreft.

Abstract

MicroRNAs (miRNAs) are endogenous non-coding RNAs, which play an essential role in the regulation of gene expression. This study was carried out to analyze miRNA expression profile in benign breast tumours, malignant breast tumours and normal breast tissue. Through the use of miRNA microarray technology, we demonstrated that benign breast tumours are more similar to malignant breast tumours than normal breast tissue according to their miRNA profile. RNA was isolated from 20 biopsies from women with benign breast tumours, 13 tissue samples from women with breast cancer, and 29 samples of normal tissue from healthy women in order to perform miRNA microarray analysis. Unsupervised hierarchical clustering using expression information for 342 miRNAs produced 3 major clusters that separated the different histological groups.

A subsequent three class SAM analysis identified 81 miRNAs that were differentially expressed between benign- and malignant tumours and normal tissue. Amongst the miRNAs that were most differentially expressed are members of the let-7 family, miR-125b, miR 145, miR-155, members of the miR-200 family (miR 200b, miR-200c, and miR 141), miR-205 and miR-21. These miRNAs have previously been identified to have tumorigenic potential in several cancers, including breast cancer. We have also identified 31 miRNAs that have a similar expression in benign- and malignant tumours and are most differentially expressed from normal tissue. Amongst them were miR-21 and let-7. Mir-21 targets tumour suppressor genes while let-7 targets several oncogenes, amongst them is the oncogene *RAS* which is found to be deregulated in many human cancers.

The development of clinically validated biomarkers for cancer has remained an impossible task despite advances in the field of cancer molecular biology. MiRNAs have many characteristics of an ideal biomarker, especially due to their inherent stability and flexibility. The finding of tumorigenic miRNAs in benign tumours indicates that these miRNAs may be probable factors involved in the pathogenesis of breast cancer and might be potential diagnosis biomarkers and therapeutic targets in the future.

Contents

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is a heterogeneous disease comprising multiple tumour entities associated with distinctive histological patterns and different biological features and clinical behaviours (Resi-Filho et al., 2008). Worldwide more than one million women are diagnosed with breast cancer every year, making it the most commonly diagnosed cancer among women (WHO, Globocan 2002). In Norway, breast cancer is the most common type of cancer amongst women and the incidence of breast cancer is increasing for each year (Engoholm et al., 2009). The human breast consists of several types of tissue and cells. The major components of the breast are the stroma, the terminal ductal lobular units (TLDUs), the ducts and the nipple (Fig.1A). The stroma is composed of different types of cells such as endothelial cells, macrophages, adipocytes and fibroblasts, and are collectively called mammary fat pad (Visvader J.E., 2009). The ducts contain two layers of cells, one contain myoepithelial cells that are able to contract and luminal cells (Fig.1B). The TLDUs have a cell composition similar to the ducts (Fig.1C).

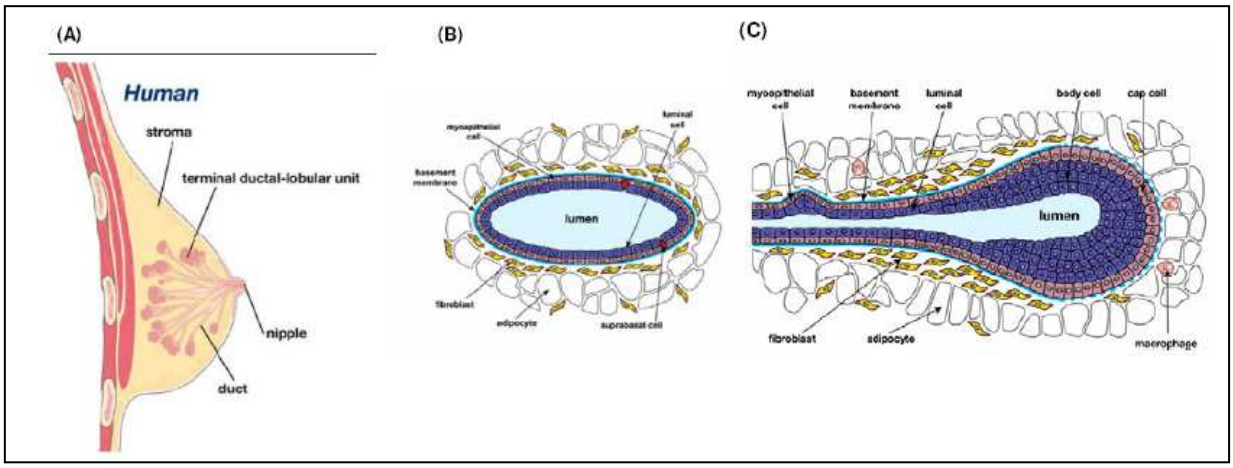

Figure 1: **The human female breast.** Schematic representation of the main components of the female breast (A), the duct (B) and the terminal end bud (TLDU) (C). (Picture taken from Visvader J.E. (2009)).

Most breast cancers are derived from the epithelium lining the ducts or lobules and are called ductal and lobular breast cancer, respectively. Traditionally, invasive breast cancers are classified into subgroups based on histological grade, histological type, presence of

lymphovascular invasion and lymph-node metastasis, and on the expression of a few predictive markers, including hormone receptors, especially estrogen receptor (ER), and Human Epithelial Receptor 2 (HER-2) (Geyer and Reis- Filho, 2008). Through the use of mammography, ultrasound, and magnetic resonance imaging of the breast and the extensive use of needle biopsies, the diagnosis of breast can be accomplished at an early stage.

1.1.1 Oncogenesis: Oncogenes and Tumour suppressor genes

Breast cancer and other types of malignancies result from genetic changes in the cells leading to abnormal growth of cells by influencing cellular pathways involved in growth and development. The step towards tumour formation involves the accumulation of various genetic changes such as amplification of oncogenes and mutation or loss of tumour suppressor genes (Beckmann et al., 1997). Oncogenes and tumour suppressor genes have opposite effects. In normal cells many genes are proto-oncogenes, meaning genes that turn into when mutated or expressed at high levels. Oncogenes helps normal cells turn into tumour cells through loss of growth control (Fig. 2B). A tumour suppressor gene on the other hand protects cells from becoming cancerous. If both alleles of the gene are mutated to reduce function of the gene, the cell can grow uncontrolled and thus lead to cancer (Fig. 2A). In certain cancers, such as breast, lung and colon cancers, both homologous chromosomes show deletions of specific regions where tumour suppressor genes are located that inhibit cell growth and division.

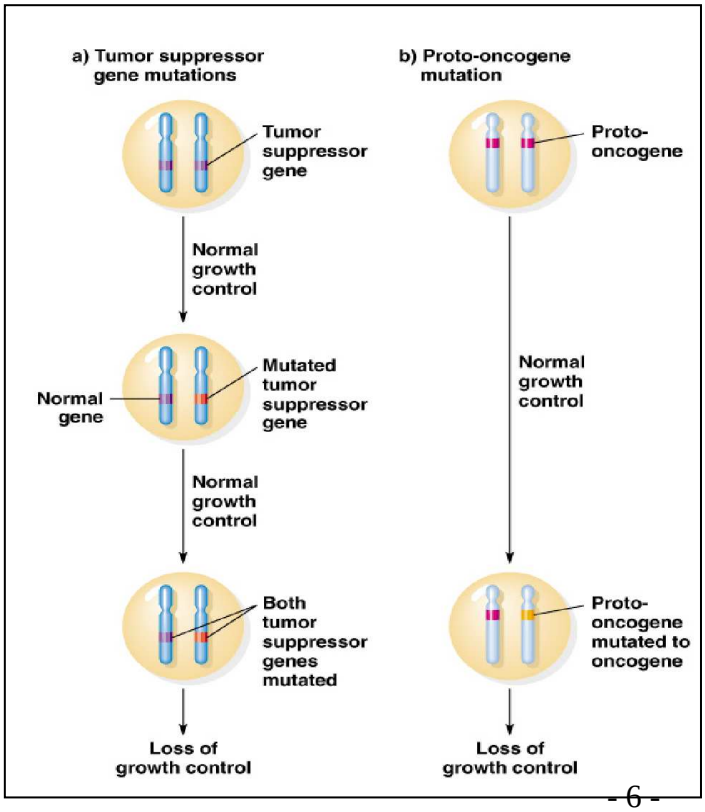

Figure 2: **Oncogenes and tumour suppressor genes.** a) Both tumor suppressor genes must be lost for unregulated growth to occur (recessive), b) while only one mutation is needed to change a proto-oncogene to an oncogene (dominant). Oncogenes and tumour suppressor genes are implicated in the development of cancer. (Picture taken from Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.)

Oncogenes are regulatory genes present in all normal cells and activation of oncogenes can contribute to the development of cancer. Activation can occur through mutations or gene amplification such that more of the protein encoded by the gene is present; hence, its function is enhanced (Osborne et al., 2004). An example of activation of oncogenes is that of the *HER-2* gene, which is identified to be amplified and over-expressed in 20-30% of primary breast cancer cases (Slamon et al., 1987). Her2/neu oncogene is a member of the erbB-like oncogene family. It is a membrane surface-bound receptor involved in signal transduction leading to cell growth and division. Her2 receptor is an orphan receptor and is only activated when a ligand bind to ErbB receptors and induce dimerisation with Her2. Upon ligand binding, a cascade of intracellular signals is activated which leads to cell growth and division (Fig.3a). The binding of growth inhibiting factors has the opposite effect of growth factors, contributing to inhibition of cell division and growth (Fig. 3b). Any deregulation of the gene encoding for Her2 results in abnormal cell division and growth, which may contribute to the development of cancer.

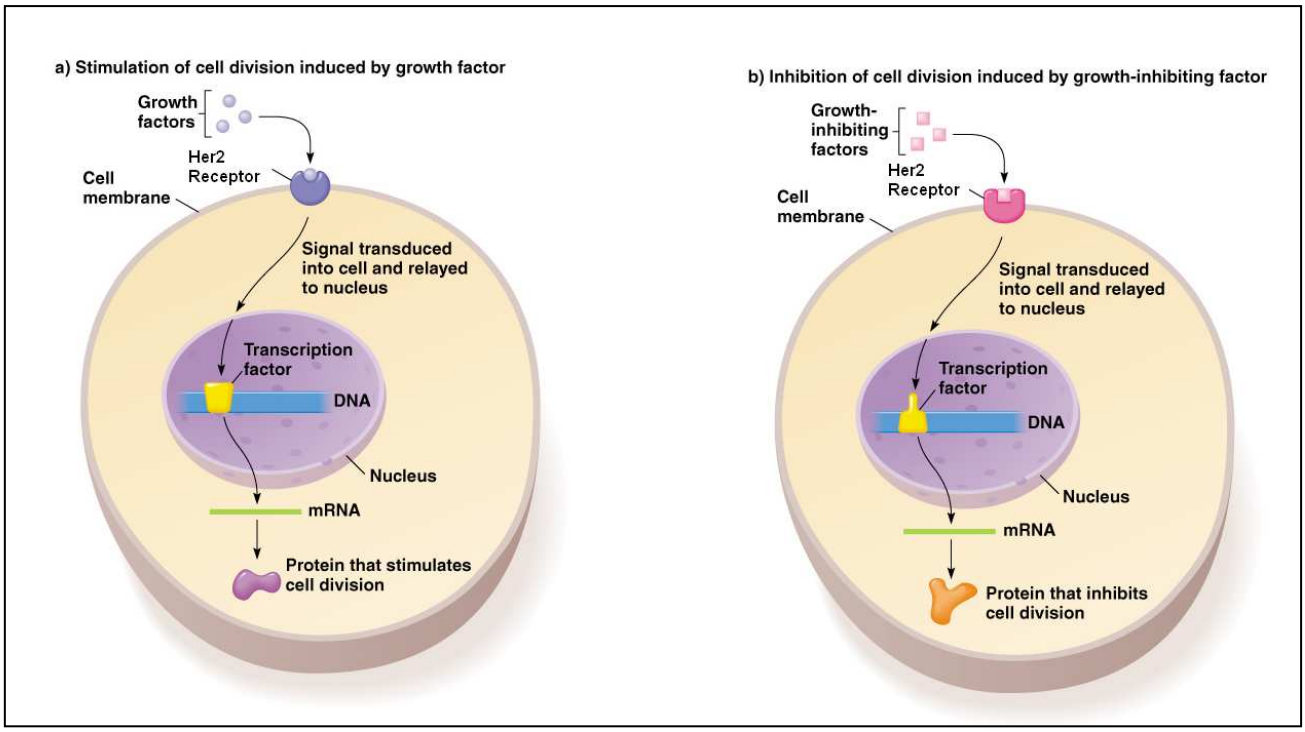

Figure 3: Regulation of cell division in normal cells via Her2 receptor. Upon ligand binding, the cell can either produce proteins that (a) stimulate cell division or (b) inhibit cell division, depending on the type of ligand binding to the receptor. (Modified after Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.)

Tumour suppressor genes usually follow the “two hit” hypothesis which implies that both alleles that code for a particular gene must be affected for a malignant phenotype to be established (Knudson et al., 1971). The most frequently mutated tumour suppressor gene in human cancer is *TP53* (tumour protein 53). *TP53* is involved in many processes, including DNA repair and apoptosis. In the regulation of DNA repair, *TP53* is stabilized in an unknown way. Upon radiation *TP53* is activated, which leads to activation of a signaling cascade resulting in G1 arrest (Fig. 4). In one way, *TP53* halts time so that the cell can repair any DNA damage. Its main task is to maintain stability and prevent mutations to occur in the cells. In breast cancer approximately 20%–30% of all cases involve mutations in this gene (Hollstein et al., 1991). Since p53 is a multifunctional protein and is involved in several types of cancer, it is regarded as a prognostic marker.

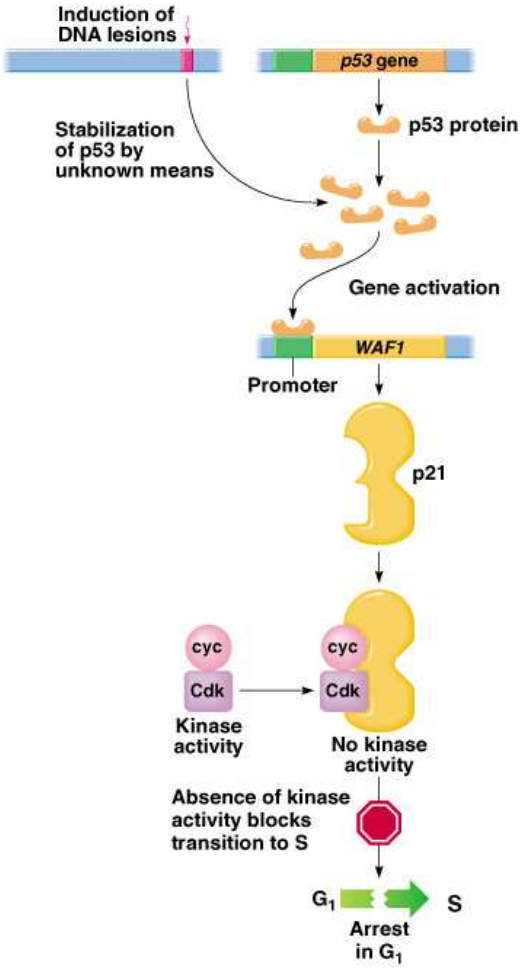

Figure 4: P53 involvement in DNA repair. Damage to cellular DNA (e.g., by irradiation) causes *p53* to initiate a signaling cascade leading to G1 arrest**.** (Picture taken from Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings)

1.1.2 Inherited Mutations and Breast Cancer

Some cancer types are inheritable and are mainly due to genetic changes in the germcells inherited from the parents. In breast cancer, there are two genes, *BRCA-1 and BRCA-2*, that are directly related to increased risk of breast cancer and any mutations in these genes inherited from the parents may lead to breast cancer. *BRCA-1* mutations have been estimated to account for >5% of all breast cancer cases in women below 40 years and increase significantly with increasing age. The risk of getting cancer in a young age (< 40 years) increases significantly in a family that has a history of breast cancer and ovarian cancer (Ford et al., 1995). Many mutations in the *BRCA-1* gene have been identified, including deletions, insertions and substitutions. The severity of the disease can be linked to the location of the mutation and mutations in some certain areas leads to higher proliferation rates in tumours (Sobol et al., 1996).

The *BRCA-2* gene is similar to *BRCA-1*, although its structure is dissimilar. In the *BRCA-2* gene, same as with the *BRCA-1* gene, many unique mutations have been identified causing truncation of the protein. The life-time risk for developing breast cancer is similar for women with mutations in *BRCA-2* as with *BRCA-1*, but mutations in *BRCA-2* also confers increased risk in other types of cancers including melanoma, gastric cancer, prostate cancer and biliary tree cancer (Breast Cancer Linkage Consortium). Therefore, patients with family histories suggestive of *BRCA-1* or *BRCA-2* mutations are usually recommended to do a pre-screening to assess the risk of getting breast cancer. Although many genetic factors can explain the risk of getting breast cancer, genetics only accounts for 5-10 % of all factors involved in the progression of breast cancer (Rebbeck, 2002) (Fig. 5).

