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microRNAs AS PROGNOSTICATORS IN BREAST CANCER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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Abstract

Breast cancer is a heterogeneous disease. It is the leading cause of death due to cancer among women in the western world. With the increase in incidence of breast cancer and mostly the lymph node negative breast cancer, accurate prognosis is necessary to make the treatment effective and to avoid under and over treatment. In attempt to provide reliable diagnosis, and to find out which therapy is most suitable for a particular patient various prognostic biomarkers have been developed. microRNA is one among the potential biomarkers. These small non-coding RNA molecules exerts its effect through mRNA either by binding directly and specifically to 3'-UTR region (imperfect match) and silencing them or by enabling the degradation of target mRNA (perfect match).

In this study, expression level of nine different miRNAs (let-7b, miR-18b, miR-21, miR-25, miR-29c, miR-106b, miR375, miR505 and miR-150) was investigated using quantitative real time PCR (qPCR). One hundred and twenty-three formaldehyde fixed paraffin embedded (FFPE) tissues from the MMMCP project were used to isolate total RNA. Tumor infiltrating lymphocytes (TILs) were assessed from the samples to observe its prognostic value. Among the miRNAs studied, the biological function of let-7b was examined in ER negative (MDA-MB-231) and positive (MCF-7) breast cancer cell lines. For this, PNATM inhibitor was transfected and the corresponding miRNA was analyzed by using qPCR. The rate of proliferation and migration potential of the transfected cells was studied by wound healing assay. A immunohistochemical screening was performed to see the expression of markers in the let-7b inhibited. Furthermore, metabolic functions of the cells were studied by mitochondrial mito-stress test using a Seahorse XF analyzer. Independent t-test was used to detect significant correlations between miRNA expression and different clinical features of breast cancer

Kaplan- Meier survival analysis showed that high MAI (>10) and high histological grade (≥ 2) were the most important prognostic factors for distance metastasis free survival. Among the nine miRNAs studied, miR-18b, miR-21, miR-25 and miR-150 are associated with high grade while miR-18b and miR-106b are associated with higher proliferation in breast tumor cells. Similarly let-7b and miR-150 were found to be associated with low and high TILs respectively while let-7b, miR-375 and miR-150 were found to be associated with older age (>50yrs). The expression of let-7b in both MCF-7 and MDA-MB-231 cells was successfully knocked out with an inhibitor concentration of 0.5 μ M. However, a wound healing assay performed in order to see the rate of proliferation, in absence of let-7b, did not provide any conclusive results. No distinct difference in the expression of markers was obtained except for Cyclin D1, Cyclin D1 was seen to be expressed higher in the cells transfected with inhibitor in both MCF-7 and MDA-MB-231 cells. Mitochondrial mito stress test done to study the cellular metabolism showed that, the mitochondrial parameters as well as ATP production was lowered in MCF-7 cell transfected with let-7b in comparison to the control. While MDA-MB-cell do not show any significant change the the mitochondrial respiration in absence of let-7b inhibitor. our results confirm the role of Let7b as a tumor suppressor in breast cancer. Furthermore, we confirm the strong prognostic value of proliferation in lymph node negative breast cancer, also the prognostic value of TILs is confirmed.

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Table of Contents

ABSTRACT	III
ACKNOWLEDGEMENT	IV
ABBREVIATIONS	VII
LIST OF FIGURES	VIII
LIST OF TABLES	IX
INTRODUCTION	1
CANCER	1
BREAST CANCER	1
<i>Epidemiology</i>	1
<i>Classification of breast cancer</i>	2
<i>Predictive and prognostic markers of breast cancer</i>	3
<i>Potential prognostic and predictive factors</i>	6
<i>miRNA</i>	6
Biogenesis of microRNA	7
miRNA in cancer	8
miRNA in breast cancer	9
<i>Mitochondrial mito-stress test</i>	11
OBJECTIVE OF STUDY	13
MATERIALS AND METHODS	14
PATIENTS AND SAMPLES	14
RNA ISOLATION	14
CDNA SYNTHESIS	15
REAL TIME PCR	16
TILS EVALUATION	17
CELL LINE EXPERIMENT	18
WOUND HEALING ASSAY	20
CELL BLOCK PREPARATION	21
MITOCHONDRIAL MITO-STRESS TEST	22
DATA NORMALIZATION AND STATISTICAL ANALYSIS	23
RESULTS	24
DETECTION OF TILS	24
MICRORNA EXPRESSION IN TUMOR MATERIAL	26
INHIBITION OF MIRNA LET-7B IN BREAST CANCER CELL LINES	29
WOUND HEALING ASSAY	30
IMMUNOHISTOCHEMISTRY	33
MITOCHONDRIAL MITO-STRESS TEST	36
INGENUITY PATHWAY ANALYSIS	41
DISCUSSION	43
CORRELATION BETWEEN TILS AND CLINICAL FEATURES OF BREAST CANCER	43
CORRELATION BETWEEN MIRNAS AND CLINICAL FEATURES OF BREAST CANCER	43
INHIBITION OF LET-7B IN BREAST CANCER CELL LINES	45
FUTURE DIRECTIONS	47
CONCLUSION	48
REFERENCES	49

APPENDICES57

Abbreviations

ALNs	Axillary lymph nodes
BCL-2	B-cell lymphoma 2
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A
cDNA	Complementary Deoxyribonucleic acid
CK5/6	Cytokeratin 5 and 6
DGCR8	DiGeorge syndrome chromosomal region 8
ECAR	Extracellular acidification rate
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
HER2	Human epidermal growth fact
MAPK	Mitogen-activated protein kinase
miRNA	micro RNA
OCR	Oxygen consumption rat
PBS	Phosphate buffered saline
pCR	Pathological complete response
PCR	Polymerase chain reaction
PDCD4	Programmed cell death
PI3K	Phosphatidylinositol 3-kinase
PPH3	Phosphorylated phosphohistone H3
PR	Progesterone receptor
Pre-microRNA	Precursor microRNA
Pri-microRNA	Primary microRNA
PTEN	Phosphate and tensin homolog
qRT-PCR	Quantitative real-time polymerase chain reaction
RAS	Rat sarcoma
RISC	RNA induced silencing complex
ROC	Receiver-operating curve
stRNA	Small temporal RNA
TGF β	Transforming growth factor beta
TGF β R2	Transforming growth factor beta receptor 2
TILs	Tumor Infiltrating Lymphocytes
TKRs	Tyrosine kinase receptors
TNM	Tumor-node-metastasis
UTR	Untranslated region
WHA	Wound healing assay
WHO	World Health Organization