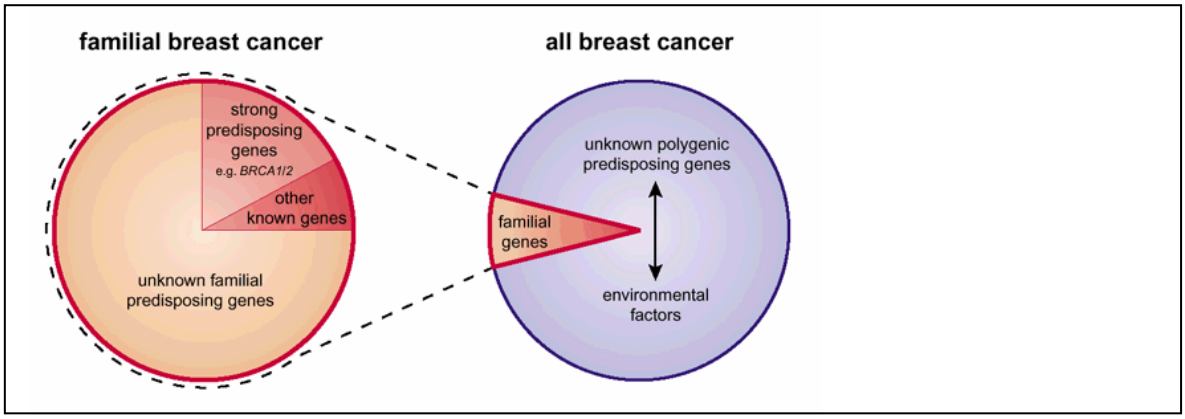

Figure 5: Breast Cancer Susceptibility Genes. Familial breast cancer (left) accounts for about ~10 % of the total breast cancer cases. Mutations in the high penetrance genes *BRCA1* and *BRCA2* account for about 20% of the familial risk. The low penetrance genes that contribute to the risk of developing sporadic breast cancer are still unknown. (Picture taken from Balmain et al., 2003)

1.1.3 Other Risk factors of Breast Cancer

A small percentage of the factors involved in breast cancer progression is due to genetic factors. Some other factors related to increased risk of breast cancer include gender, age, race and ethnicity and breast density (Table 1, right panel). Breast cancer, like other cancers, is an age-related disease meaning that the risk of getting cancer increases with increasing age and breast cancer is more frequent in women than men. Statistics verify that only ~1% of all breast cancer cases are observed in men, and the prognosis and treatment is the same in men as in women (Giordano et al., 2002). Female breast cancer incidence rates vary across racial and ethnic groups. African-American (AA) women get diagnosed with breast cancer at earlier age and experience a 15 % higher mortality than Caucasians-Americans (CA) (Newman et al., 2006). However, the incidence of breast cancer is now increasing in CA but stabilising in AA. The prevalence of several risk factors differs across racial and ethnic groups and these risk factors may contribute to higher breast cancer incidence rates in whites compared to others (Ghafoor et al., 2003).

Human breast tissue is composed of epithelial tissue, stromal tissue, and adipose tissue, of which the proportions vary widely between women. Looking at mammographic images of a breast, epithelial and stromal tissue appears as radio-dense, while adipose tissue appears as non-dense (Oza and Boyd, 1993). It has been suggested for a long time that women with increased mammographic density are at increased risk of getting breast cancer, and interestingly, exogenous hormones (estrogen-plus-progestin HRT), tamoxifen, or endogenous hormones (PRL) have shown to increase both mammographic density and breast cancer risk (Becker and Kaaks, 2009). It is therefore hard to establish mammographic density as a measure of risk without taking into account the effects of exogenous and/or endogenous hormones on breast tumour development.

Although some risk factors are determined from birth and are not reversible, there are also many factors associated with increased risk of breast cancer that are associated with life style choices (Table 1, left panel). Several risk factors including exogenous hormone use, relation to nulliparity, age at menarche, birth of first child, age at menopause, alcohol consumption, smoking, lack of physical activity and working night shifts appear to increase risk of breast cancer in women (McPherson et al., 2000).

Table 1: **Breast Cancer Risk Factors**

Risk factors you CAN change	Risk factors you CANNOT change
- Oral contraceptive use - Nulliparity - Post menopausal hormone therapy - Estrogen replacement therapy - Breast feeding - Alcohol - Obesity - Physical activity - Work at night - Smoking	- Gender - Age - Genetic factors (5-10%): BRCA1 and BRCA2 mutations + other genes - Race and ethnicity - Breast density

1.1.4 Benign Breast Disease

Much attention has been given to malignant lesions of the breast as it is the most common malignancy in women; however, the majority of lesion that occur in the breast are benign and are far more frequent than the malignant ones (Cole et al., 1978). The term "benign breast diseases" (BBDs) encompasses a heterogeneous group of lesions, usually subdivided into non-proliferative lesions, proliferative lesions without atypia, and atypical hyperplasia, with an increased risk of breast cancer associated with proliferative or atypical lesions (Fitzgibbons et al., 1998). As BBD is an important risk factor for developing breast cancer later in life, the identification of BBD has become more important and the understanding of the molecular mechanisms underlying these early changes may give some good insight into the first steps of cancer development. A retrospective study performed by Dupont et al. in 1994 identified fibroadenomas as a long-term risk factor for breast cancer with increased risk in women with complex fibroadenomas, proliferative disease, or a family history of breast cancer. Fibroadenoma is a benign breast tumour composed of epithelial and stromal components exhibiting a wide range of cytologic and histologic patterns and the increased risk of breast cancer persists for more than 20 years after the initial diagnosis of fibroadenoma (Dupont et al., 1994). As proliferative disease is an established risk factor for breast cancer, the molecular changes occurring at an early stage will be of great diagnostic and therapeutic values.

1.1.5 Biomarkers for early detection of Breast cancer

Early detection of cancer will be of great value as it gives a better prognosis for patients and less extensive treatment is needed. The society is less burdened when considering the economical benefits gained if cancer could be detected at an early stage. To detect cancer at an early stage, biomarkers that can separate tumour tissue from normal tissue must be

identified. A biomarker is a substance that indicates the risk of getting a particular disease and can be present in biological material. Biomarkers are being extensively studied to find out how they can be of more use in cancer screening. Several technologies have been developed to give a comprehensive understanding of cancer by characterizing tumours at the molecular level. Up till today, several breast cancer biomarkers have been analysed and identified (Levenson V.V., 2007, Review). However, although many show promising results, only mammographic screening and HER2/neu testing has been incorporated into standard practice. The value of current prognostic markers in predicting the course of the disease is weak and the molecular mechanisms leading to cancer progression is still poorly understood. Despite many efforts, there has been an overall deficit in identifying valuable prognostic biomarkers. This has however led to significant interest in the search for novel predictive biomarkers for breast cancer.

1.2 Micro-Ribonucleic Acid (miRNA)

Less than a decade ago, miRNAs were considered relatively unimportant. Today, miRNAs have been identified as important gene regulators that are involved in how and when genes are turned on and off. Suddenly, gene studies became less interesting while the things that influence their behavior gained more attention. The first miRNA, lin-4, was discovered in 1993 by Lee and his colleagues in *C. elegans* (Lee et al., 1993). Seven years later, a second small RNA was identified in *C. elegans*, named let-7 (Reinhardt et al., 2000). When the genes were cloned they were identified to code for two 21-22 nt long RNAs with an important role in developmental timing (Reinhardt et al., 2000). Let-7 was thereafter identified in several vertebrates, including humans.

MiRNAs are a recently discovered class of small nucleic acids (approximately 18-24 nt) that negatively regulate gene expression by binding to the 3'-untranslated regions (3'-UTR) of specific mRNAs. They have been identified in plants and animals and are highly conserved (Lewis et al., 2005). Up to date, more than 700 miRNAs are identified in human (Griffiths-Jones et al., 2008). The name of miRNAs found in the miR database includes 3 or 4 letters that designates the species (e.g., "hsa" for *Homo sapiens*), while the core of the miRNA name is the designation "miR" (denoting a mature sequence) followed by a unique identifying number.

MiRNA regulate mRNA by targeting them at the post-transcriptional level and leads us to believe that miRNA expression profiles can be used to define a biological profile better than gene expression analysis as it represent the transcriptional program better. Elucidating the mechanism of miRNAs is still at its beginning, but these molecules have shown to play important regulatory role in a range of biological and pathological processes (Heneghan et al., 2010). MiRNA profiles are highly informative as they reflect the developmental lineage and differentiation state of tumours (Lu et al., 2005). A study performed by Lu et al. (2005) has identified miRNAs that are significantly differentially expressed between tumour tissue and normal tissue. This study has shown that the miRNA profile is more reliable in classifying tumours compared to the mRNA profile (Lu et al.,2005). Up to date, several miRNAs have been implicated in tumorigenesis and many of them are thought to behave as tumour suppressors or oncogenes and are either up- or down-regulated (Zhang et al., 2007). Furthermore, altered miRNA profiles have been found in a variety of cancers indicating their significant role in cancer development.

1.2.1. MiRNA Biogenesis

The miRNA biogenesis is a multistep complex procees in humans that has only recently been elucidated. MiRNA genes are located mainly within introns of protein-coding and non-coding sequences, and also in intergenic regions (Rodrigues et al., 2004, Zeng et al., 2006). Some miRNAs are also identified in DNA repetitive sequences, including LINE-2 transposable elements (Smallheiser et al., 2005). MiRNAs are transcribed by RNA polymerase II in the nucleus producing primary transcripts, called “pri-miRNA”. From a long primary miRNA transcript to a short mature miRNA, the RNA must go through a series of processing steps (reviewed by Kim, 2005) (Fig.6). The miRNA within the pri-miRNA structure forms a stem-loop hairpin structure, which is excised by RNase III endonucelase Drosha to form a 65-75 bp long precursors named pre-miRNA (Lee et al., 2003). Drosha forms a complex with the double stranded RNA-binding protein DGCR8/Pasha in order to be able to process pri-miRNA to pre-miRNA (Gregory et al., 2004). Pre-miRNA is exported out of the nucleus to the cytoplasm by Ran-GTP-dependent Exporting -5 complex and the transcript is processed further to ~22 bp long RNA-hairpin duplexes by RNase III endonuclease Dicer (Kolb et al., 2005). Dicer removes the loop of pre-miRNA to produce a duplex made up of the mature miRNA sequence and a fragment of similar size (miRNA*) (Bartels and Tsongalis, 2009).

Finally, the RNA strand with the least stable 5'- end is chosen to form a complex with the RNA- induced silencing complex (RISC) while the other strand, miRNA*, is degraded. Argonaute (Ago) proteins are crucial components of the RISC complex that direct both short interfering (siRNA)- and miRNA-mediated gene regulation. There are four Ago proteins identified in humans (Ago1-Ago4) that are associated with miRNA (Meister et al., 2004).

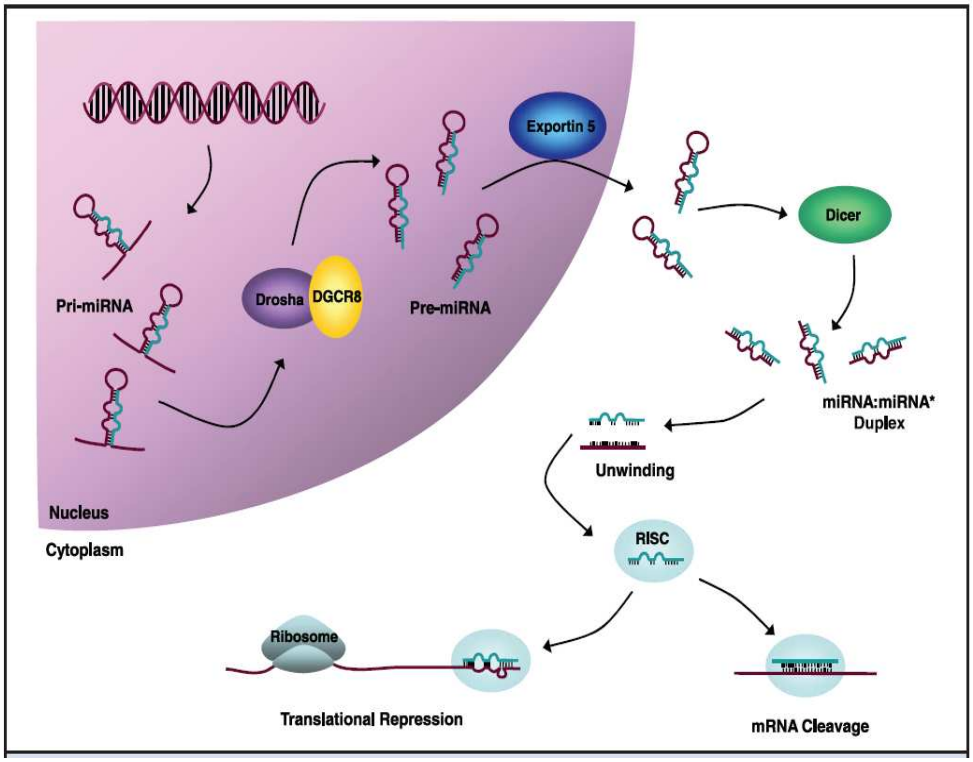

Figure 6: **MiRNA biogenesis and mode of action.** MiRNA biogenesis occurs in several steps. The primary transcript (pri-miRNA) is processed in the nucleus by Drosha to produce pre-miRNA which is exported to the cytoplasm of the cell. Splicing of pre-miRNA by DICER in the cytoplasm, results in binding of miRNA to the RISC complex, which aid binding of miRNA to mRNA transcripts. (Figure from Bartels and Tsongalis (2009))

1.2.2. MiRNA as Gene Regulators

In mammals, the mature miRNA negatively regulates target mRNA by either binding to imperfect complementary sites within the 3'- UTR of mRNA-targets, or by targeting specific cleavage of homologous mRNAs. However, studies have indicated that miRNAs can also downregulate the expression of genes by base-pairing to the coding region or 5'-UTR of some mRNAs (Forman et al., 2008; Lytle et al., 2007). Perfect or almost perfect complementarity to the target site can induce gene silencing via the RNA interference pathway. By binding to imperfect complementary sites on the mRNA-target strand, miRNAs inhibit translation and reduce protein levels of target genes without affecting mRNA levels of these genes (Dennis

C., 2000). This makes gene regulation more flexible as the opportunity to reactivate the gene is present. However, through complementary pairing of miRNA and ssRNA, the mRNA is targeted for degradation by RISC. Several Studies have suggested that the 5'end of the miRNA is important for biological functioning and is vital for the stability and loading of miRNA into the RISC complex (Reviewed by Esquela-Kerscher and Slack, 2006). To find complementary target sites at the 3'UTR of expressed genes targeted by miRNA, bioinformatics use a "miRNA seed" comprising of 2-8 bases of the mature miRNA. However, research has shown that other variables than the "miRNA seed" dictate miRNA regulation (Lewis et al., 2005).Each miRNA is thought to regulate several hundred genes and they have shown to be important in regulating many processes such as development, cell proliferation, apoptosis, fat metabolism, muscle differentiation and stem cell division (Mattick and Makunin, 2006).

1.2.3. MiRNA and Cancer

MiRNAs are involved in many important processes of the cell and it is therefore not surprising that the interest for miRNAs has grown within cancer research environments. The processes regulated by miRNA are commonly deregulated in cancer, implicating miRNA in carcinogenesis. miRNAs affect different pathways in the cells by having either a tumour suppressor function or oncogenic function. Tumour suppressor miRNA inhibit cell proliferation and stimulate apoptosis by targeting oncogenes, while oncogenic miRNAs stimulate cell proliferation and inhibit apoptosis by targeting tumour suppressor genes (Zhang et al., 2007). The first evidence of involvement of miRNAs in malignancy came from identification of a translocation-induced deletion in miRNA miR-15a and miR-16-1 at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia (BCLL) (Calin et al, 2002). When a cells genome is exposed to deletions or duplications, this will also affect the miRNA coding regions. miRNAs have also been identified in frequently cancer-associated genomic regions, or fragile sites, reinforcing miRNAs role in cancer (Calin et al., 2002). For example, miR-125b, which is underexpressed in cancer, is located at chromosome 11q23-24. This is a region that is frequently deleted in breast cancer, ovarian cancer, and lung cancer (Negrini et al, 1995). This has given new insights within cancer diagnostics and it has been shown that specific miRNAs are related to specific cancer types and diagnosis (Tomaru and Hayashizaki, 2006). In breast cancer, miR-10b, miR-125b, miR-145, miR-21and miR-155 are the most consistently deregulated miRNAs. MiR-21 and miR-155 are up-regulated and the remaining three are down-regulated. miR-21 together with miRNAs from the let-7 family are strong

tumorigenic miRNAs as they have been identified to be deregulated in several types of cancers, including breast cancer (Reviewed by Esquela-Kerscher and Slack, 2006). MiRNA expression studies in cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field.

1.2.4 MiRNAs as novel therapeutic targets

The importance of miRNAs in carcinogenesis illustrates the possibility of using miRNAs as targets of therapeutic interventions to block tumour progression. The way to do this is either by either antagonizing or restoring miRNAs through RNA nucleotides that are stable in serum and can be readily taken up by cells. Oligonucleotides that are complementary to mature miRNAs are called anti-miRNA oligonucleotides (AMOs). By binding to miRNAs they might inhibit or inactivate oncogenic miRNAs in tumours. So far, inactivation of miRNA has been accomplished by continuous delivery of 2'-O-methyl or locked nucleic acid antisense oligonucleotides in order to target oncomirs such as miR-21. A study by Si and colleagues identified the importance of AMO in cancer by transfecting breast cancer MCF-7 cells with anti-miR-21 oligonucleotides. It was found that anti-miR-21 suppressed both cell growth in vitro and tumour growth in xenograft mouse model (Si et al., 2007).

The induction of tumour suppressor miRNA however could be done using viral or liposomal delivery of tissue specific tumour suppressors to prevent progression of tumours. The induction of tumour suppressor miRNAs is however influenced by epigenetics. A study by Saito and colleagues (2006) showed that treatment with chromatin modifying drugs led to upregulation of certain miRNAs in cancer cells but not in normal cells. Amongst them was miR-127 which was downregulated in cancer cells, but was significantly upregulated after treatment. MiR-127 showed to downregulate *BCL6*, which is a protooncogene. This suggests that DNA demethylation and histone deacetylase inhibition can activate expression of miRNAs that may act as tumour suppressors (Saito et al., 2006).
The only obstacle with antisense miRNA therapy so far has been the identification of oligonucleotides that are capable of using the RNAi pathway for delivery of gene therapy and there is a restricted number of cells that can be targeted. However, studies show promising results that miRNAs are valuable as therapeutic targets.

1.3 MiRNA Microarray Technology

Global gene expression analysis based on microarray technology has facilitated a new molecular taxonomy for classification of breast cancer (Perou et al Nature 2000). Through the use of microarray technology the existence of several molecular subtypes of breast cancer has been identified, distinguished by pervasive differences in gene expression pattern (Perou et al., 2000; Sotiriou et el., 2003). Gene-expression profiling study led to the development of a molecular classification of breast cancer that comprised several molecular subtypes; basal-like, HER2, normal breast-like and luminal (Perou et al., 2000). In particular for diagnosis of breast cancer, gene signatures have been reported that allow stratification of patients in, for example, a good and a poor prognosis group (ven't Veer et al.,2001). Such gene signatures, however, have not yet entered clinical practice, which suggests that the need for even better and more accurate molecular tumor markers is needed. The unique structure and size of miRNAs has made it necessary to identify a technique that facilitates their analysis. Previously, miRNA expression profiling have been used to distinguish tumour tissue from adjacent normal tissue (Iorio et al., 2005), predict outcome of cancer patients (Lu et al., 2004), and successfully classify breast cancer into prognostic molecular subtypes (Blenkiron et al., 2007; Enerly,2009 submitted, Takamizave et al., 2004).

To study the expression of miRNAs in breast tissue, several methods can be applied. MiRNAs are involved in many different biological processes in humans and to understand how miRNAs are functioning, it is crucial to determine when and where they are expressed. Microarray technology has been extensively used by researchers to gain more insight into fundamental aspects of growth and development and to identify the underlying causes of several human diseases. Microarray allows for the identification of a large number of genes, or miRNAs, in a quick and efficient manner, and is useful when the amount of sample to be studied is small. Microarray technology has been modified to facilitate miRNA expression profiling. DNA oligonucleotide microarrays are used for mRNA profiling, and in order to study the miRNA expression in tissue samples, the DNA microarray has been adapted to miRNAs. In contrast to mRNA profiling, miRNA profiling requires to distinguish between mature miRNA, precursor miRNA and miRNAs that differ with as little as one nucleotide (Shingara et al., 2005).

MiRNA microarray technology is mainly based on hybridization between target molecules and their corresponding complementary probes. Designing a miRNA array requires the

construction of complementary probes that are attached to the array. These probes are either synthetic oligonucleotides or cDNA fragments and are made based on previously identified and verified mature miRNAs. The glass slides used for microarrays are amine-reactive glass and the probes are amine-modified miRNA probes consisting of two parts: a “linker” sequence and a “capture” sequence. The “linker” sequence consists of poly(dT) or poly(dA) with an amine-modified terminus, while the “capture” sequence is usually complementary to the miRNA molecules (Li & Kangcheng, 2009) (Fig.7A). The length of the probes is determined by the melting temperature (T_m) of the probes. If the hybridization is set at a certain temperature, the probes with Tm lower than that temperature will give a lower binding efficiency while probes with a higher Tm will have increased unspecific binding, resulting in disturbance in fluorescent signals. Tm normalization is therefore required. Studies have found out that normalization of Tm is achieved through adjusting probe length (Li & Kangcheng, 2009). The G-C interactions of mature miRNAs have a Tm > 55 ℃ during hybridisation. For those miRNAs that have a Tm > 57,5 ℃, the length of the complementary probes are reduced so that these miRNAs also hybridize at 55 ℃ without affecting specificity (Wang et al., 2007) (Fig.7B)

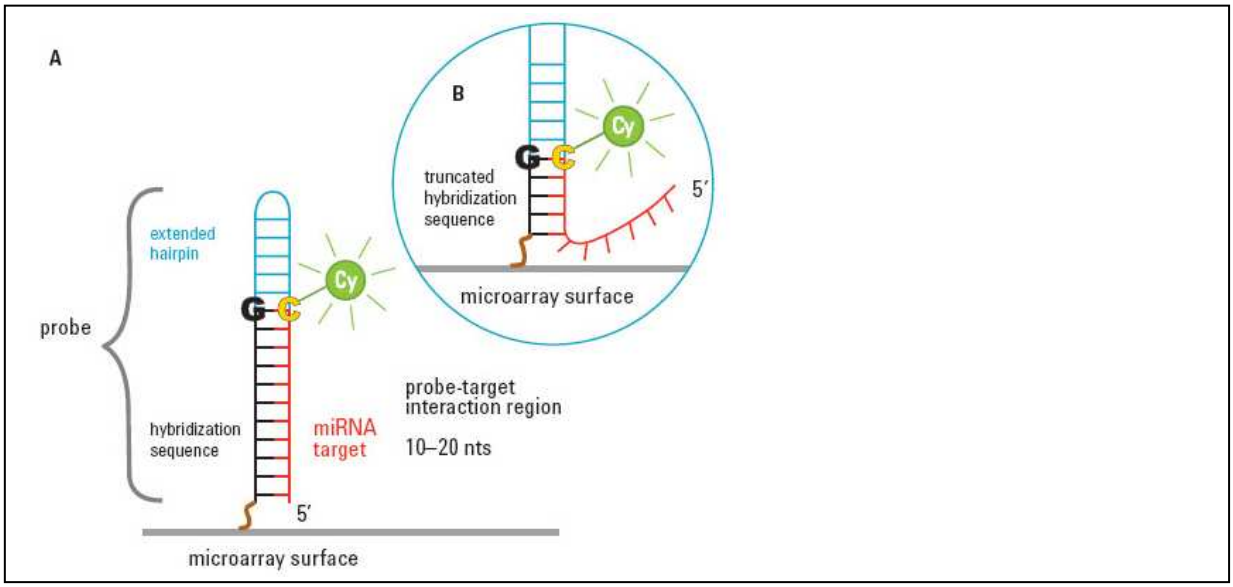

Figure 7: miRNA binding to microarrays. A microarray probe (black) hybridizes to target miRNA (red). A) Inclusion of a G residue (black) to the 5' end complements the 3' end C residue (yellow) introduced in labeling. The G-C pair in the probe-target interaction region stabilizes targeted miRNAs. All probes contain a 5' hairpin (blue), abutting the probe-target region, to increase target and size miRNA specificity. B) Destabilization of probes that are too stable. For probes requiring it, reduction of probe-target base-pairing is achieved through sequential elimination of base pairing from the 5' end of the miRNA. (Picture from www.agilent.com)

Labeling of miRNAs is a challenging task as the miRNA molecules are very small and are similar in sequence. Two main labelling techniques have been developed: 1) direct labelling, in which miRNA molecules are directly conjugated with fluorescent dye; 2) indirect labelling, in which the reverse transcript of miRNA or the in vitro transcript of miRNA are labelled instead of the miRNA molecule itself (Li & Kangcheng, 2009). The miRNA molecules are labeled with fluorescent dye, such as cyanine (pCp-Cy3), and then hybridized with the miRNA microarray, resulting in specific complementary binding of the labelled miRNAs to the corresponding probes. There are several ways in which labelling can occur, but in this section only labelling through T4 RNA ligase will be described, as this is the method used in this study and is preferred by Agilent Technologies. The enzyme T4 RNA ligase catalyzes the formation of phosphodiester bindings and is used to label the 3'-end of RNA. Cy3 is attached to 3'phosphate on 3',5'cytosine bisphophate. T4 RNA ligase adds a fluorescence-modified (di) nucleotide (pCp-Cy3) onto the 3′ end of microRNA (Wang et al., 2007). Hybridisation of miRNA with the probe occurs through attachment of guanine, G, to the 5'-end of each probe sequence that is complementary to cytosine, C, that is attached to the 3'end of miRNA during labelling.

Up to date, there are several commercial miRNAs microarrays manufactured with different design strategies. Some arrays are hybridized with samples labelled with two different fluorescent colours (Fig. 8A), mainly Cy3 (green) and Cy5 (red), while others use single-colour array format with only one sample hybridized per array (Fig. 8B). The labelled samples fluoresce at certain wavelengths and will give the spots on the arrays a red or green colour, depending on the labelling, after scanning. Agilent Technologies has developed a single-colour array format with a highly efficient labelling method and novel probe design. The arrays have little sequence bias, small amount of sample is needed, and provides both sequence and size discrimination for mature miRNAs (Wang et al., 2007). In this study, Agilent Technologies miRNA microarrays were used. The format of the array is 8x15K and consists of 8 arrays with each containing ~15000 probes. These 15000 probes are divided between 866 human miRNA and 89 viral miRNA, meaning that each spot is duplicated 20-40 times. The probes are 40–60-mer oligonucleotide synthesized directly on the array (*in situ*). First, a 10-mer nucleotide is attached to the array in which the probe gets attached to base by base through ink-jet printing until it becomes a 40-60-mer nucleotide (www.agilent.com).

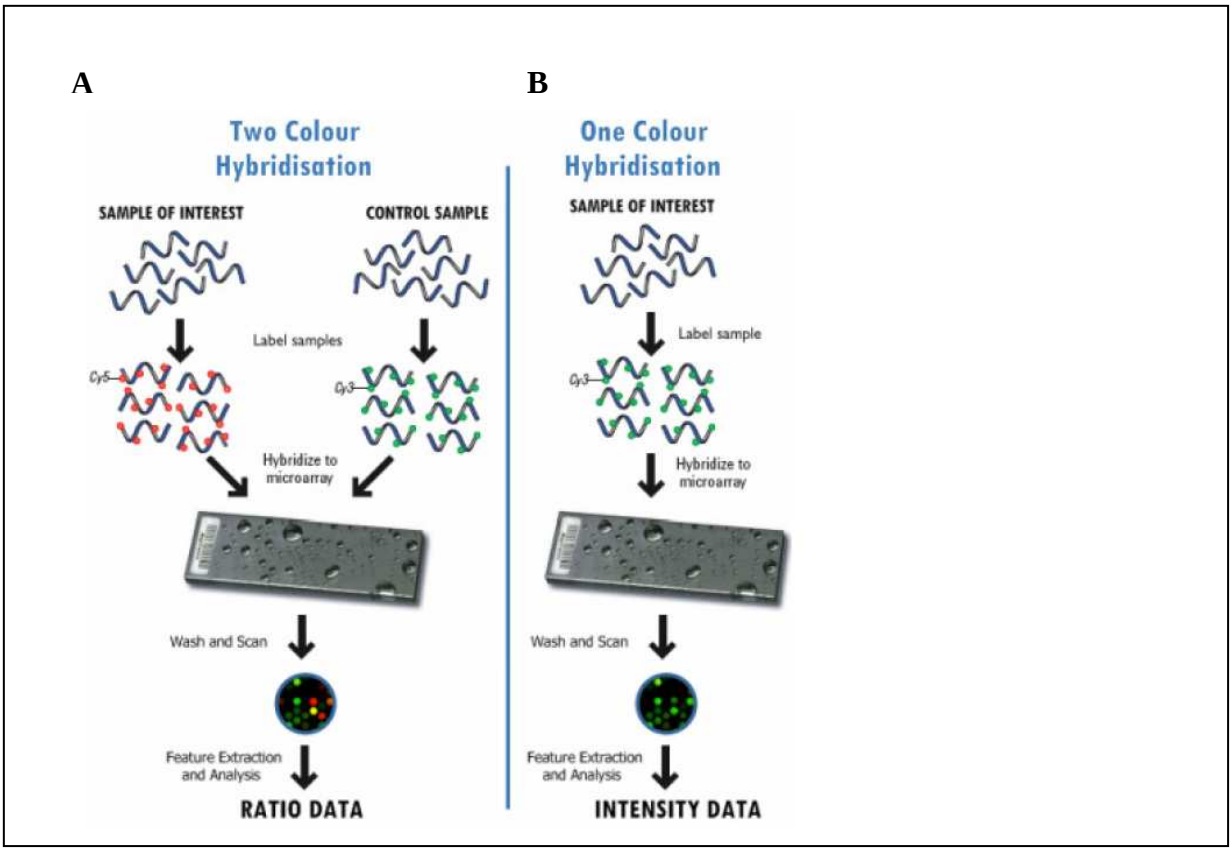

Figure 8: Two-colour vs One-colour Microarray. A) In a two-colour experiment, DNA/mRNA/miRNA from two samples is extracted and labelled with two different fluorophores (e.g., Cy3 and Cy5). Labelled samples are hybridized to the microarray and scanned to visualize fluorescence. Over the entire microarray, the signal ratios at each feature are calculated and used in ratio-based analysis to identify up-regulated and down-regulated genes. B) In one-coulor experiments samples are only labeled with one fluorophore (such as Cy3). The arrays provide intensity data for each probe or probe set indicating a relative level of hybridization with the labeled target. (Picture taken from Passos et al.,2009)

Chapter 2

Aim of the study

Although global expression analysis has been extensively used for taxonomy or prognostication, it has revealed little of the original deregulation leading to malignant growth as the studied specimens provide a molecular snapshot at the time of diagnosis. The extensive search for novel biomarkers has identified miRNAs as ideal biomarkers due to their inherent stability and flexibility. With this in mind we studied the miRNA expression in benign tumours, mainly fibroadenomas and fibroadenomatosis, compared to malignant tumours and normal tissue using miRNA microarrays.
Given that women with fibroadenomas or benign breast disease are at increased risk of developing breast cancer, we hypothesize that molecular profiles in these changes may reflect early changes in regulation leading towards proliferation and malignancy.

The aim of the study is to identify molecular profiles for benign tumours that in the future may help to detect women with benign breast disease at higher risk of developing malignant tumours.

Chapter 3

Materials & Methods

3.1 Samples

All tumour tissue specimens, biopsies and associated clinical data were collected at Akershus University Hospital (Lørenskog, Norway), while all normal tissue samples were collected at the Colloseum Clinic (Oslo, Norway). All samples were collected between years 2003-2009 and all cases have been histologically confirmed by a pathologist. In total, 62 samples were used in this study (Table 2). 29 normal tissue samples from women that have undergone reduction mammoplasty, 13 malignant tumour tissue samples from women operated for breast cancer and 20 core needle biopsies from women who have presented with suspicion for breast cancer but not diagnosed with the disease. The biopsies represented fibroadenomas and fibroadenomatosis which are regarded to be benign tumours. Tissue samples and biopsies were maintained in RNAlater and stored at -80°C.