List of figures

Figure 1. Hallmarks of cancer	1
Figure 2. Seahorse XF Cell Mito Stress	11
Figure 3. Modulators of Electron transport chain(ETC) used in seahorse mito stress test. ...	12
Figure 4 Pattern of cDNA and miRNA primers used for PCR.....	16
Figure 5. Mechanism of fluorescence from SYBR green dye during qPCR	17
Figure 6: Standardization and guidelines for TILs evaluation	18
Figure 7. A chamber of hemocytometer with (B) Enlarged view of one major square	19
Figure 8. Wound healing assay	21
Figure 9. Different parameters and the rate measurement equations used by the report generator for mite-stress test by XF analyzer.	23
Figure 10. Distant metastasis free survival curve for ER expression and TILs.....	26
Figure 11. Expression level of miRNAs for different prognostic features	28
Figure 12. Amplification curve obtained in qPCR of MCF-7 and MDA-MB-231.....	29
Figure 13. Images of wound healing assay onMCF-7 cell line at different interval of time. ...	31
Figure 14. Images of wound healing assay on MDA-MB-231 cell line at different interval of time.	32
Figure 15. Wound healing assay of MCF-7 cells.	32
Figure 16. Images from immunohistochemical staining of different markers on FFPE MDA-MB-231 cell block.....	35
Figure 17. Amplification curve obtained in qPCR of MCF-7 and MDA-MB-231 from the cell block material.	35
Figure 18. Differences in OCR response to mitochondria stress test between control and inhibitor in MCF-7 cells.....	37
Figure 19. Different parameters measured in Seahorse XF Cell Mito Stress Test	38
Figure 20. Extracellular acidification rate (ECAR, mpH/min) measured by XF analyzer in MCF-7 cells.....	39
Figure 21. Differences in OCR response to mitochondria stress test between control and inhibitor in MDA-MB-231 cells.	39
Figure 22. Different parameters measured in Seahorse XF Cell Mito Stress Test	41
Figure 23. Extracellular acidification rate (ECAR) measured by XF analyzer in MDA-MB-231 cells.	41
Figure 24. Top target network for experimentally observed targets of let-7b obtained from IPA.	42

List of tables

Table 1 The TNM classification for staging of breast cancer	3
Table 2. Histological grading system recommended by WHO.....	4
Table 3. Grading of breast cancer using the Nottingham grading system.	4
Table 4. miRNAs involved in breast cancer	9
Table 5. SeahorseXF cell mito stress test reagents.	12
Table 6: Reagents used for cDNA preparation.....	15
Table 7. Volumes of reagents and cDNA used during the real time PCR.	17
Table 8: Volume of medium and inhibitors used for the cell block formation.	22
Table 9. Distant metastasis free survival in lymph node negative breast cancer with Kaplan-Meier analysis.	24
Table 10: Independent t-test between TILs and clinical features	25
Table 11. Kaplan-Meier survival analysis for ER and TILs.	25
Table 12. Independent t-test between miRNAs and different clinical features of the breast cancer.	26
Table 13. Proliferation markers in FFPE cell blocks of MCF-7 and MDA-MB-231.	36
Table 14. list of experimentally observed targets of miR-let-7b common in IPA, miRtarbase and exiqon.....	42
IV. Table 15. Data for quantification of wound area in MCF-7 and MDA-MB-231 cells: ..	62
Table S1. comparison of nature of samples between two experiments.	64

Introduction

Cancer

Cancer is one of the most researched topics in the last decades, however it is still a poorly understood disease. Much debate is still on its origin either it arises from a single cell or is it a result of aberrant behavior of a group of cells. Different models are proposed to describe the origin and progression of cancer. The clonal evolution model by Nowel *et al.* (1), first describe development of malignant tumor with time. This model describes cancer either as monoclonal or polyclonal in origin. The progression of tumor is due to Genetic variability among tumor cell population leading to clonal expansion of more aggressive tumor cell population (2).

For a tumor to arise and develop it must bypass a number of processes that controls normal tissue homeostasis (3), for example evasion of programmed cell death, insensitivity to antigrowth signals, avoiding immune destruction, sustained angiogenesis, tissue invasion and metastasis which are described as the hallmarks of cancer by Hanahan and Weinberg (4).

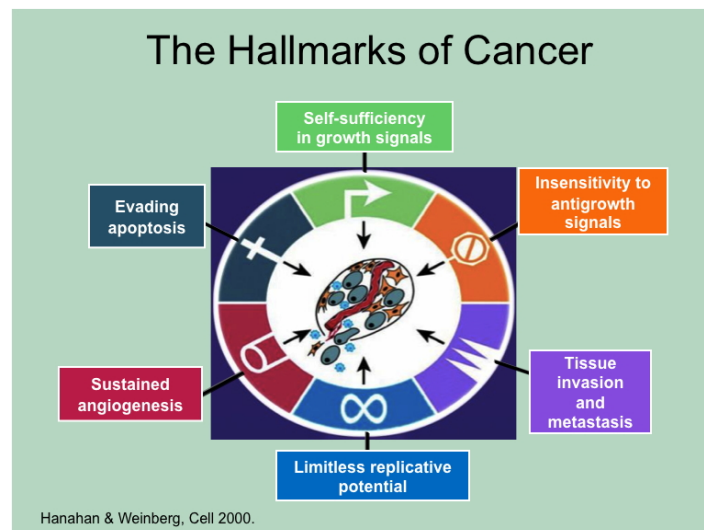


Figure 1. Hallmarks of cancer (4)