Table 2: Samples used in this study

Normal Tissue (RP)	Benign tumours (CMB)	Malignant tumours (CM)
- 29 Breast reduction plastic tissue samples	- 13 Fibroadenomas - 7 Fibroadenomatosis	- 13 Breast cancer tissue samples

3.2 RNA extraction

Total RNA, including miRNA, from tissues and biopsies was isolated by TRIzol (Invitrogen) in combination with RNeasy Mini Kit from Qiagen. The method combines phenol/guanidine-based lysis and siliconmembrane-based purification of total RNA (Wei et al.,2002) . Briefly, 5-50 mg of biopsies and ~100 mg of malignant- and normal tissue was homogenised in 1-2 mL TRIZOL (Invitrogen) using a bench-top homogenizer (Mixer Mill MM 301). By adding chloroform, the homogenate was separated into three phases. The upper phase of clear liquid

contained RNA and was used for further extraction of RNA. The addition of ethanol optimizes the binding conditions of all RNA molecules above 18 nucleotides. All the samples were run through an RNeasy Mini spin column where total RNA binds to the column membrane while phenol and other impurities are washed away. Contaminating DNA was removed by on-column Dnase digestion. RNA was eluated from the column using 30 µl of RNase-free water. A small volume of the purified RNA was used for quantitative and qualitative analysis.

3.3 RNA analysis with NanoDrop and Bioanalyzer

Quantification and purity of total RNA was assessed by using NanoDrop1000® spectrophotometer (Supplementary table 1). RNA absorbs at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity. Pure RNA has an A260/A280 value of 1.8-2.0, depending on how the measurement is performed and the source of putative contaminants. The quality of each sample was controlled on the Agilent 2100 bioanalyzer to assure that RNA was not degraded and that miRNAs were present in the sample. Agilent 2100 bioanalyzer uses labchip technology to integrate sample preparation, detection, quantification and data analaysis. The anlaysis of an intact total RNA sample is shown in figure 8 A and is regarded as a good sample. The 18S and 28S ribosomal RNA (rRNA) peaks are identified by the Agilent 2100 bioanalyzer. The first peak, which indicates the amount of small RNA in the sample, can include 5.8S and 5S ribosomal peaks and tRNA, depending on the protocol. Figure 8 B shows a sample that contains degraded RNA. RNase degradation of total RNA results in a shift in RNA size distribution toward smaller RNA products and the 18S and 28S peak is no longer identifiable. Only samples with good quality as in fig. 8 A were used for further analysis in this study. The integrity of total RNA was also assessed using the RNA 6000 Nano Kit assay (Agilent Technologies). The RNA integrity number (RIN) obtained for each sample is based on the ratio of ribosomal bands and the presence/absence of degradation products. Only RNA extracts with RIN values >6 were included in further analysis (Suppl.table 1). The presence of small RNAs (miRNAs) was analysed using the Small RNA Assay with the Agilent 2100 Bionanalyzer.

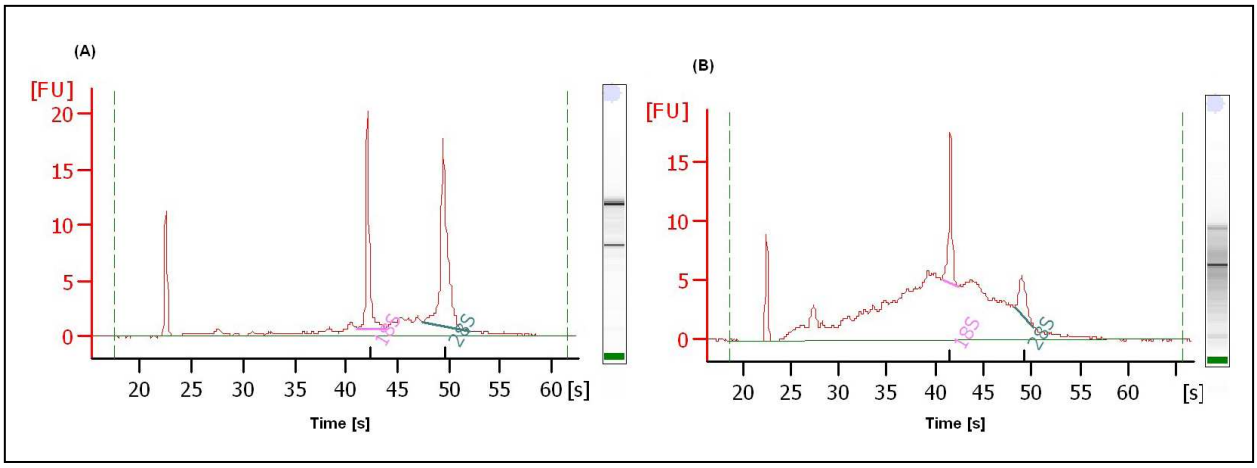

Figure 8:Total RNA quality analysed with Agilent 2100 Bioanalyzer. A) Good quality of total RNA using Agilent 2100 bioanalyzer. B) Degradation of total RNA. Samples were analysed using RNA 6000 nano Kit assay. Agilent 2100 bioanalyzer provides an electropherogram (left) and a gel-like image (right).

3.4 MiRNA microarrays

MiRNA profiling from total RNA was performed using Agilent Technologies "Human miRNA Microarray Kit (V3)". The microarray contained probes for 866 human and 89 human viral microRNAs from the Sanger database v12.0. Sample labeling, hybridisation and scanning were performed according to manufacturer's protocol. Briefly, all samples were diluted to 25 ng/ul of total RNA and treated with calf intestine alkaline phosphatase for 30 minutes at 37°C before labeling. Samples (7 µl) were diluted with 5 µl of DMSO, denatured for 10 minutes at 100°C and labeled in a total volume of 20 µl at 16°C for 2 hours using pCp-Cy3 and T4 RNA ligation buffer supplied in the kit (Agilent). After purification with Bio-Rad MicroBio-Spin 6 columns, 45 ng/ul of labeled and purified RNA was used for hybridization on microarray slides. The microarray option on the NanoDrop spectrophotometer was used to measure the optical density of the purified samples at 550 nm allowing us to asses the labeling efficiency of Cy3 (Suppl. Table 1). The slides were hybridized at 55°C for at least 20 hours in an Agilent SureHyb chamber rotated at 20 rpm. The arrays were washed with Gene Expression Wash Buffer (Agilent) at room temperature and 37°C before scanning with an Agilent microarray scanner. Processed slides were scanned using Agilent scanner G2505C. The microarray scanner visualizes fluorescence of the fluorophores after excitation with a laser beam at wavelengths that fluoresce green light. For each spot, the levels of intensity are measured and are used to identify the relative expression of each probe on the array. Features and local background were detected and analysed with Agilent Feature Extraction (FE) Software version 10.7.3.1.

3.5 Statistical Analysis

Unsupervised hierarchical clustering, differentially expressed miRNAs among the histological groups and significance analyses of microarrays (SAM) were preformed using J-express 2009. After filtration of missing values and normalization of the samples using J-express 2009, we were left with 342 miRNAs (probes) that were used for further analysis. Parameters used for filtration of probes are listed in the table below (Table 3). Hierarchical Clustering was performed on samples and miRNAs using Euclidean correlation as a distance measure. Correspondence analysis (CA) was performed using J-express 2009. This type of analysis is useful in the study of associations between variables. CA displays a low-dimensional projection of the data into a plane for two variables simultaneously, thus revealing associations between them.

Table 3: Filtration Parameters used for miRNA Microarray Analysis

Parameters	Threshold	Function
gIsFeatNonUnifOL	1.0	Boolean flag indicates if a feature is non-uniform. Non-uniform means when the pixel noise of a feature exceeds threshold established for a "uniform" feature
gIsBGNonUnifOL	1.0	Same as above, but for background. Indicates whether local background is a non-uniform outlier or not.
gIsSaturated	1.0	Boolean flag indicates if a feature is saturated or not. A feature is satured if 50% of the pixels in a feature are above saturation threshold.
gIsFeatPopnOL	1.0	Boolean flag indicates if a feature is a population outlier or not. Probes with more than one feature are examined using statistics. Feature is a population outlier if its signal is less lower threshold or above upper threshold determined by statistical measures.
gIsBGPopnOL	1.0	Same as above, but for background. Indicative of whether local background is a population outlier
gIsWellAboveBG	0.0	Boolean flag indicates whether a feature is well above background or not.

For supervised analysis, SAM was used (Tusher et al., 2001). The signal intensity of each probe was log2 transformed and for probes without a signal, a subsidised value was calculated by the program. The false discovery rate is the proportion of miRNAs claimed to be differentially expressed that are expected to be false positives. Therefore, in this study, only miRNAs with false discovery rate (FDR) = 0.0 and q-value = 0.0 were chosen for further analysis. The differentially expressed miRNAs were selected and hierarchical clustering was carried out on samples and miRNAs using Euclidean correlation as a distance measure. 139 probes (81 miRNAs) were included in the clustering and used for further analyses.

Chemicals and reagents used for the experimental procedures are listed in Table 4.

Table 4: Chemical and Reagents used for experimental procedures

Experimental Procedure	Chemical / kit	Catalogue no.	Producer
RNA extraction	TriZol / TriReagent	T9424-100ML	Sigma-Aldrich PO Box 14508 St. Louis, MO 63178 UNITED STATES
	Chloroform	C2432	Sigma-Aldrich PO Box 14508 St. Louis, MO 63178 UNITED STATES
	Ethanol 99%	459844	Sigma-Aldrich PO Box 14508 St. Louis, MO 63178 United States
	RNeasy Mini Kit (250)	74106	Qiagen, Hilden, Germany
	RNase-Free, DNase set	79254	Qiagen, Hilden, Germany
RNA quality analysis	RNA 6000 Nano Chip Kit		Agilent Technologies 5301 Stevens Creek Blvd Santa Clara CA 95051 United States
	Small RNA kit	5067-1548	Agilent Technologies 5301 Stevens Creek Blvd Santa Clara CA 95051 United States
miRNA microarray	miRNA labeling Reagent and hybridization kit	5190-0408	Agilent Technologies 5301 Stevens Creek Blvd Santa Clara CA 95051 United States
	Calf intestine Alkaline phosphatase and 10X Alkaline phosphatase (CIP) Buffer	2250A	TaKaRa Bio Inc. Seta 3-4-1, Otsu, Shiga, 520-2193, Japan
	T4 RNA ligase 5U/µl	AM2140	Applied Biosystems/ Ambion 2130 Woodward St. Austin, TX 78744-1832 USA
	Micro Bio-Spin 6 Columns	732-6221	BioRad Laboratories 1000 Alfred Nobel Drive Hercules, CA 94547
	Dimethyl Sulfoxide (DMSO) ≥ 99.9%	D8779	Sigma-Aldrich PO Box 14508 St. Louis, MO 63178 UNITED STATES
	Human miRNA V3 Oligo Microarray Kit	G4470C	Agilent Technologies 5301 Stevens Creek Blvd Santa Clara CA 95051 United States
	Gene expression Wash Buffer Kit	5188-5327	Agilent Technologies 5301 Stevens Creek Blvd Santa Clara CA 95051 United States

Chapter 4

Results

MiRNAs regulate mRNA expression in animals and plants by targeting mRNAs for either cleavage or translational repression. They have various expression patterns and are involved in various developmental and physiological processes. Profiling miRNA expression is very helpful for studying their biological functions. The most commonly used high-throughput technique for genome-wide assessment of miRNA expression levels when dealing with a high number of samples is represented by miRNA microarrays (Liu et al., 2004). In this study, miRNA microarray technology was applied to identify miRNA profiles in malignant tumours, benign tumours and normal tissue as described previously in materials and methods. The miRNA microarray contains probes for 866 human and 89 human viral microRNAs from the Sanger database v12.0.

4.1 Quality Control of Microarrays

Data extracted from the microarrays were analysed using Feature extraction (FE). FE is a software that flags out bad features on the microarray such as artefacts (scratches on the slide), features with low intensity compared to the background and saturated features. FE will normalize all data to exclude any systematic errors and an array is regarded as bad if >10% of the features on the array are bad. FE generates a quality report (QC report) for each sample on the array (Fig.9). The QC report consists of different feature statistics that helps us to assess the performance of the microarray system.

First, features in the four corners of the microarray have to be identified if they are located properly. If the spots are off-centre in one or more corners, the extraction must be run again with a new grid. If the QC report shows a greater number of non- uniform or population outliers than expected, there might be something wrong with the hybridization/wash step. The spatial distribution of all outliers on the array shows both population and non-uniformity outliers, whose positions are distributed across the microarray. The plot allows us to distinguish the background population and non-uniform outliers from one another through colour coding. The histogram of signals plot shows the level of signal and the shape of the

signal distribution. The histogram is a line plot of the number of points in the intensity bins vs. the log of the processed signal.

MiRNA microarrays are designed with replicated non-control probes, meaning that multiple features on the microarray contain the same probe sequence. The QC report uses these probes to evaluate reproducibility of the signals and the log ratios. If a non- control probe has a minimum number of inliers, a %CV (percent coefficient of variation) of the background-corrected signal is calculated for each channel (average of signals). This calculation is done for each replicated probe, and the median of those %CV's is reported in the table for each channel. A low median %CV value indicates better reproducibility of signal across the microarray. In our study, only samples with a %CV below 10 have been chosen for further analysis.

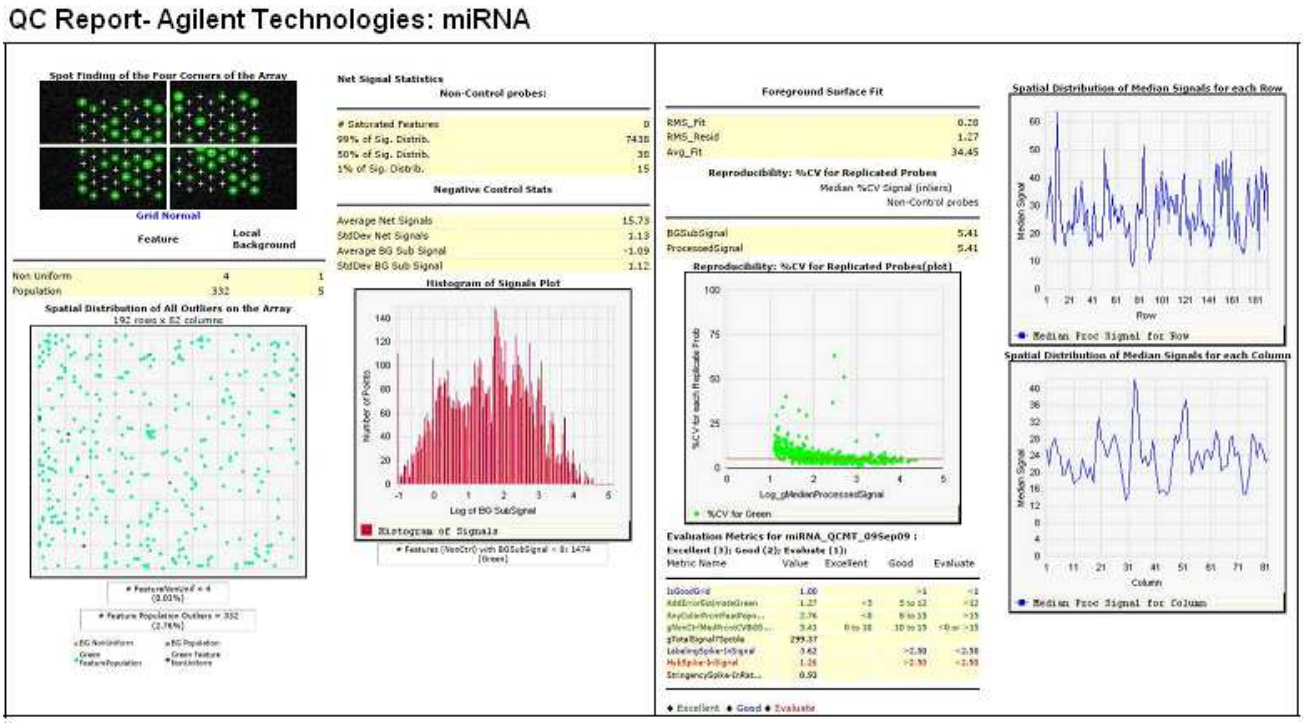

Figure 9: Example of a QC report of 8 X 15 K microarray generated by Feature extraction Software. The report is helpful in the assessment of whether the array is of good quality or not**.**

4.2 Sample Classification using Unsupervised Hierarchical Clustering And Correspondence Analysis

To analyze miRNA expression in the three different tissue types, the software J-express was used. After filtration of missing miRNA probes and normalization, we ended up with 342 miRNAs (probes) that were considered for further analyses. Unsupervised hierarchical clustering of all 342 miRNAs using Eucledian correlation as a distance measure separated the three histological groups into 3 major clusters based on their miRNA profile, with a few exceptions (Fig.10). One cluster consisted of mainly malignant tumour samples, the second cluster consisting of mainly benign tumours and a third cluster consisting of mainly normal tissue. The first cluster with malignant tumours included a few benign tumour samples. The second cluster of mainly benign tumour samples also included a few malignant tumour samples and a few samples of normal tissue (RP 35, RP 38, RP 40, RP 46, and RP 66). The third cluster of mainly normal tissue included a few benign tumour samples (CMB 11, CMB 50, and CMB 209) and one malignant tumour tissue sample (CM 125T *).

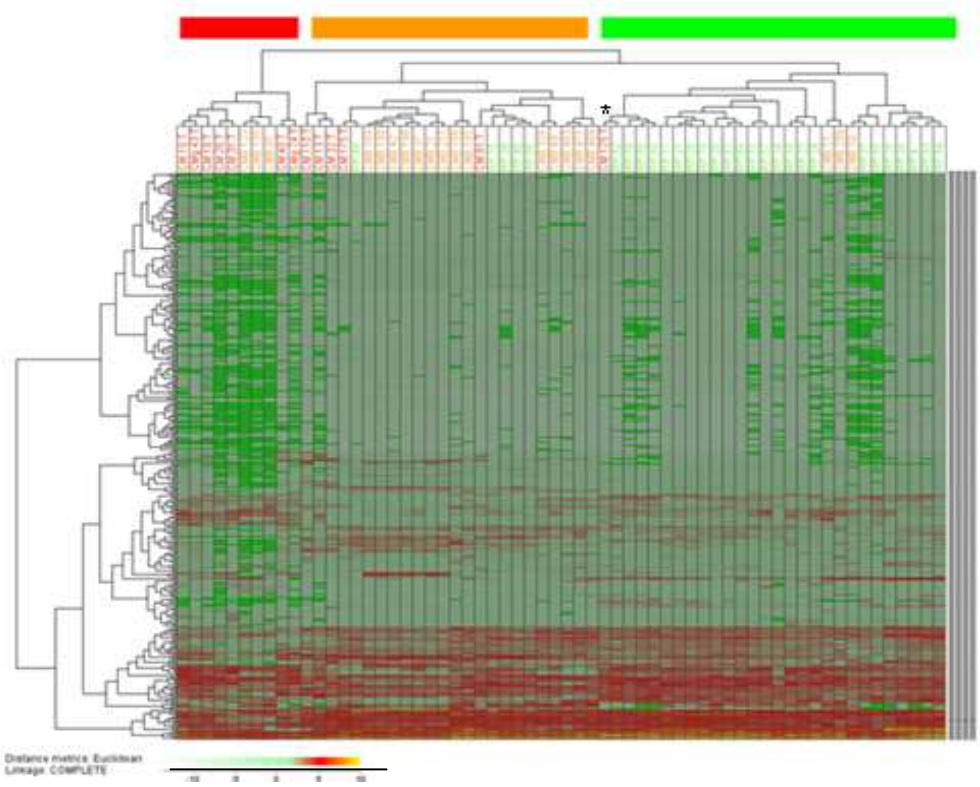

Figure 10: **Unsupervised hierarchical clustering**. Clustering of 342 miRNAs across 29 normal tissue samples (RP- Green), 20 benign tumour samples (CMB -orange) and 13 malignant tumour samples (CM and CMg-Red). The colour bar beneath the heat map illustrates the link between expression level of the different miRNAs and colour intensity. * Regarded as normal sample

Correspondence Analysis (CA) is a technique that generates graphical representations of the interactions between categories of two categorical variables, and which does not require any prior choice of parameters. A CA plot was therefore generated to show the distribution of the samples and the variance between the three different tissue types based on their miRNA profile (Fig.11). As with the cluster analysis, there was a clear division of the normal tissue samples compared to benign tumour samples and malignant tumour samples. Five normal tissue samples were assigned to the benign tumour sample group, whereas one malignant tumour sample was assigned to the group of normal tissue samples, as observed earlier. Malignant tumours and benign tumours were more similar to each other based on the CA plot and hierarchical clustering when considering their miRNA profile.

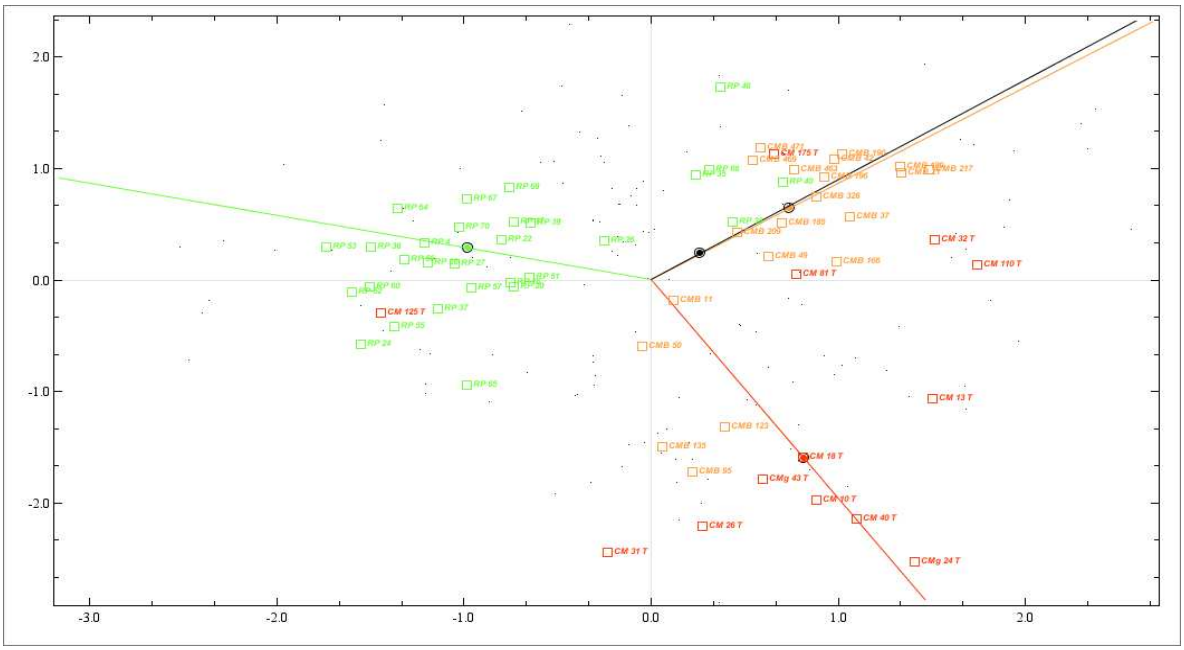

Figure 11: **CA mapping based on 342 miRNAs**. Sample separation shown in a CA plot according to the miRNA profile in the three different histological groups. Green= normal tissue, Red= malignant tumours, Orange: Benign tumours.

4.3 Significantly differentially expressed miRNAs in Normal tissue and Benign and Malignant tumours

In addition to unsupervised sample classification, we also performed supervised analysis to identify miRNAs that were differentially expressed among the three different tissue types. To identify which miRNAs that were differentially expressed between malignant tumours, benign tumours and normal tissue, a three class SAM analysis was performed. SAM identified miRNAs that were significantly differentially expressed between the histological groups analysed.

Only miRNAs with q-value = 0.0 and FDR = 0.0 were chosen for further analysis as most statistically significant. The differentially expressed miRNAs were selected and hierarchical clustering was carried out on samples and miRNAs using Euclidean correlation as a distance measure. 139 probes (81 miRNAs) were included in this clustering. We observed a cluster which clearly divided the normal samples from the benign and tumour samples with a few exceptions (Fig.12). As previously seen, a few normal tissue samples (RP 35, RP 38, RP 40, RP 46, RP 65, RP 66) clustered together with benign and malignant tumour samples, and the one malignant tumour sample (CM 125 T*) clustered with normal tissue samples. However, the three samples of benign tumours (CMB 50, CMB 11 and CMB 209) that earlier clustered together with the normal tissue samples, did not cluster with the normal tissue samples any longer.

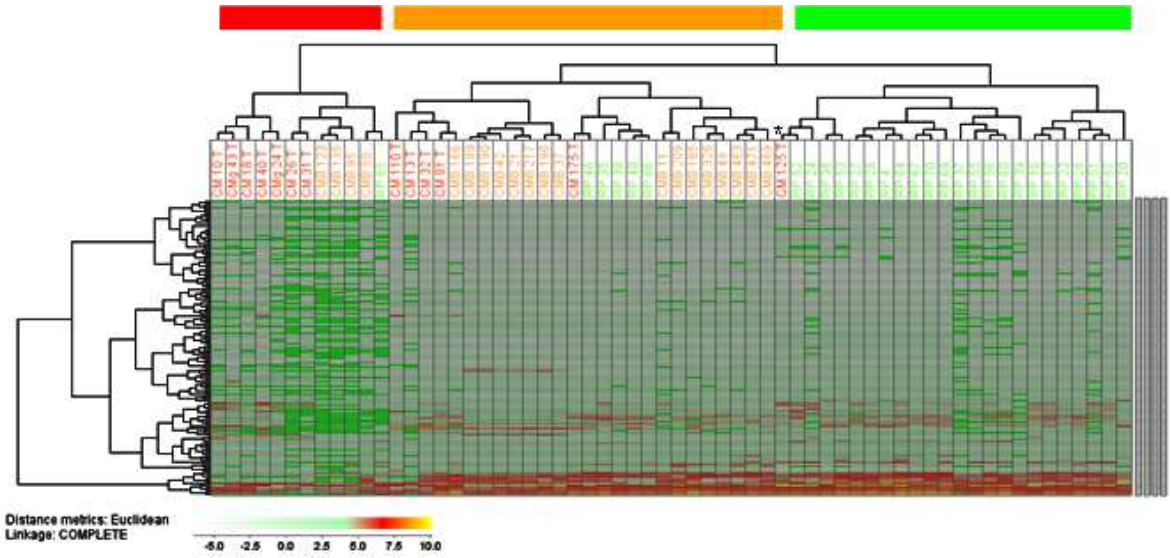

Figure 12: Supervised hierarchical clustering of the 81 most variable miRNAs at FDR=0 across 29 normal tissue samples (RP- Green), 20 benign tumour samples (CMB -orange) and 13 malignant tumour samples (CM and CMg- Red). The colour bar beneath the heat map illustrates the link between expression level of the different miRNAs and colour intensity. ***** Regarded as normal sample

Through the use of SAM, the expression level of all 81 miRNAs at FDR=0.0 were identified (Supplementary table 2). Some miRNAs were represented several times on the array and therefore, an average value of each probe was calculated, reflecting the relative expression level in normal, benign and malignant tissue samples (Supplementary Fig. 1).

In this study, 8 members of the let-7 family have been identified to be differentially expressed, with all of them being significantly downregulated in malignant tumours compared

to normal tissue (Fig. 13). However, there were also members of the let-7 family that were downregulated in benign tumours compared to normal tissue. Most significantly downregulated members of the let-7 family in both benign and malignant tumours compared to normal tissue were let-7a-e. The remaining let-7 family members (let-7f, let-7g, and let-7i) were upregulated in both benign tumours and normal tissue compared to malignant tumours.

We also identified miR-21 to be differentially expressed amongst the three tissue types studied (Fig.13). The expression of miR-21 was significantly higher in malignant tumours compared to normal tissue, but its expression was also significantly upregulated in benign tumours compared to normal tissue. The discovery of two oncomirs having the same expression in benign tumours as in malignant tumours led to the question of whether there were more miRNAs expressed in both benign and malignant tumours compared to normal tissue that are of tumorigenic character?

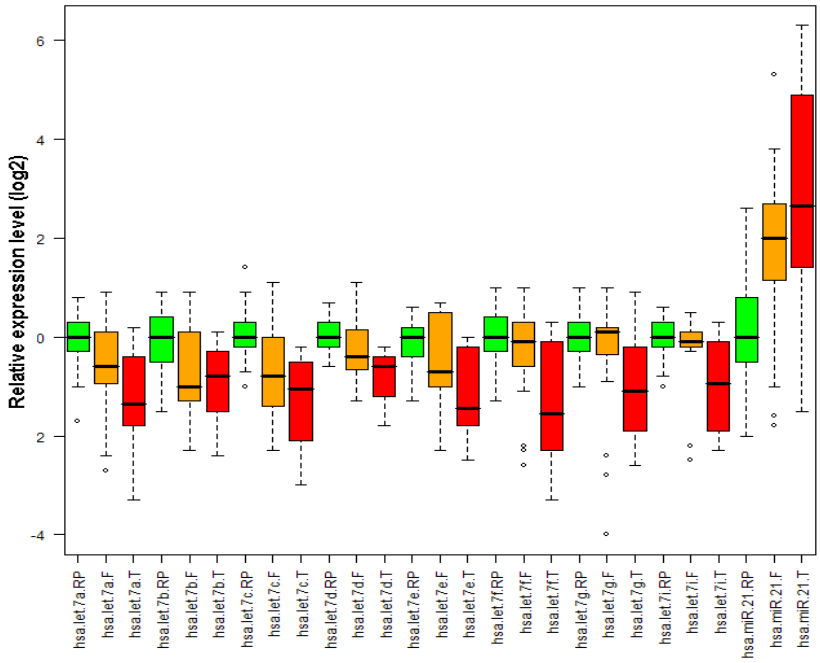

Figure 13: Box plot of the relative expression of let-7 and miR-21 in benign tumours (orange), malignant tumours (red) and normal tissue (green).

4.4 MiRNA expression in Benign and Malignant tumours

Interestingly, many miRNAs were identified to have similar expression in benign and malignant tumours compared to normal tissue. Two-class SAM analysis of malignant tumours against normal tissue, and benign tumours against normal tissue were performed to identify miRNAs that were expressed in both benign and malignant tumours. In total, 32 miRNAs were identified to be similarly expressed in both benign and malignant tumours compared to

normal tissue. Among the 32 miRNAs, 5 of them (miR-155, miR-183, miR-551b, miR-663 and miR-99) were also identified to be most dissimilar between benign and malignant tumours. For most of them, the expression level in benign tumours was intermediate between normal tissue and malignant tumours. For miR-663 however, the normal samples had an expression level in between malignant and benign tumours, and has therefore not been included in table 5.

Out of the 31 miRNAs identified, 18 of them were down regulated, whereas the remaining 13 miRNAs were upregulated. MiR- 923 was also identified to be similarly expressed among benign and malignant tumours, but in reality, this miRNA is a fragment of the 28S rRNA and is no longer considered as a miRNA. The rest of miRNAs identified to be similarly expressed in benign and malignant tumours can be of great importance in identifying potential diagnostic miRNA markers as many of these miRNAs appears to be involved in several types of human cancers, including breast cancer (Table 5).