Breast Cancer

Epidemiology

Breast cancer is the second most common cancer after Lung cancer worldwide and most frequent cancer among women comprising 25% of all cancers. But because of its relatively favorable prognosis it falls in the fifth rank in terms of cause of overall cancer death and second cause of cancer death among women (5). Incidence rates varies highly from underdeveloped, developing and developed country. It is 19.3 per 100,000 in women in Eastern Africa and 89.7 per 100,000 in Western Europe, and below 40 per 100,000 in other developing countries (6). Similarly the survival rate also varies greatly being 80% or more in high income countries, around 60% in middle- income and below 40% in low income countries (7). This fact can be related to the lack of early detection programs in the less developed country which results in

the diagnosis of breast cancer in the late stage (6). Besides region and income, breast cancer incidence also varies on age, race and ethnicity. The rate of death due to breast cancer increases generally with age. Research from American Cancer Society shows that higher percentages of death (88%) and new cases (79%) are seen in women with the age of 50 years or more while in 2006-2010 median age was 61 years at the time of cancer diagnosis (8). Racial difference in the epidemiology is considered to be due to the endogenous hormones. Pinheiro and colleagues showed that higher estrogen and growth factor level may lead to higher risk of breast cancer (9).

In Norway and other European countries, mammography screening program is carried out and a lots of researches has been done to see the impact of those screening programs on the cancer mortality. It is found that breast cancer mortality was reduced with 26-28% for the women involved in a screening program with follow up of 6-11 years (10,11). The incidence of breast cancer is found to increase by two fold since the beginning of registration of Mammography screening program till 2014, this is because of constantly increasing awareness of breast cancer, introduction of breast cancer screening program to all women and better diagnostic methods (12). Breast cancer is the second most common cause of cancer death in Norwegian women with 663 deaths in 2014 (12). Long term survival is seen higher in patients diagnosed at the age of 50-59 than among patients diagnosed under the age of 50, this is due to a more aggressive tumor type in the younger age and impact of screening in the older age (12). It is estimated that more than 200,000 new cases of invasive breast cancer will be diagnosed only in U.S in 2016, and 1 in 8 U.S women will develop invasive breast cancer in her life time (13).

Classification of breast cancer

Breast cancer is a heterogeneous disease because of its variable clinical prognosis and biological profiles (14). The most intensively identified and studied subtypes of breast cancer in the latest time are Luminal A, Luminal B, epidermal growth factor negative (HER2)-enriched, Basal-like and Claudine-low along with normal breast like group (15).

Luminal A is the most common subtype including about 40% of breast cancer (16). These are Estrogen receptor positive (ER+) and/or Progesterone receptor positive (PR+) and Human (HER2-) which makes them responsive to hormonal therapy. These are slow growing and less aggressive. They have good short term prognosis but long term survival does not differ from other subtypes (17). Luminal B subtype constitutes about 10-20% of breast cancer and are ER+ and/or PR+ like luminal A. Luminal A and B are distinguished on the basis of high proliferation rate (18).

Basal like subtype resembles triple negative subtypes. These are about 10-20% of breast cancer and common in African American women, and those with BRCA1 mutation (16). Triple negative breast cancer (TNBC) are so called because they lack Estrogen receptor (ER), Progesterone receptor (PR) and human epidermal growth factor2 receptor (HER2/neu) expression and do not have any viable targeted therapy (19,20). Because of the lack of hormonal receptors, TNBC patients have relatively poor outcome and cannot be treated with hormonal therapies targeted to HER2. Approximately 12-17% of breast cancer patients have triple

negative breast cancer. and associated with 30-50% of mortality in lymph node negative breast cancer (21).

Predictive and prognostic markers of breast cancer

Predictive and prognostic factors are used in the management of breast cancer. It is necessary to know the the differences between these two factors. Prognostic factor is any feature or variable which is associated with the natural history of disease, while predictive factors defines the effectiveness of the given therapy (22,23). The predictive and prognostic factors that are in the Norwegian Breast Cancer Guidelines (<http://nbcg.no/>) are described below.

Axillary lymph node status

Axillary lymph node involvement is one of the most important prognostic factor in early stage breast cancer. During the time of surgery, one or more axillary nodes are surgically removed and are examined for metastases. Micro metastasis less than 0.2 cm is considered to have clear prognostic significance and is defined as lymph node negative. The number of positive lymph nodes is correlated with the risk of distant recurrence (24) and used in determination of patients for systemic therapy (22).

Tumor size

Tumor size is considered both as independent prognostic factor and as a predictor of axillary node status. large sized tumor has worse prognosis with high chances of nodal metastasis (22). On the basis of three characteristics i.e., tumor size (T), spread to regional lymph node (N) and distant metastasis (M), TNM (tumor-node-metastasis) staging system is developed to characterize the nature of tumor as illustrated in table1.

Table 1 The TNM classification for staging of breast cancer (25)

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
T1	Tumor \leq 20 mm in greatest dimension
T1mi	Tumor \leq 1 mm in greatest dimension
T1a	Tumor $>$ 1 mm but \leq 5 mm in greatest dimension
T1b	Tumor $>$ 5 mm but \leq 10 mm in greatest dimension
T1c	Tumor $>$ 10 mm but \leq 20 mm in greatest dimension

T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)

Histologic grade

Histological tumor grade is measured based on degree of differentiation of the tumor tissue. It is simple, inexpensive and easy method which can be performed by well-trained pathologists with the help of standard protocol (26). Nottingham grading system is internationally accepted grading system (23) which is based on the three parameters, tubular formation, nuclear pleomorphism and mitotic count in which dividing cancer cells are counted (26). These parameters are scored differently and are shown in table 2.

Table 2. Histological grading system recommended by WHO (27). *counting with an objective with a field diameter of 0.59mm

Tubule Formation (% of Carcinoma Composed of Tubular Structures)	Score
> 75%	1
10-75%	2
< 10%	3
Nuclear Pleomorphism (Change in Cells)	Score
Small, uniform cells	1
Moderate increase in size and variation	2
Marked variation	3
Mitosis Count (Cell Division)*	Score
1-9 mitoses	1
10-19 mitoses	2
≥ 20 mitoses	3

By adding the scores from each category three grades are made as presented in the table 3.

Table 3. Grading of breast cancer using the Nottingham grading system (23).

Grade	Description	Total Score
Grade 1(low)	Well differentiated breast cancer cells	3-5
Grade 2 (Intermediate)	Moderately differentiated breast cancer cells	6 or 7

Grade 3 (high)	Poorly differentiated breast cancer cells	8 or 9
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Age

Age is one of the most important risk factor in breast cancer patient, and more prevalence is seen in elderly than in younger patients (28). The application of adjuvant chemotherapy is also influenced by age. Younger patient (<35 years) have higher chances of relapses because their tumor often have high proliferation and other more aggressive clinicopathological features, in comparison to older patients (29). In women with age less than 50 years of age tumors with ER-, Basal like HER2, overexpression are more common while in the patients with the age more than 70 ER+, luminal A and B are more common (30).

Hormonal receptors (ER/PR)

ER is the receptor for estrogen hormone and PR is the receptor for progesterone hormone, both of which are steroid hormones. Both of these hormones plays role in maturation of sex organs and control menstruation cycle. Presence of ER and PR are used both as predictive and prognostic factors, but the prognostic effect is limited and should be assessed in the absence of adjuvant tamoxifen (24). During administration of adjuvant tamoxifen, ER and PR presence are used as predictive factor to observe the benefit of therapy (24). It is shown that 70-80% of breast cancer expressing both ER and PR regressed with hormonal therapy. ER and PR status are analyzed by the use of immunohistochemistry (31).

HER2

Human epidermal growth factor receptor 2 (HER2) which belongs to the family of epidermal growth factor receptor (EGFRs) is amplified and/or overexpressed in 20-30% of all breast cancers. Its overexpression is associated with negative prognostic and predictive factors as well as worse clinical outcomes. Patients with HER2 positivity have increased resistance to endocrine therapy due to greater likelihood of decreased ER receptor. The disease may also be more aggressive in Her2 positive patients (32). Routine evaluation of HER2/neu protein overexpression is evaluated with immunohistochemistry. Trastuzumab, a recombinant humanized IgG monoclonal antibody is used to control the concentration of HER2. By the down modulation of HER2 Trastuzumab arrest the cell cycle by inhibiting phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (33,34).

Ki67

Ki67 is an antigen which is present in the nuclei of the cell in all phases (G₁, S, G₂ and M) of cell cycle except the G₀ phase. Since it is present in all proliferating cells, its expression is the marker to determine the proliferating cells (35). Interpretation of Ki67 is done by IHC staining on the tissue samples with MBM1 antibody and scored in percentage. Some studies have suggested that high Ki67 expression is related to worse distance metastasis free-survival and overall survival in both node-negative and node-positive patients and better response to chemotherapy (36).

Potential prognostic and predictive factors

Proliferation measurement

MAI one of the simple, well-reproducible proliferation associated prognostic factor (37). Large number of retrospective and prospective studies has been carried out to ensure its prognostic value (38–41). Among them is Multicenter Morphometric Mammary Carcinoma Project (MMMCP) which is large prospective multicenter study carried out with the follow up of longer than 10 years (42). According to MMMCP, patient with high MAI (>10) had low survival rate (70%) than patients with low MAI (<10) which was 92% (37). Although it is simple and reproducible, pathologists are not keenly interested in MAI counting because of time it consumes, and poor section quality sometimes makes difficulties in its reproducibility Mitotic figure identification can be difficult if it get more condensed due to hypoxia, fixation delay and suboptimal fixation (42) Phosphorylated Histone H3 (PPH3) is expressed during mitotic chromatin condensation. Its expression is having good correlation with MAI so it could be a reliable prognostic marker for invasive lymph node negative breast cancer. Another cell cycle-related protein, Cyclin-D can also be developed as suitable prognostic biomarker. however further investigation and validation is necessary to establish PPH3 and Cyclin D as potential biomarkers.

Tumor infiltrating lymphocytes

In recent days Tumor infiltrating lymphocytes (TILs) is been evaluated as an important predictive and prognostic biomarker in breast cancer. According to Gu-Trantien *et al* the leucocyte infiltrate in breast tumor composed of 75% T-lymphocytes, 20% B-lymphocytes, less than 10% monocytes around 5% of natural killer and natural killer T-cells (43). TILs may be stromal infiltrating the stroma or intra-epithelial (intra-tumoral) infiltrating tumor cell islets (44). Patients with triple negative breast cancer are found with high TILs (45)

In 2013, an international working group of breast cancer experts recommend a model for evaluating of TILs, focusing on (i) what areas to examine in the tumor, (ii) how to score TILs and (iii) why TILs are clinically important (46).

Change in number of circulating T-Lymphocytes is also used as the indicator of systemic immune activation after the administration of systemic chemotherapy (47) so TILs are used as predictors of response to neoadjuvant systemic treatments in breast cancer (44).

miRNA

miRNAs are naturally occurring small, 20- to 24-nucleotide long RNAs which regulate a number of physiological processes, including cell proliferation, differentiation, apoptosis and development. They are found in a number of eukaryotes including plants and animals (48) and regulates the gene expression by post-transcriptional modification (49) either by catalyzing the cleavage of messenger RNA or by repressing its translation (48). A single miRNA molecule can target up to 200 mRNAs molecules and a single mRNA can be targeted by more than a miRNA molecules (50,51).

Members of miRNA family were first discovered in nematode *Caenorhabditis elegans* as small temporal RNAs (stRNAs) which regulates their developmental transition (49). And later they

were found and studied in different eukaryotes including human. miRNA is predicted to regulate at least 30% of protein coding genes and constitutes almost 1.5% of total human genome (52–55).