Table 5: MiRNAs identified in Benign and Malignant tumours compared to Normal tissue

miR systematic name	Down- or up regulated in benign- and malignant tumours	Involvement in Cancer	Reference
Let -7c	Down	Tumour suppressor miRNA. Target genes are oncogenes *RAS* and *HMGA2* that are involved in many human cancers. Down regulated in breast and prostate cancer compared to normal tissue.	Blenkiron et al., 2007 Iorio et al., 2005 Ozen et al., 2007
has-miR-126	Down	Little expression in primary breast tumours from patients who relapse. Loss of expression is associated with poor distal metastasis-free survival. Expression also lost in colon cancer tissue compared to normal tissue. Thought to suppress growth by targeting PI3K signaling.	Guo et al., 2008 Tavazoie et al., 2008
miR-1274b	Up	No previous important finding in cancer	
hsa-miR-1280	Up	Upregulated in renal cell carcinoma compared to normal tissue	Yi et al., 2009
hsa-miR-130b	Up	Upregulated in glioblastoma and gastric tumours compared to matched normal tissue. Contributes to Cancer by increasing cell viability and reduce cell death via regulating RUNX3 tumour suppressor gene.	Tomaru et al., 2006 Lai et al., 2010
hsa-miR-139-5p	Down	Down regulated in gastric cancer compared to normal tissue	Guo et al., 2009
miR-140-3p	Down	No previous important finding in cancer	
miR-141	Up	Important regulators of stem cell functions that control both EMT and self-renewal, and/or proliferation in normal mammary stem cells and breast cancer stem cells	Gregory et al., 2008 Shimono et al., 2009
miR-145	Down	Significantly downregulated or completely eliminated in breast cancer. Can be related to early progression of breast	Iorio et al., 2005,

		cancer Also down regulated in prostate cancer compared to normal tissue.	Sempere et al., 2007 Ozen et al., 2007
miR-149	Up	Downregulated in prostate carcinoma compared to adjacent normal tissue and in squamous cell carcinoma of tongue compared to normal tissue.	Schaefer et al., 2010 Wong et al., 2008
miR-155	Up	Upregulated in breast cancer compared to normal tissue. Upregulated in pancreatic intraepithelial neoplasia compared to non-neoplastic ductal epithelium.	Iorio et al., 2005 Ruy et al., 2010
miR-183	Up	Upregulated in prostate carcinoma compared to adjacent normal tissue.	Schaefer et al., 2010
hsa-miR-193a-3p	Down	Upregulated in bladder cancer compared to normal tissue	Dyrskjøt et al., 2009
hsa-miR-193b	Down	Downregulated in breast cancer. Downregulated miR-193b increases the invasive, migratory and proliferative capacity of breast cancer cell lines by upregulating uPA	Li et al., 2009
hsa-miR-196a	Up	High levels found in colon cancer. Promote the oncogenic phenotype of cancer cells via activation of the AKT pathway.	Schimanski et al., 2009
miR-199a-5p	Down	No previous important finding in cancer	
hsa-miR-200b	Up	Important regulators of stem cell functions that control both EMT and self-renewal, and/or proliferation in normal mammary stem cells and breast cancer stem cells	Gregory et al., 2008 Shimono et al., 2009
miR-21	Up	Targets tumour suppressor genes such as *PTEN, PDCD4, TPM1*, and *MASPIN*. Over expression is associated with an advanced clinical stage and lymph node metastasis in breast cancer and is also associated with low sensitivity and a poor response to chemotherapy	Baffa et al., 2009 Yan et al., 2008 Krichevsky et al., 2009
miR-224	Down	Down regulated in lung cancer compared to non-cancer tissue.	Yanaihara et al., 2006
miR-23a	Down	No previous important finding in cancer	
miR-29a	Down	Downregulated in breast cancer with vascular invasion.	Iorio et al., 2005
has-miR-34a	Up	Upregulated in different cancers. It is regarded as a tumour-suppressor as it is a direct pro-apoptotic transcriptional target of p53	Shapira et al., 2007
miR-365	Down	Up regulated in breast cancer compared to normal adjacent tissue. Potential target genes include members of the RAS family, such as *RAB1B* and *RAB22A*, and ubiquitin specific peptidase 33.	Yan et al., 2008
hsa-miR-551b	Down	Down-regulated in colon cancer compared to normal colon tissue	Sarver et al., 2009
hsa-miR-575	Down	Downregulated in gastric cancer compared to normal gastric tissue	Luo et al., 2009
hsa-miR-652	Down	Downregulated in gastric cancer compared to normal gastric tissue	Luo et al., 2009
hsa-miR-720	Up	No previous important finding in cancer	
miR-766	Up	No previous important finding in cancer	
hsa-miR-923	Down	A fragment of the 28S rRNA and is no longer considered as a miRNA	
miR-99a	Down	Downregulated in squamous cell carcinoma of tongue compared to normal tissue.	Wong et al., 2008

4.5 MiRNA expression in Benign vs. Malignant tumours

Although many miRNAs were identified to be similarly expressed in benign tumours and malignant tumours, there were also some miRNAs that were differentially expressed between the two tissue types. Two class SAM analyses identified 31 miRNAs (51) probes at FDR=0.0 that were differentially expressed between malignant tumours and benign tumours (Supplementary Table 3). In total, 19 of the miRNAs identified were up regulated, and the remaining 15 miRNAs were down regulated in malignant tumours compared to benign tumours (Table 6).

Table 6: Differentially expressed miRNAs in malignant tumours compared to benign tumours

Upregulated miRNAs in Malignant tumours compared to Benign tumours	miR-10, miR-1202, miR-1225-5p, miR-1228, miR-1300, miR-1308, miR-135a, miR-150, miR-155, miR-15b, miR-183, miR-188-5p, miR-210, miR-22, miR-324-3p, miR-331-3p, miR-425, miR-551b, miR-663
Downregulated miRNAs in Malignant tumours compared to Benign tumours	Let-7d, miR-100, miR-125b, miR1260, miR-127-3p, miR-130a, miR-132, miR-205, miR-223, miR-30b, miR-423-5p, miR-451, miR-497, miR-572, miR-99a

Chapter 5

Discussion

5.1 miRNAs as potential clinical biomarkers

Microarray technology has been extensively used in hope to create a gene signature that will allow us to predict cancer. Even though many of such gene signatures have been identified, none of them have been applied in to clinical practice due to inconsistency in the way they were preformed in the different studies when considering treatment regimes, sample sizes, the patient population studied and the definition of pathological complete response (Weigelt et al., 2010). However, the emergence of miRNA microarrays and their use in prognostic research have made it possible to believe that miRNAs give a more reliable view of the transcriptional program as it regulates mRNA at the post-transcriptional level and has been proven to give more reliable results than mRNA profiling regarding classification of tumours (Lu et al.,2005). In this study we have demonstrated that benign tumours are more similar to malignant tumours than to normal tissue, considering their miRNA expression profile.

Based on unsupervised hierarchical clustering, a clear division between normal samples, benign tumour samples and malignant tumour samples was observed, with the exception of a few samples. Normal tissue from healthy women clustered together in one cluster with some of the benign tumours clustering in between the samples (CMB 11, CMB 209 and CMB 50). The two other clusters consisted mostly of benign- and malignant tumours, with the exception of some samples of normal tissue samples that clustered within benign and malignant tumour samples (RP 46, RP 35, RP 38, RP 40 and RP 66). The reason for this is yet unclear. The normal tissue that was used in this study was from healthy women that have undergone breast reduction, and there are no records of whether these women have developed any sort of benign or malignant lesions in their breasts after the surgery. Also, sample CM 125T, which is a malignant tumour sample, clustered together with the normal samples. Considering that none of the other malignant tumour samples cluster with normal samples, the tissue specimen used for analyses has probably been taken from an area consisting of mostly normal tissue. This is consistent with results from another ongoing study (results not shown). Looking at the mRNA expression profile of some the same samples as used for miRNA expression profiling,

the same type of clustering occurred, with some of the normal samples clustering together with the benign and malignant tumour samples, and CM 125 T clustering with the normal samples (data not shown). This indicates that the observed differences are truly biological and not due to the way the experiment was performed.

5.2 miRNA in Cancer Development

A subsequent three class SAM analysis identified 81 miRNAs (139 probes) that were differentially expressed between normal tissue, benign tumours and malignant tumours at FDR = 0. We observed a cluster which clearly divided the normal samples from the benign and tumour samples when considering the miRNA profile. In figure 14, we have designated specific miRNAs to each histological group depending on their power to distinguish a specific group from the two others.

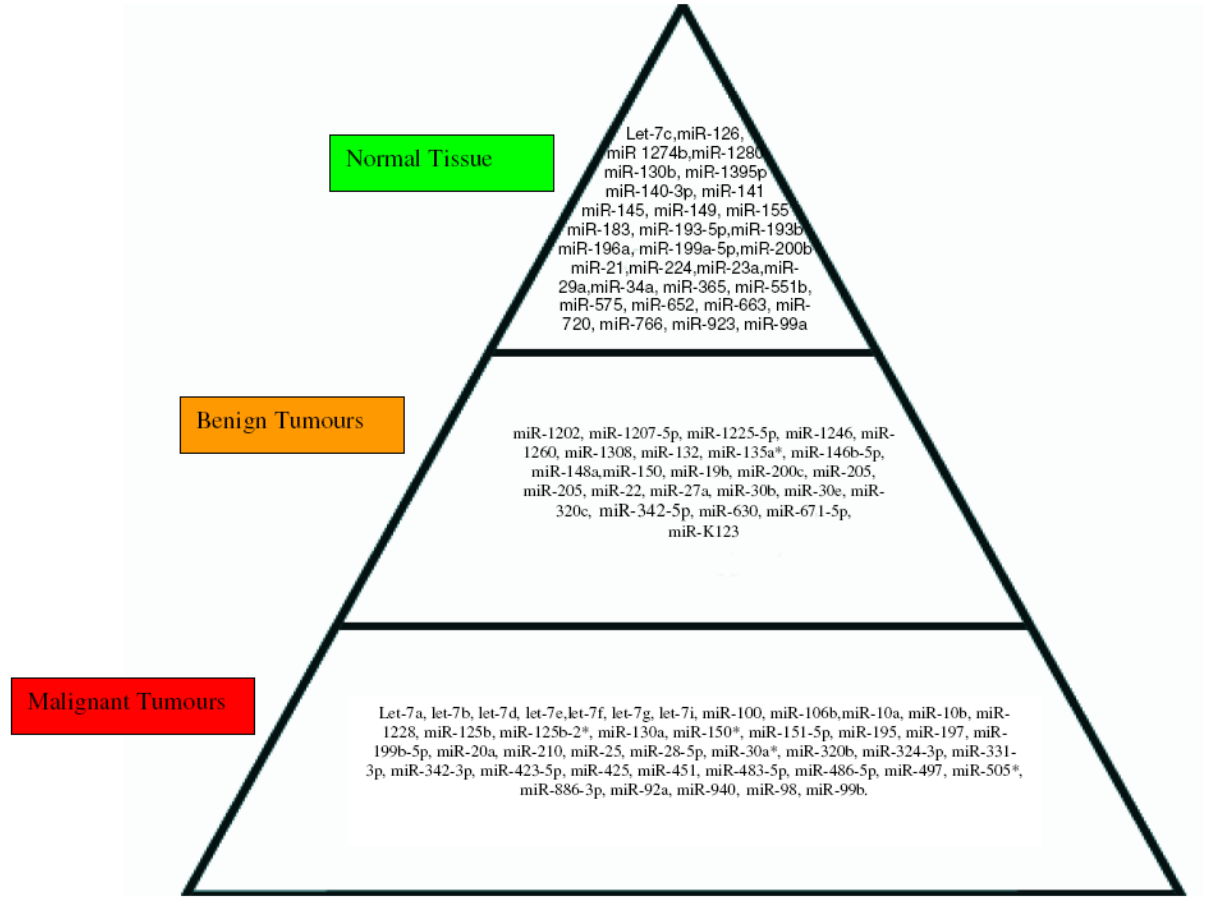

Figure 14: miRNAs that separates one histological group from the others.

There are many miRNAs that are thought to play a role in tumorigenesis with some of them acting as oncogenes and promote cancer, whereas others function as tumour suppressors and

preventing cancer (Zhang et al., 2007). In breast cancer, miR-10b, miR-125b, miR-145, miR-21 and miR-155 are the most consistently deregulated miRNAs. MiR-21 and miR-155 being upregulated, whereas miR-10b, miR-125b, miR-145 being downregulated (Iorio et al., 2005). The findings of Iorio et al. (2005) are consistent with our findings where miR-21 and miR-155 are upregulated in both malignant- and benign tumours compared to normal tissue. MiR-10b was not found to be differentially expressed between the three groups studied, but it was down regulated in malignant tumours compared to normal tissue. MiR-125b and miR-145 were identified to be differentially expressed in all the three tissue types studied, and in accordance with what has been observed earlier (Iorio et al., 2005; Sempere et al., 2007), the expression of these miRNAs was downregulated in malignant breast tumours compared to normal tissue. However, miR-145 was also identified to be downregulated in benign tumours compared to normal tissue (Table 5). It has been claimed that loss of miR-145 expression can be related to early progression of breast cancer, and thereby act as an important biomarker and target for cancer therapy (Sempere et al., 2007).

MiR-125a and miR-125b have been identified to be significantly down regulated in cases of breast cancer where HER2 is over-represented. MiR-125b is a potential tumour suppressor as a study with cell lines showed that overexpression of miR-125a and miR-125b suppresses HER2 expression. As the HER2 gene is associated with an aggressive form of breast cancer and high risk of relapse and bad prognosis, the suppression of HER2 by miR-125a and miR-125b reduces invasive growth (Verghese et al., 2008). This suggests that these miRNAs might have a protective function as our findings supports the notion that at least miR-125b is significantly less expressed in malignant tumours compared to benign tumours and normal tissue (Suppl. Fig. 1).

MiR-155 is a multifunctional miRNA involved in both physiological and pathological processes such as haematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases (Faraoni et al., 2009). MiR-155 is overexpressed in invasive breast cancer tissue and contributes to mammary epithelial cell plasticity by targeting RhoA. RhoA protein expression drives epithelial-to-mesenchymal- transition (EMT) progression and is an important modulator of cell junction formation and stability (Kong et al., 2008).A recent study by Ryu et al. (2010) identified miR-155 to be significantly overexpressed in pancreatic intraepithelial neoplasia, which is a non-invasive precursor to invasive pancreatic adenocarcinoma, suggesting that overexpression of miR-155 is an early event in the

progression of pancreatic adenocarcinoma (Ryu et al., 2010). Mir-155 has also been identified in higher levels in the serum of patients with diffuse large B-cell lymphoma (DLBCL) and women with progesterone receptor (PR) positive breast tumours compared to control subjects (Lawrie et al., 2008; Zhu et al., 2009). The significance of miR-155 in PR positive tumours does need further exploration, but the fact that we have identified miR-155 to be upregulated in both malignant and benign tumours compared to normal tissue, suggest that miR-155 has potentials to be an important biomarker in breast cancer diagnostics.

Other miRNAs that might be of interest are members of the miR-200 family and miR-205. MiR-200 family expression is necessary for maintenance of the epithelial phenotype. All the members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) have been identified as important regulators of stem cell functions that control EMT, self-renewal, and/or proliferation in normal mammary stem cells and breast cancer stem cells (Gregory et al., 2008, Shimono et al., 2009). MiR-200c has been found in breast cancer stem cells and down regulation of this miRNA links breast cancer stem cells with normal stem cells by modulating a known stem cell regulator gene called BMI1 (Shimono et al., 2009). BMI1 epigenetically represses transcription of HOX genes and also represses apoptotic, senescence and differentiation pathways in stem cells (Park et al., 2003). The same pathways are also modulated by miR-200c in part through BMI1 (Shimono et al.,2009). The difference between normal stem cells and cancer stem cells is that normal stem cells have a cell cycle machinery that is highly controlled through each step, while cancer stem cells lose this control and subsequently uncontrolled proliferation of cells occur, leading to tumour formation. These observations lead us to believe that there must be some elements that are shared between cancer stem cells and normal stem cells. The miRNA profile identified by Shimono et al., show remarkable similarities between breast cancer stem cells and normal mammary stem cells, and amongst them is miR-200b, miR-200c and miR-141 (Shimono et al., 2009). In our study, we have identified miR-200b, miR-200c and miR-141 to be differentially expressed between the three different histological groups studied. All three miRNAs are significantly upregulated in malignant- and benign tumours compared to normal tissue. Also, miR-200b and miR-141 have similar expression in malignant- and benign tumours and their expression is most different from normal tissue (Table 5). Previously, miR-141 has been identified in the serum of patients with prostate cancer, and the levels of miR-141 can distinguish patients with prostate cancer form healthy controls (Mitchell et al., 2008). The identification of miR-141 in

the blood of patients with prostate cancer makes this miRNA a promising marker for cancer classification and prognostication.

Together with the miR-200 family, miR-205 is also found to be involved in EMT (Gregory et al., 2008). In this study, miR-205 is significantly downregulated in malignant tumours compared to normal tissue, but in benign tumours this miRNA is significantly upregulated**.** Expression of miR-200 family members and miR-205 are lost in invasive breast cancer cell lines with mesenchymal phenotype suggesting that down regulation of these miRNAs may be an important step in tumour progression (Gregory et al., 2008). Since miR-205 is down regulated in malignant tumours but upregulated in benign tumours and in normal tissue, this miRNA might be of great diagnostic value and one might postulate that miR-205 serves a protective effect in the cells.

5.3 miR-21 and let-7

Interestingly, many miRNAs were identified to have similar expression in benign and malignant tumours compared to normal tissue. Amongst the miRNAs that were similarly expressed are let-7c and miR-21, miRNAs well known to be deregulated in several types of cancer, including breast cancer. MiR-21 is usually upregulated in cancers and regulates genes that are involved in important cellular processes and thereby leading to cancer. MiR-21 overexpression has been associated with an advanced clinical stage and lymph node metastasis in human breast cancer and is also associated with low sensitivity and a poor response to chemotherapy (Yan et al., 2008; Krichevsky et al., 2009). Amongst the genes targeted by miR-21 are several tumour suppressor genes such as *PTEN, PDCD4, TPM1*, and *MASPIN* which all regulate important processes, including cell growth, proliferation and apoptosis (Baffa et al., 2009). The gene *PDCD4* targets translation by inhibiting eukaryotic initiation factor 4 and has been implicated in cell transformation, tumour progression, tumour invasion and tumour growth (Schmid et al., 2008). It has also been suggested as an independent risk factor in colorectal and lung cancers (Baffa et al., 2009). Deregulation of tumour suppressor genes are often involved in the progression of tumours and previous findings establish miR-21 as an oncogenic miRNA that might play an important role in tumour growth, invasion and metastasis. High levels of miR-21 have been reported in the serum of patients with DLBCL compared to control subjects. The high expression of miR-21 is also associated with relapse free survival in these patients suggesting that miR-21 has a

potential as non-invasive diagnostic markers for DLBCL and possibly other cancers (Lawrie et al., 2008).