Biogenesis of microRNA

The biogenesis of miRNA in human is two-step process involving different events within the nucleus and cytoplasm in which long primary miRNA (Pri-miRNA) transcript is processed to form mature miRNA through different stages of enzyme catalysis trimming and transport (54,56).

Primary miRNA precursor molecule is generated in nucleus from miRNA gene transcription by RNA Pol II which then undergo nuclear cleavage by microprocessor complex forming precursor miRNA (pre-miRNA) which is 70-bp long. The microprocessor complex consists of DGCR8 and Drosha. Thus formed pre-miRNA is exported to cytoplasm by nucleocytoplasmic transporter containing Exportin 5 and Ran-GTP (54).

In cytoplasm, the pre-miRNA is cleaved by RNase III, Dicer forming miRNA duplex (miRNA:miRNA*). The duplex consists of two two strands among which one act as a guide strand and the next get degraded later. The duplex unwind and guide strand combine with RISC complex which contain GW182 and Argonaute (AGO) protein (55). Helicase enzyme aids in unwinding of the duplex.

The mature miRNA in combination with RISC complex repress the mRNA by binding to complementary site in 3' untranslated region of the target mRNA (54–56,58). The repression of mRNA by miRNA complex occurs in two ways; imperfect match lead to blockage of the protein synthesis and perfect base pairing causes cleavage of the target mRNA (55).

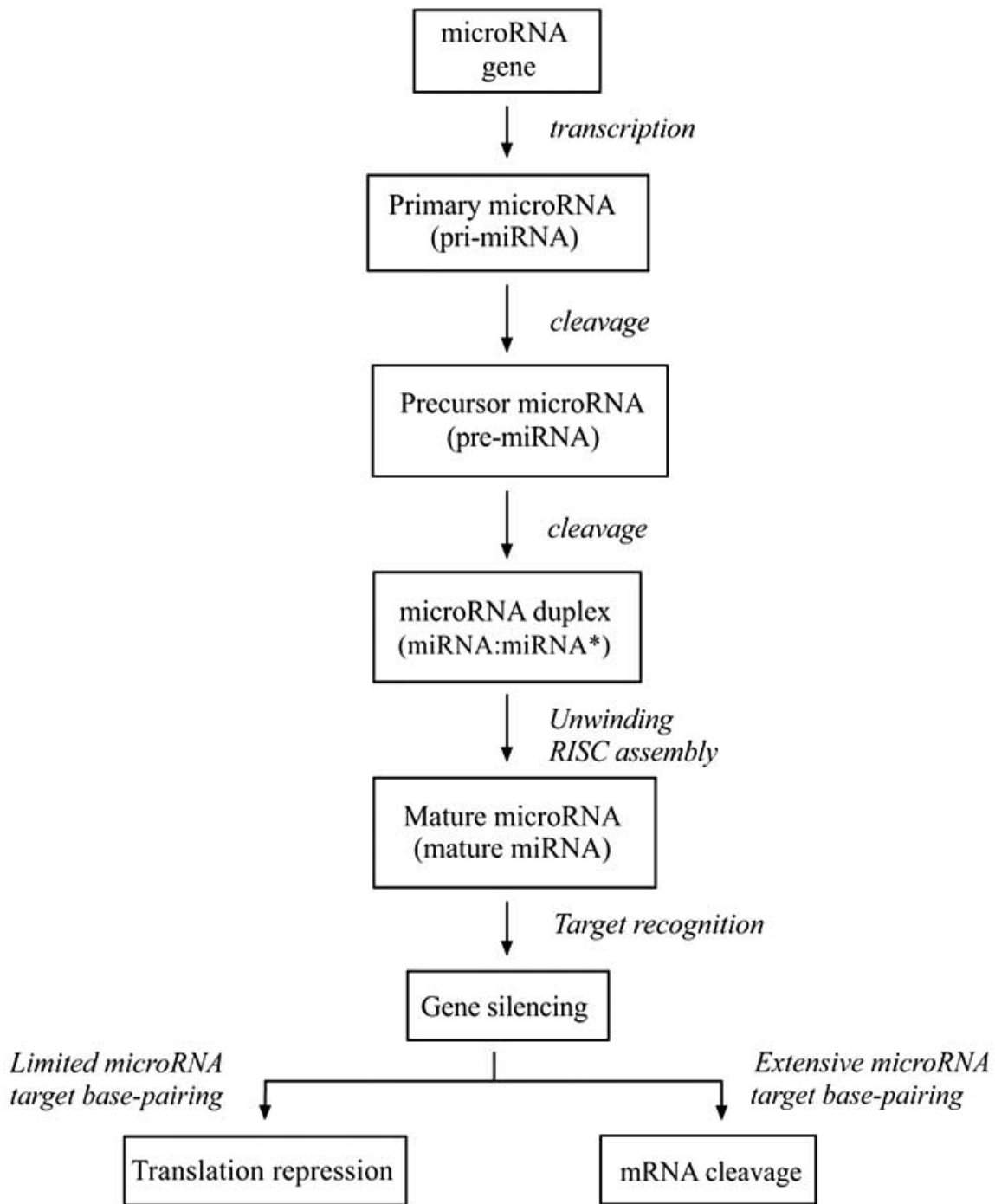


Fig. MicroRNA maturation and function (54).

miRNA in cancer

Cancer relevant processes includes proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism and all these processes are influenced by miRNAs (59). A number of genes are involved in the cell proliferations, differentiation and apoptosis, and a cancer to occur some dysregulation in these should occur. The genes are classified as oncogenes and tumor suppressor genes. Overexpression of oncogenes leads to cancer development, while the

loss of function or under expression of tumor suppressor genes leads to the development of cancer (60–62). Some miRNAs also exhibit anti-metastatic properties (63). As there is involvement of miRNA as regulator of gene expression in the control of stem cell development, this theory can be linked to the development of tumor by miRNA with self-renewal of stem cells. Additionally, disturbance in the miRNA processing and alteration on miRNA expression may result in generation or maintenance of tumors (55).

miRNA in breast cancer

Despite of intensive study and lots of progress in breast cancer researches in the last decades. early diagnosis and management of breast cancer patient along with the management of unwanted response and resistance to the adjuvant therapies is still a challenge. Because of all these challenges, new and prominent biomarkers are needed to improve diagnosis and prognosis. In the recent years miRNA is studied as one of the probable candidate for this (64). The dysregulation of miRNA in human breast cancer was first demonstrated by Iorio and colleagues by using miRNA microarray method suggesting that miRNA can act both as potential tumor suppressor or oncogenes in which they found miR-10b, miR-125b and miR-145 were down-regulated and miR-21 and miR-155 were upregulated (65). Different miRNAs plays important roles in breast cancer progressions involving in anti-apoptosis, cell cycle dysregulation, metastasis (66).

Table 4. miRNAs involved in breast cancer

miRNAs	Targets	Functional Pathways
Tumor Suppressor miRNAs		
Let-7	H-RAS, HMGA2, LIN28, PEBP1	Proliferation, Differentiation
miR-29c	TNFAIP3	Inflammation and immune response
Oncogenic miRNAs		
miR-18b	ER α	Proliferation
miR-21	TPM1, PDCD4, PTEN, MASPIN	Increase tumor growth and decrease apoptosis
miR-25	BIM	Proliferation and apoptosis
miR-106b	P21	Proliferation and EMT transition
miR-505	ASF/FS2	unknown

After the discovery miRNA as small temporal RNA in *C.elegans*, *let-7* family is the second miRNA to be discovered (66). *Let-7* family which consists of thirteen members in human is the one the first identified tumor suppressor miRNA. *Let-7* are found in many species and its altered expression in human is associated with various cancer types (67). *Let-7* miRNAs regulate the RAS oncogene. The expression of *let-7* is inversely proportional to the expression of RAS oncogenes in lung tumors, so low expression of *let-7* helps in tumorigenesis, due to increased expression RAS oncogene(8). In breast cancer initiating cells (BT-IC) RAS is the direct target of *let-7*. Some studies suggest that self-renewal of breast cancer stem cells takes place in lack of *let-7* (66). Downregulation of *let-7b* expression in breast cancer samples with lymph node metastasis or higher proliferation index is found which suggest the association of its reduced expression with poor prognosis (67).

miRNA-21 is reported to be involved in all phase of breast cancer tumorigenesis (66). Ioror *et al.*, 2005 in their experiment found that aberrant expression of miR-21 is directly correlated with breast cancer and predict the TGFB gene as the target of miR-21 explaining its oncogenic property (65). Transforming Growth Factor beta (TGFB) is a cytokine which functions in cell growth, cell proliferation cell differentiation and apoptosis. Thus by targeting on this polypeptide miR-21 helps in oncogenesis. High expression of miR-21 is also found to be associated either with lymph node positivity or with the development of metastasis. An experiment in xenograft carcinoma mouse model shows the inhibition of tumor growth with anti-miR-21, where MCF-cell transfected with anti-miR-21 grow slower compared to the control, while the results of immunostaining with anti-Ki-67 was also weaker in anti-miR-21 suggesting its important role in tumorigenesis (68). Anti-miR-21 is also reported to increase cell apoptosis by downregulating the expression of bcl-2 (68). Expression of tumor suppressor PDCD4 (programmed cell death-4) is also found to be inhibited by miR-21 (69). A tumor suppressor gene, PTEN is also an important target for miR-21 (70).

miR-29c belongs to the human miR-29 family which has three members including miR-29a, miR29b and miR-29c. Mature form of these miRNAs are highly conserved in human and mouse and rat (71). Many studies shows that downregulation of miR-29 family results in aggressive form of cancer or relapse (72–75). Jonsdottir *et al.*, from their result mention that miR-25 is found to be up-regulated in highly proliferative tumor without ER α or with CK5/6 all of which are the indicators of low apoptosis (76). miR-25 is also found to suppress the tumor growth in colon cancer by targeting Smad7 (77). miR-150 is associated with microenvironment in breast cancer and associated with better prognosis due to linkage with gene for immune response (78). miR-18b is among those miRNAs which directly targets ER α but its expression is not found to have correlation with ER α protein expression. Low expression of miR-18b is found to have association with improved survival in HER2-negative breast cancer (79) miR-505 is associated with apoptosis (76,80) and it can be observed when the cell lines are transfected with miR-505 for this miR-505 targets alternative splicing factor/splicing factor 2(ASF/SF2) (80).very few information and data can be can be found on miR-375 in breast cancer. Some studies shows that miR-375 expression is high in ER α positive and CK5/6 negative tumors (76).

Mitochondrial mito-stress test

Normal cells primarily use energy obtained from mitochondrial oxidative phosphorylation for their cellular processes, while most cancer cells are dependent on aerobic glycolysis. This phenomenon is defined as “Warburg effect”. Metabolism of glucose through oxidative phosphorylation produces 36 ATPs, while the glycolysis generates only 2 ATP molecules from a glucose molecule. Despite of very low ATP production by aerobic glycolysis in comparison to mitochondrial oxidative phosphorylation, the actual reason behind the utilization of this pathway by cancer cells is still unclear (81). This phenomenon observed in cancer cells is utilized in cancer researches and one of them is mitochondrial mito-stress test performed in XF-analyzer.