Members of the let-7 family are usually poorly expressed or even deleted in several human cancers. They function as tumour suppressors and may inhibit cancers by regulating oncogenes and/or genes that control cell differentiation or apoptosis (Zhang et al., 2007). Some might believe that low let-7 expression could be considered a diagnostic feature of certain stem cell populations as it acts as a key regulator deciding cell proliferation and differentiation at the transition from stem cells to more differentiated cells (Büssing et al., 2008). In this study we have identified 8 members of the Let-7 family to be differentially expressed between normal tissue and benign and malignant tumours. Let-7a-i are significantly downregulated in malignant and benign tumours compared to normal tissue, while let-7f, let-7g and let7-i are upregulated in normal tissue and benign tumours compared to malignant tumours. The reason for this is not known, but one might speculate that these three miRNAs are more indicative of malignant tumours than benign tumours, and they might serve a protective role in the cells. Previous studies have observed that miRNAs located in deleted regions of the chromosome, such as let-7, have low levels of expression in cancer tissue compared to normal tissue (Calin et al., 2004, Verghese et al., 2008). This is consistent with our observations where all members of let-7 are significantly downregulated in breast cancer tissue compared to normal tissue. In lung cancer, let-7 homologues have been proven to be significantly down regulated and are correlated with a poor prognosis and a short post-operative survival (Takamizawa et al., 2004), while in breast cancer, the let-7 family appears as the most consequent and heavily down regulated miRNAs within potential breast tumour initiating cells (BT-IC) (Verghese et al., 2008). BT-ICs have stem cell-like properties which are suggested to be regulated by let-7. Let-7 is able to reduce proliferation, mammosphere formation, tumour formation and metastasis in BT-IC *in vivo* (Yu et al., 2007). Considering that the target genes of let-7 are the oncogenes *RAS* and *HMGA2*, any deregulation of these genes by loss of regulation through let-7 family might contribute to human cancer (Blenkiron et al., 2007). Recently, let- 7a was identified in high levels in the serum of breast cancer patients compared to control subjects, and the levels of let-7a decreased significantly postoperatively (Heneghan et al., 2010). Members of let-7, including let-7c, are generally downregulated in breast cancer and other types of cancer, suggesting that let-7 expression could be associated with poor prognosis (Iorio et al., 2005, Takamizawa et al., 2004).

5.4 miRNAs and breast cancer

Many of the miRNAs identified to be similarly expressed in both malignant and benign tumours have also previously been identified to be involved in breast cancer, such as miR-126, miR-193b and miR-200b. The expression of miR-126 is lost in the majority of primary breast tumours from patients who relapse, and loss of expression is associated with poor distal metastasis-free survival (Tavazoie et al., 2008). MiR-126 is also frequently lost in colon cancer tissue compared to normal tissue and is thought to suppress growth by targeting phosphatidylinositol 3-kinase (PI3K) signalling. Loss of this miRNA may therefore promote growth during carcinogenesis (Guo et al., 2008). Previous findings are consistent with the findings in this study, where miR-126 has low expression in both malignant- and benign tumours compared to normal tissue.

In our study we have identified miR-193b to be down regulated in benign- and malignant tumours compared to normal tissue. Previous studies have identified this miRNA to be down regulated in breast cancer. MiR-193b downregulation increases the invasive, migratory and proliferative capacity of breast cancer cell lines by upregulating urokinase-type plasminogen activator (uPA) (Li et al., 2009). uPA is a serine protease that is involved in extracellular matrix degradation and increased levels of this protease therefore increases migratory capacity of breast cancer cells.

MiR-200b is part of the miR-200 family that are important regulators of stem cell functions that control EMT, self-renewal, and/or proliferation in normal mammary stem cells and breast cancer stem cells (Gregory et al., 2008, Shimono et al., 2009). This miRNA is highly upregulated in malignant and benign tumours compared to normal tissue.

Other miRNAs that are interesting for future breast cancer research are miR-196a, miR-34a and miR-365. In our study, miR-196a is highly up regulated in benign- and malignant tumours compared to normal tissue. It has been hypothesized that miR-196a might promote tumour progression in colorectal cancer through directed cleavage of specific homeobox genes. High levels of this miRNA promote the oncogenic phenotype of colorectal cancer cells via activation of the AKT pathway (Schimanski et al., 2009). However, the exact mode of function of miR-196a needs further elucidation. MiR-34a has previously been shown to be downregulated in different type of cancers and its expression is induced by p53 in response to DNA damage and oncogenic stress (He et al.,2007). MiR-34a is regarded as a tumour-

suppressor as it is a direct pro-apoptotic transcriptional target of p53 that can mediate some of p53's biological effects. Perturbation of miR-34a expression, as occurs in some human cancers, may thus contribute to tumorigenesis by attenuating p53-dependent apoptosis (Raver-Shapira et al., 2007). In this study however, miR-34a is found to be slightly upregulated in benign- and malignant tumours compared to normal tissue. Overexpression of miR-34a is shown to exhibit antiproliferative effects and augment apoptosis (Raver-Shapira et al., 2007). Since the p53 status is not known in our patient samples, it is difficult to know whether the p53 status might be of major importance in the results obtained in this study compared to previous studies.

In this study, miR- 365 is identified to be downregulated in both malignant and benign tumours compared to normal tissue, while a study performed in 2008 (Yan et al., 2008) identified this miRNA to be significantly upregulated in breast cancer compared to adjacent normal tissue. Potential gene targets for this miRNA are members of the *RAS* oncogene family, such as *RAB1B* and *RAB22A*, and ubiquitin specific peptidase 33 (Yan et al., 2008). This addresses the issue of whether normal adjacent tissue can be regarded as normal tissue or not. This miRNA does however need further elucidation in order to confirm its function regarding cancer pathogenesis.

5.5 miRNA that differs between benign and malignant tumours

Although many miRNAs were identified to be similar in both benign and malignant tumours, we also identified miRNAs that were most different from the two tumour types using two-class SAM analysis. 34 miRNAs were identified to be differentially expressed between the two groups; 19 miRNAs were up regulated and the remaining 15 were down regulated. Only one member of the let-7 family, let-7d, was differentially expressed between benign and malignant tumours with the expression of let-7d being significantly lower in malignant tumours compared to benign tumours. Altered expression of let-7d is found in head and neck squamous cell carcinoma compared to adjacent normal tissue, and low expression of let-7d together with miR-205 is associated with poor head and neck cancer survival, and these miRNAs can therefore be used as prognostic markers, at least for head and neck cancer (Childs et al., 2009). In this study, both let-7d and miR-205 were significantly less expressed in malignant tumours compared to benign tumours and normal tissue, making them important as prognostic markers in breast cancer also.

Chapter 5

Conclusion

Association between deregulated miRNAs and their involvement in cancer has been proposed for a long time. However, the molecular mechanisms by which miRNA can modulate tumour growth still remain unknown. Identifying miRNA expression profiles that separates different subtypes of breast cancer and identifying the molecular pathways that miRNAs affect will make miRNAs useful as biomarkers for cancer diagnostics and also important in cancer prevention and therapeutics.

In this study we identified 81 miRNAs that were differentially expressed between benign and malignant tumours and normal tissue. The expression of the miRNAs identified to be differentially expressed among the different histological tissue types studied showed that benign tumours have a miRNA expression level that lies in between normal tissue and cancerous tissue. Many of the miRNAs identified are shown to be involved in breast cancer. In addition, we found 30 miRNAs that have a similar expression in malignant tumours as in benign tumours compared to normal tissue. Some of these miRNAs have previously been implied in different types of cancer, including breast cancer.

The finding of cancer promoting miRNAs such as, let-7 and miR-21 in benign tumours indicate that these miRNAs may be potential diagnosis biomarkers and probable factors involved in the pathogenesis of breast cancer. However, some of the miRNA identified needs further elucidation as their involvement in breast cancer has not been revealed yet, but their involvement in different types of cancer suggests that they might be potential biomarkers and therapeutic targets in the future.

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Supplementary Material

Supplementary Table 1	Information Summary of samples used
Supplementary Table 2	SAM identified miRNAs in Malignant tumours, Benign tumours and Normal tissue at FDR=0
Supplementary Table 3	SAM identified miRNAs in Malignant tumours vs Benign tumours at FDR= 0.
Supplementary Fig. 1	Relative expression of 81differentially expressed miRNAs in Benign tumours, Malignant tumours and Normal tissue.

Suppl.Table 1: Information summary of the samples used

Type of Tissue	Sample ID	Mass (mg)	RNA conc (ng/ul)	RNA (ug)	260/280	260/230	RIN RNA	miRNA %	Microarray slide no.	Scanning date
Fibroadenom	37	-	64	1,92	2,08	1,32	7,8	25	0469	27.10.2009
Fibroadenom	42	-	65	1,95	2,01	0,53	7,4	32	0469	27.10.2009
Fibroadenom	95	-	527	15,81	2	2,07	8,9	46	0467	29.09.2009
Fibroadenom	123	-	222	6,66	2,07	1,71	8,6	8	0467	29.09.2009
Fibroadenom	135	-	208	6,24	2,04	1,98	8,7	8	0469	27.10.2009
Fibroadenom	189	-	78	2,34	2,07	1,81	8,1	29	0470	27.10.2009
Fibroadenom	190	-	71	2,13	2,08	1,48	8,3	21	0470	27.10.2009
Fibroadenom	196	-	64	1,92	1,97	0,23	8,5	27	0470	27.10.2009
Fibroadenom	209	37	45	1,35	2,03	0,64	8,2	25	0471	04.11.2009
Fibroadenom	217	-	108	3,24	2,11	6,2	8,1	56	0466	16.09.2009
Fibroadenom	326	32	97	2,91	2,03	1,63	8,1	23	0466	16.09.2009
Fibroadenom	463	23	43	1,29	1,95	1,01	7,8	70	1400	23.12.2009
Fibroadenom	469	42	57	1,71	2,07	1,42	7,7	25	1399	23.12.2009
Fibroadenom	471	19	44	1,32	1,98	0,31	7,3	23	1399	23.12.2009
Fibroadenomatose	11	21	91	2,73	1,99	0,87	8,9	32	0465	03.09.2009
Fibroadenomatose	21	-	101	3,03	2,03	1,57	7,5	25	0466	16.09.2009
Fibroadenomatose	49	33	160	4,80	2,09	0,82	N/A	28	0469	27.10.2009
Fibroadenomatose	50	30	66	1,98	1,98	1,65	8,3	23	0467	29.09.2009
Fibroadenomatose	166	38	434	13,02	2,07	1,94	9	66	0466	16.09.2009
Fibroadenomatose	185	17	182	5,46	2,05	1,73	8,7	23	0466	16.09.2009
Reduction Plastic	20	99	111	3,33	1,98	1,4	7,7	33	0465	03.09.2009
Reduction Plastic	22	115	100	3,00	2,04	0,98	8,1	33	0465	03.09.2009
Reduction Plastic	24	88	88	2,64	2,02	1,04	7,1	38	0520	13.11.2009
Reduction Plastic	26	110	284	8,52	2,05	1,7	6,8	32	0466	16.09.2009
Reduction Plastic	27	80	68	2,04	2,02	1,64	7,8	33	0466	16.09.2009
Reduction Plastic	28	120	115	3,45	2,03	0,92	7,8	28	0466	16.09.2009
Reduction Plastic	37	123	189	5,67	2,01	0,58	8,2	33	0467	29.09.2009
Reduction Plastic	38	130	855	25,65	2,1	1,91	8,1	28	0467	29.09.2009
Reduction Plastic	39	140	121	3,63	2	1,75	8,5	13	0467	29.09.2009
Reduction Plastic	40	120	515	15,45	2,06	2,05	6,3	33	0469	27.10.2009
Reduction Plastic	51	101	145	4,35	2,01	1,09	7,7	30	0467	29.09.2009
Reduction Plastic	52	202	72	2,16	1,9	1,16	7	28	0469	27.10.2009
Reduction Plastic	53	137	62	1,86	2	0,57	8,3	27	1399	23.12.2009
Reduction Plastic	55	110	129	3,87	2	1,17	9	26	0469	27.10.2009
Reduction Plastic	56	101	83	2,49	2,08	1,74	8,8	22	0470	27.10.2009
Reduction Plastic	57	120	158	4,74	1,97	0,95	5,6	26	0470	27.10.2009
Reduction Plastic	60	90	66	1,98	2,06	0,99	8,3	18	0470	27.10.2009
Reduction Plastic	64	119	47	1,41	2,01	1,54	7,6	34	1400	23.12.2009
Reduction Plastic	65	102	76	2,28	2,03	0,64	8,3	13	0470	27.10.2009
Reduction Plastic	66	124	335	10,05	2,05	1,82	6,9	35	0471	04.11.2009
Reduction Plastic	67	86	68	2,04	1,93	1,4	7,3	33	0471	04.11.2009
Reduction Plastic	69	101	89	2,67	2,05	1,59	8,2	33	0471	04.11.2009
Reduction Plastic	70	123	124	3,72	2,06	1,68	7,5	22	0471	04.11.2009
Reduction Plastic	4		77	2,31	2,13	1,82	8,4	48	0520	13.11.2009
Reduction Plastic	16		83	2,49	2,07	1,9	6.3	37	0520	13.11.2009
Reduction Plastic	17		85	2,55	2,03	2,06	8.3	17	0520	13.11.2009
Reduction Plastic	36		76	2,28	2,06	0,42	8.6	27	0520	13.11.2009
Reduction Plastic	35		97	2,91	2,06	1,93	6.6	30	0520	13.11.2009
Reduction Plastic	46		422	12,66	2,07	2,16	8.2	24	0520	13.11.2009

CM	32 T	104	1295	38,85	2,13	2,25	8,6	44	1399	23.12.2009
CM	10T	70	356	10,68	2,09	2,19	7.1	41	1401	29.01.2010
CM	13 T	55	2102	63,07	2,11	2,24	8.1	22	1401	29.01.2010
CM	18 T	40	115	3,44	2,12	1,26	6.7	33	1401	29.01.2010
CM	31 T	79	88	2,64	2,04	1,96	6.8	35	1202	29.01.2010
CM	26 T	86	114	3,43	2,07	2,06	6.8	25	1202	29.01.2010
CMG	24 T	64	812,4	24,37	2,13	1,28	7.4	39	1203	09.02.2010
CMG	43 T	100	171,5	5,15	2,09	1,30	6.9	32	1204	09.02.2010
CM	110 T	79	1907	57.2	2.00	2.07	7.7	35	0523	16.12.2009
CM	81 T	62	133	4.0	2.05	1.94	7.6	45	0523	16.12.2009
CM	125 T		71	2.1	2.08	1.26	8.1		0522	16.12.2009
CM	175 T		376	11.3	2.07	2.00	4.6		0522	16.12.2009
CM	40 T	102	1697	50.9	2.10	2.24	6.5	47	1203	09.02.2010

Suppl. Table 2: Differentially expressed miRNAs in Normal Tissue (RP), Benign Tumours (CMB) and Malignant Tumours (CM) at FDR=0