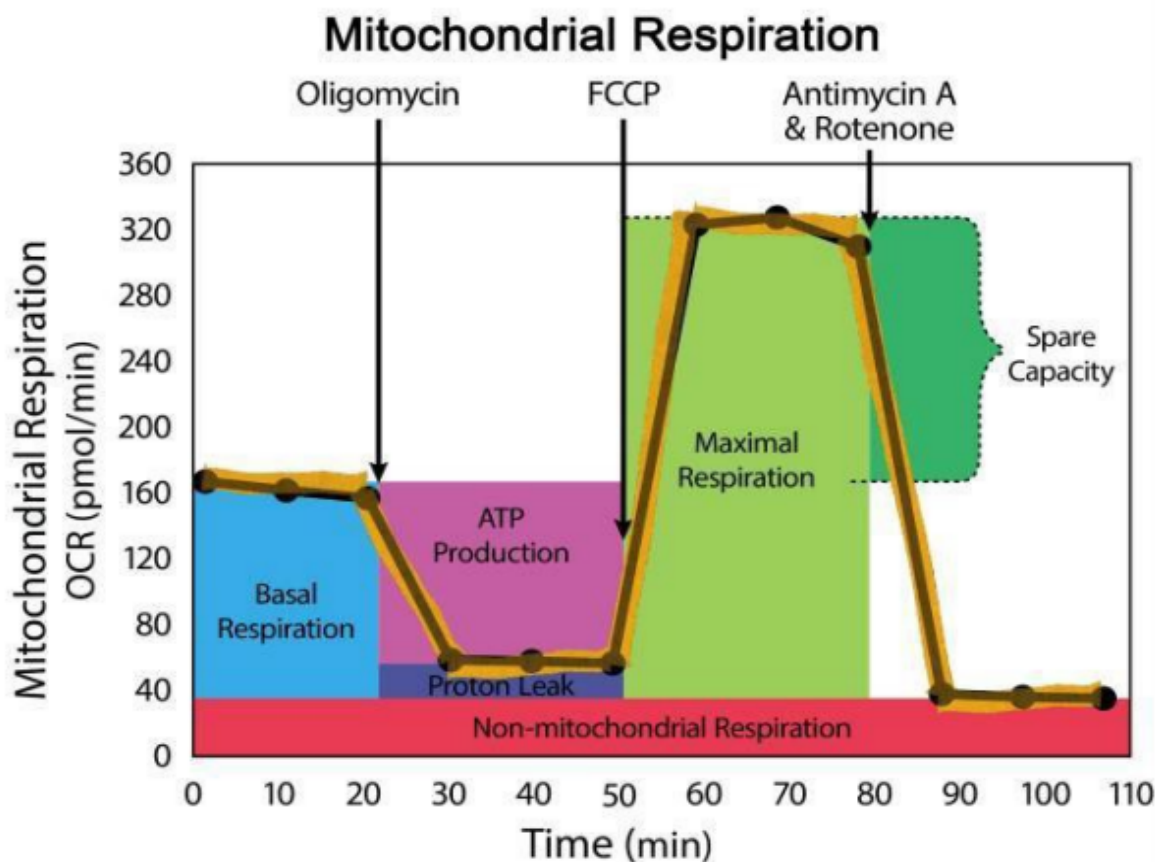


Figure 2. Seahorse XF Cell Mito Stress: Test profile of the key parameters of mitochondrial respiration. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration (82).

XF analyzer monitors the above mentioned two major energy pathways of the cells by measuring the O_2 consumption and proton release in the medium surrounding the cells. The rate of oxygen consumption from the medium is measured as respiration kinetics which is presented as Oxygen Consumption Rate (OCR) (82). During lactic acid production by glycolysis the proton (H^+) is released in the medium, this released proton is measured by the XF analyzer as Extracellular Acidification Rate (ECAR). By measuring the respiration and glycolysis simultaneously in real time and the shift between the two pathways during

pathological conditions, XF analyzer provides greater insights in the cancer researches (82). In addition, by using different mitochondrial modulators, Oligomycin, FCCP and a mix of rotenone and antimycin A, different parameters are measured (82).

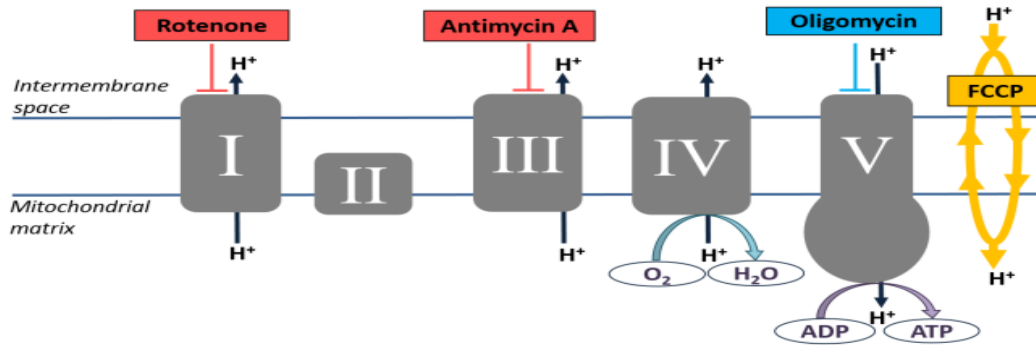


Figure 3. Modulators of Electron transport chain(ETC) used in seahorse mito stress test. Oligomycin inhibits ATP synthase (complex V), FCCP uncoupled oxygen consumption from ATP production and rotenone and antimycin A inhibit complexes I and III respectively (82).

Table 5. SeahorseXF cell mito stress test reagents (in order of injection) (82).

compounds	ETC target	Effect of OCR
Oligomycin	ATP Synthase (Complex V)	Decrease
FCCP	Inner Mitochondrial Membrane	Increase
Rotenone/antimycin in A	Complex I and III (respectively)	Decrease

Objective of study

Breast cancer is a leading cause of mortality due to cancer among women. Around 80% of all breast cancer are lymph node negative. Due to variability in the accuracy of commonly used prognosticators, it results in an unbalanced treatment of the breast cancer patients. Due to which the patients who are not in need of chemotherapy are treated with high dose while those who needs to be treated are getting under treatment. Patients showing almost same clinical and pathological signs may also have different tumor profile and required different treatments. To minimize these inaccuracies in treatment and to find a better solution and define it properly, a suitable prognosticator is necessary. The main aim of this study was:

1. To observe the expression pattern of nine different miRNAs in FFPE tissues by means of qPCR and to identify their prognostic value in combination with different traditional prognosticators in lymph node negative breast cancer.
2. To determine the optimal concentration of let-7b PNATM inhibitor that could knock down the expression of let-7b, in order to examine the biological function of let-7b in ER negative and positive breast cancer cell line.
3. To perform and to analyze wound healing assay for both ER+ and ER- breast cancer cells transfected with inhibitor of let-7b and study the effect of let-7b in proliferation rate and compare with controls.
4. To perform immunohistochemical screening in order to observe the effect of inhibition of let-7b in possible biological target.
5. To access the tumor infiltrating lymphocytes (TILs) in the FFPE slides and study the correlations between the clinical features of breast cancer and the presence of immunological cells to observe relevance of TILs as immunological biomarker in cancer therapy.