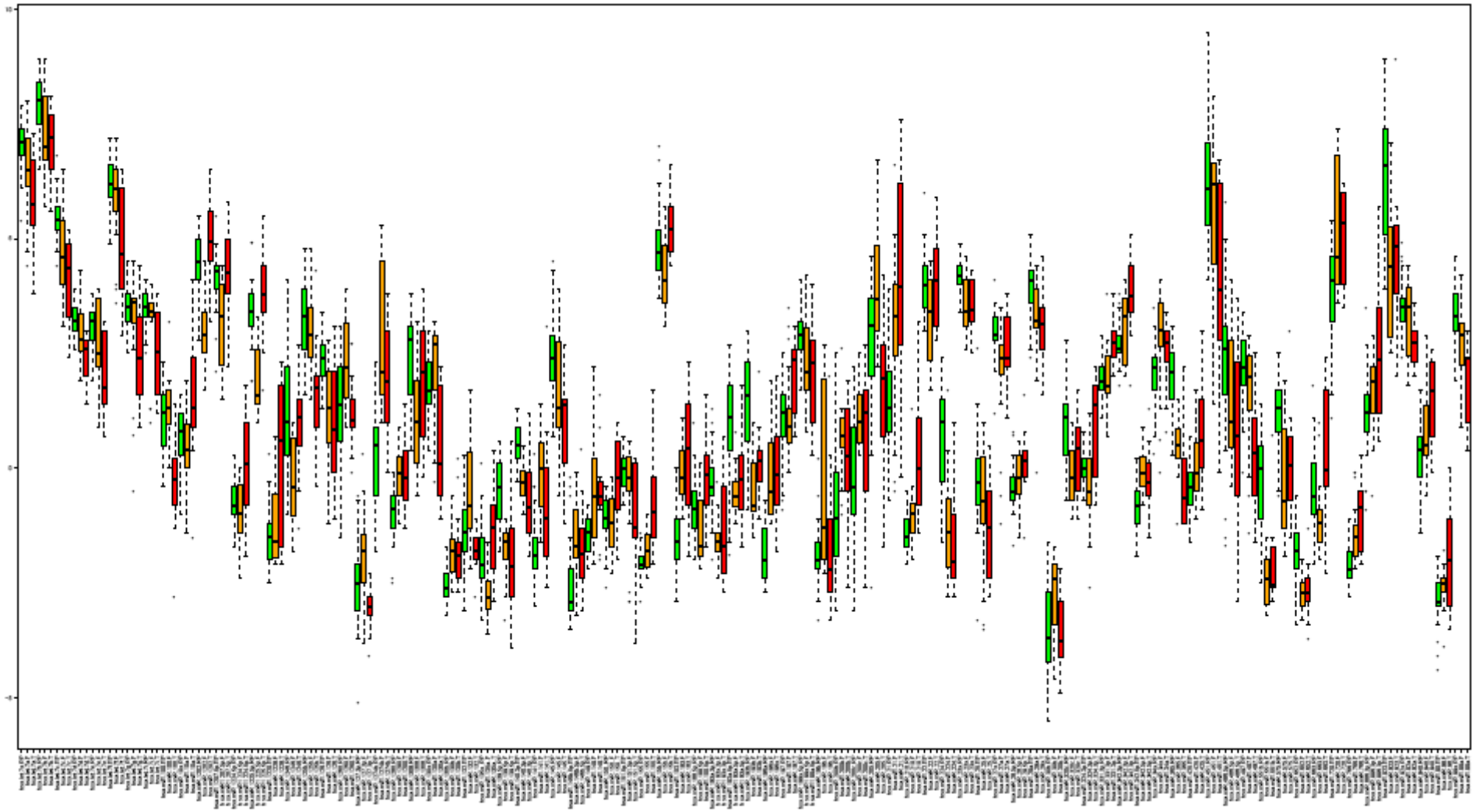
ProbeName	SystematicName	Average RP	Average CMB	Average Tumour
A_25_P00015076	hsa-miR-1202	4,26	2,87	4,85
A_25_P00010995	hsa-miR-210	-1,05	-0,55	1,37
A_25_P00015075	hsa-miR-1202	4,33	2,95	4,86
A_25_P00014920	hsa-miR-1225-5p	3,29	1,90	3,71
A_25_P00014921	hsa-miR-1225-5p	3,15	1,74	3,54
A_25_P00010799	hsa-miR-663	0,18	-0,38	1,39
A_25_P00010976	hsa-miR-21	-0,57	1,07	2,62
A_25_P00010469	hsa-miR-10a	0,44	0,02	1,52
A_25_P00014937	hsa-miR-1228	-1,70	-1,37	0,05
A_25_P00013406	hsa-miR-135a*	-1,67	-2,40	-0,99
A_25_P00014936	hsa-miR-1228	-1,60	-1,28	0,11
A_25_P00013405	hsa-miR-135a*	-3,69	-4,43	-3,07
A_25_P00012271	hsa-miR-155	-1,49	-0,78	0,50
A_25_P00010540	hsa-miR-324-3p	-0,23	-0,42	0,72
A_25_P00012246	hsa-miR-188-5p	-1,14	-1,65	-0,53
A_25_P00010800	hsa-miR-663	-1,30	-1,97	-0,85
A_25_P00010975	hsa-miR-21	2,96	4,47	5,56
A_25_P00012247	hsa-miR-188-5p	-0,85	-1,30	-0,21
A_25_P00010204	hsa-miR-22	3,75	2,98	4,06
A_25_P00013453	hsa-miR-150*	-1,02	-1,19	-0,13
A_25_P00014913	hsa-miR-886-3p	0,94	1,41	2,44
A_25_P00013452	hsa-miR-150*	-1,45	-1,61	-0,58
A_25_P00013407	hsa-miR-135a*	-0,93	-1,54	-0,51
A_25_P00013450	hsa-miR-150*	-1,07	-1,16	-0,14
A_25_P00015087	hsa-miR-1207-5p	3,75	2,87	3,88
A_25_P00011101	hsa-miR-15b	4,62	4,16	5,16
A_25_P00015143	hsa-miR-1246	1,09	-0,27	0,69
A_25_P00013451	hsa-miR-150*	-1,49	-1,41	-0,45
A_25_P00010835	hsa-miR-197	0,84	0,97	1,92
A_25_P00012098	hsa-miR-183	-1,16	0,32	1,26
A_25_P00014894	hsa-miR-551b	1,42	-1,09	-0,16
A_25_P00015088	hsa-miR-1207-5p	4,04	3,12	4,04

ProbeName	SystematicName	Average RP	Avererage CMB	Average Tumour
A_25_P00012099	hsa-miR-183	-1,00	0,51	1,42
A_25_P00015035	hsa-miR-320b	3,78	3,69	4,57
A_25_P00010977	hsa-miR-425	-0,67	-0,18	0,69
A_25_P00010996	hsa-miR-210	-2,12	-1,70	-0,84
A_25_P00010205	hsa-miR-22	0,61	-0,16	0,69
A_25_P00014027	hsa-miR-331-3p	1,64	1,82	2,66
A_25_P00012358	hsa-miR-342-3p	2,43	2,92	3,72
A_25_P00010539	hsa-miR-324-3p	1,49	1,33	2,12
A_25_P00015250	hsa-miR-1308	2,24	1,10	1,81
A_25_P00013089	hsa-miR-940	-0,65	-0,01	0,66
A_25_P00010437	hsa-miR-130b	-1,90	-1,19	-0,53
A_25_P00010797	hsa-miR-27a	1,55	0,66	1,28
A_25_P00010048	hsa-miR-98	-3,00	-2,74	-2,13
A_25_P00012270	hsa-miR-155	-2,98	-2,78	-2,21
A_25_P00013850	hsa-miR-28-5p	-0,66	-0,47	0,09
A_25_P00013090	hsa-miR-940	0,74	1,37	1,93
A_25_P00011965	hsa-miR-766	-2,25	-1,44	-0,92
A_25_P00012512	hsa-miR-193b	3,62	2,06	2,56
A_25_P00012254	hsa-miR-193a-5p	2,28	1,06	1,54
A_25_P00013051	hsa-miR-923_v12.0	7,60	5,63	6,04
A_25_P00012086	hsa-miR-34a	2,86	3,68	4,09
A_25_P00015036	hsa-miR-320c	0,76	-0,28	0,09
A_25_P00011964	hsa-miR-766	-2,42	-1,75	-1,42
A_25_P00012258	hsa-miR-193a-3p	1,26	-0,15	0,12
A_25_P00012834	hsa-miR-652	-0,32	-1,47	-1,20
A_25_P00012511	hsa-miR-193b	-1,40	-2,94	-2,72
A_25_P00010249	hsa-miR-630	-0,78	-1,69	-1,48
A_25_P00014896	hsa-miR-575	1,01	-0,24	-0,11
A_25_P00012053	hsa-miR-196a	-1,15	1,00	1,12
A_25_P00015264	hsa-miR-720	2,33	3,75	3,86
A_25_P00013050	hsa-miR-923_v12.0	4,79	2,74	2,84
A_25_P00010808	hsa-miR-575	0,89	-0,32	-0,23
A_25_P00012253	hsa-miR-193a-5p	-1,10	-2,39	-2,31
A_25_P00012257	hsa-miR-193a-3p	-2,34	-3,41	-3,40
A_25_P00010648	hsa-miR-551b	-2,68	-3,91	-3,92
A_25_P00015265	hsa-miR-720	4,83	6,11	6,07
A_25_P00015248	hsa-miR-1280	-1,16	-0,32	-0,38
A_25_P00012070	hsa-miR-139-5p	-2,23	-3,30	-3,40
A_25_P00012138	hsa-miR-200b	-2,51	-1,36	-1,46
A_25_P00012090	hsa-miR-181b	-0,55	0,00	-0,13
A_25_P00012052	hsa-miR-196a	-2,98	-1,58	-1,73
A_25_P00012215	hsa-miR-126	0,83	-0,36	-0,54
A_25_P00012833	hsa-miR-652	-3,72	-4,20	-4,39
A_25_P00014215	hsa-miR-551b	-0,29	-2,50	-2,69
A_25_P00014820	hsa-miR-23a	4,95	4,36	4,15
A_25_P00010471	hsa-miR-99a	4,20	3,61	3,40
A_25_P00012177	hsa-miR-140-3p	1,34	0,58	0,36
A_25_P00012354	hsa-miR-342-5p	-1,07	-0,21	-0,50
A_25_P00012071	hsa-miR-139-5p	0,65	-0,80	-1,09
A_25_P00010438	hsa-miR-130b	-3,53	-2,79	-3,12
A_25_P00010071	hsa-let-7b	6,87	6,42	6,08

ProbeName	SystematicName	Average RP	Avererage CMB	Average Tumour
A_25_P00012265	hsa-miR-200c	-2,77	-1,61	-1,96
A_25_P00010548	hsa-miR-141	-3,27	-2,03	-2,40
A_25_P00012216	hsa-miR-126	3,29	2,27	1,90
A_25_P00012139	hsa-miR-200b	0,45	2,11	1,65
A_25_P00012097	hsa-miR-183	-2,87	-1,45	-1,94
A_25_P00012012	hsa-miR-29a	2,46	1,82	1,32
A_25_P00010547	hsa-miR-141	-0,70	1,07	0,53
A_25_P00012133	hsa-miR-224	-0,41	-1,70	-2,25
A_25_P00012176	hsa-miR-140-3p	-0,84	-1,51	-2,08
A_25_P00012266	hsa-miR-200c	1,58	3,53	2,92
A_25_P00014852	hsa-miR-365	1,90	0,49	-0,12
A_25_P00012145	hsa-let-7i	3,22	2,98	2,32
A_25_P00015230	hsa-miR-1274b	-0,38	2,17	1,50
A_25_P00011584	hsa-let-7a	7,65	7,21	6,52
A_25_P00012134	hsa-miR-224	1,04	-0,62	-1,33
A_25_P00014851	hsa-miR-365	1,40	0,05	-0,67
A_25_P00012375	hsa-miR-151-5p	-0,38	-0,65	-1,37
A_25_P00012193	hsa-miR-145	3,47	2,54	1,82
A_25_P00010072	hsa-let-7c	6,61	5,93	5,19
A_25_P00010999	hsa-miR-19b	-0,71	0,60	-0,18
A_25_P00012141	hsa-let-7g	1,99	1,58	0,79
A_25_P00010086	hsa-let-7a	5,81	5,38	4,54
A_25_P00011984	hsa-let-7e	2,82	2,62	1,78
A_25_P00015231	hsa-miR-1274b	0,43	2,99	2,15
A_25_P00011980	hsa-let-7d	1,76	1,60	0,73
A_25_P00012192	hsa-miR-145	0,92	0,27	-0,61
A_25_P00010073	hsa-let-7c	3,74	3,15	2,27
A_25_P00010990	hsa-miR-25	-0,50	-0,80	-1,70
A_25_P00012085	hsa-miR-34a	0,66	1,54	0,55
A_25_P00010088	hsa-let-7f	5,97	5,64	4,63
A_25_P00014866	hsa-miR-497	3,50	2,86	1,84
A_25_P00011000	hsa-miR-19b	-3,55	-2,41	-3,49
A_25_P00013946	hsa-miR-127-3p	-2,70	-2,21	-3,30
A_25_P00014063	hsa-miR-486-5p	2,12	1,50	0,39
A_25_P00010130	hsa-miR-497	0,22	-0,45	-1,62
A_25_P00010472	hsa-miR-99a	1,80	1,18	-0,01
A_25_P00015173	hsa-miR-1260	1,09	2,07	0,87
A_25_P00010588	hsa-miR-132	-0,65	0,02	-1,18
A_25_P00010589	hsa-miR-132	-2,36	-1,71	-2,97
A_25_P00012446	hsa-miR-451	5,50	4,85	3,44
A_25_P00012447	hsa-miR-451	6,39	5,75	4,32
A_25_P00010475	hsa-miR-100	0,79	0,94	-0,55
A_25_P00010439	hsa-miR-130a	1,97	2,38	0,84
A_25_P00010440	hsa-miR-130a	0,85	1,16	-0,38
A_25_P00010979	hsa-miR-125b	5,52	5,14	3,57
A_25_P00010980	hsa-miR-125b	0,49	0,44	-1,22
A_25_P00010503	hsa-miR-205	1,66	3,05	0,52
A_25_P00010504	hsa-miR-205	2,98	4,29	1,74

Suppl. Table 5: Differentially expressed miRNAs in Malignant tumours (CM) vs Benign tumours (CMB)

ProbeName	SystematicName	Average value CMB	Average value CM
A_25_P00011980	hsa-let-7d	1,73	0,93
A_25_P00010475	hsa-miR-100	1,28	-0,35
A_25_P00010469	hsa-miR-10a	0,29	1,71
A_25_P00015076	hsa-miR-1202	3,05	5,00
A_25_P00015075	hsa-miR-1202	3,13	5,01
A_25_P00014920	hsa-miR-1225-5p	2,08	3,87
A_25_P00014921	hsa-miR-1225-5p	1,92	3,70
A_25_P00014937	hsa-miR-1228	-1,23	0,24
A_25_P00014936	hsa-miR-1228	-1,13	0,30
A_25_P00010979	hsa-miR-125b	5,43	3,74
A_25_P00010980	hsa-miR-125b	0,69	-1,03
A_25_P00015173	hsa-miR-1260	2,26	1,07
A_25_P00013946	hsa-miR-127-3p	-1,95	-3,08
A_25_P00010382	hsa-miR-127-3p	0,45	-0,52
A_25_P00015123	hsa-miR-1300_v13.0	-2,75	-2,14
A_25_P00015249	hsa-miR-1308	-1,13	-0,12
A_25_P00010439	hsa-miR-130a	2,75	1,06
A_25_P00010440	hsa-miR-130a	1,49	-0,14
A_25_P00010589	hsa-miR-132	-1,42	-2,71
A_25_P00010588	hsa-miR-132	0,33	-0,86
A_25_P00013406	hsa-miR-135a*	-2,38	-0,81
A_25_P00013405	hsa-miR-135a*	-4,17	-2,89
A_25_P00013407	hsa-miR-135a*	-1,54	-0,33
A_25_P00013453	hsa-miR-150*	-1,02	0,05
A_25_P00013452	hsa-miR-150*	-1,39	-0,41
A_25_P00013450	hsa-miR-150*	-0,98	0,03
A_25_P00013451	hsa-miR-150*	-1,20	-0,27
A_25_P00012271	hsa-miR-155	-0,72	0,69
A_25_P00011101	hsa-miR-15b	4,30	5,31
A_25_P00012099	hsa-miR-183	0,45	1,54
A_25_P00012246	hsa-miR-188-5p	-1,47	-0,34
A_25_P00012247	hsa-miR-188-5p	-1,08	-0,02
A_25_P00010504	hsa-miR-205	4,57	2,12
A_25_P00010503	hsa-miR-205	3,32	0,88
A_25_P00010995	hsa-miR-210	-0,42	1,56
A_25_P00010204	hsa-miR-22	3,22	4,22
A_25_P00012130	hsa-miR-223	0,47	-0,43
A_25_P00010390	hsa-miR-30b	-2,71	-3,64
A_25_P00010540	hsa-miR-324-3p	-0,25	0,91
A_25_P00014027	hsa-miR-331-3p	2,06	2,84
A_25_P00012419	hsa-miR-423-5p	0,48	-0,29
A_25_P00010977	hsa-miR-425	-0,03	0,86
A_25_P00012446	hsa-miR-451	5,12	3,62
A_25_P00014866	hsa-miR-497	3,18	2,03
A_25_P00010130	hsa-miR-497	-0,13	-1,42
A_25_P00014894	hsa-miR-551b	-1,11	-0,02
A_25_P00011096	hsa-miR-572	0,93	-0,07
A_25_P00010799	hsa-miR-663	-0,22	1,57
A_25_P00010800	hsa-miR-663	-1,85	-0,67
A_25_P00010472	hsa-miR-99a	1,44	0,17



Suppl. Fig. 1: Box plot of the relative expression of 131 probes (81 miRNA) in benign tumours (orange), malignant tumours (red) and normal tissue (green) at FDR=0. MiRNAs are identified through a 3 class SAM analysis.