Materials and methods

Patients and samples

All the samples were obtained from the patients enrolled in MMMCP project (83). The project was set up to investigate the prognostic value and reproducibility of routine assessments of the morphometric Multivariate Prognostic Index (MPI) and other quantitative parameters in comparison with classical prognosticators in breast cancers. The classical pathologic parameters include tumor size and lymph node status while the quantitative parameters include mean nuclear area, DNA index and MAI. Patients included in the project were diagnosed with primary breast cancers from 34 different hospitals of Netherlands. The samples were collected from October 1, 1987 through January 1, 1990. All samples included in this study were obtained from LN-negative patients which were not treated with any systemic adjuvant therapy. This may add up to study the biology of cancer.

FFPE tissues which were fixed after the measurement of post-surgical size from fresh specimen were preserved in suitable condition. All the preserved tissue samples along with H&E slides included in the study were provided from Department of pathology at Stavanger University Hospital (SUS) Stavanger Norway.

RNA isolation

Background

It is the process of isolation of all the RNA present in the sample. Since the samples used in the study were FFPE tissues the protocol varies with isolation from simple blood cells or fresh tissues without paraffin. The total RNA including mRNA and miRNA were isolated. As we are focusing on miRNAs the process may be more favorable in case of destruction of the shape of RNA since miRNAs are very small in size. However, there are few points to be considered during processing FFPE samples; age of the block, if they are fixed before 25 years and the moisture content in them affects the yield, plus how long they were sitting in formalin before they were dehydrated and embedded.

Protocol

MiRNeasy kit from Qiagen, (Germany) was used for the isolation of total RNA from the FFPE tissue samples. The first step includes the deparaffinization of the sample, for that Xylene was used. 100% ethanol was used to remove the remaining xylene. Similarly, ethanol was removed by evaporation. The unwanted cellular components except RNA which include protein, DNA, RBC and cellular debris were removed. For that, the sample was treated with adequate amount of buffer PKD followed by proteinase K, DNase booster buffer followed by DNase, buffer RBC and finally with ethanol with subsequent centrifugations and incubations. Then the lysate was passed through RNeasy MinElute spin column specially made for the isolation of total RNA from FFPE tissue. After adequate wash with Buffer RPE, total RNA was eluted with the help of 20 μ l nuclease free water. The complete protocol for the isolation of total RNA from FFPE provided by the manufacturer is attached in appendix I.

After isolation of total RNA, the concentration was measured in spectrophotometer

(Thermoscientific NanoDrop Nd-2000C with cuvette). 1.5ul of sample was used to measure the total RNA. Sample absorbance is calculated as:

$$\text{Absorbance} = -\log\{\text{intensity}(\text{sample})/\text{Intensity}(\text{blank})\}$$

The ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA or RNA. The ratio of ≈ 2 is generally accepted as pure for RNA. Lower ratio indicates the presence of proteins phenols or other contaminants that absorbs strongly at near 280nm.

cDNA synthesis

The RNA isolated above was used to synthesize cDNA for further processing. For that, miRNA LNA[™] Universal RT microRNA PCR kit (Exiqon) was used.

single step quantitative reverse transcriptase PCR reaction was done using 10ng of total RNA. First the RNA sample was diluted to 5ng/ μ l using nuclease free water, cDNA mix was made according to the protocol provided by the manufacturer (appendix II). 8 μ l of cDNA mix was added with 2 μ l of 5ng/ μ l RNA sample, mixed well and was incubated in the thermostat plus machine. The reaction parameter were set as 42°C for 60 minutes and at 95°C for 5 minutes and immediately cooled to 4°C and the prepared cDNA was stored at -20°C for downstream processing.

Table 6: Reagents used for cDNA preparation

cDNA mix Reagents	Volume(μl)
5x Reaction buffer	2
Nuclear-free water	4,5
Enzyme mix	1
RNA spike ins	0,5
Template totRNA (5 ng/ μ L)	2
Total	10

Reagents in each tube

cDNA mix	8 μ l
Template total RNA (5 ng/ μ L)	2 μ l

Incubation and heat inactivation

60 min at 42°C

5 min at 95°C

Cool to 4°C

Store at 4°C or freeze

Real Time PCR

Background

It is an advanced form of PCR in which every single steps during the amplification process can be monitored so called real time. The cDNA obtained above was amplified by using Real time PCR machine, Light cycler 480 (Roche, Zurich, Switzerland). The amplification occurs in three steps, denaturation, annealing and extension. SYBR® Green dye was used to detect the amplified DNA product. SYBR green is a fluorescent dye which binds nonspecifically with the amplified double stranded DNA. Since some primer dimers may also be formed they are also detected with this dye so it is important to use specific primers as possible.

Protocol

Roche Light cycler 480 multiwell plate 96, white was used for running the PCR. PCR mix consists of SYBR green master mix and PCR primers mix. It was made as per the volume ratio mentioned in table 7. The cDNA sample was diluted 1:80 times in RNase free water. Reaction volume in each well was 10µl containing 6ul of PCR mix and 4ul of 1:80 times diluted cDNA. Two cDNA samples were run in one 96 well plate with each 11 miRNA primers and one spike in in triplicates as in figure 4. Two housekeeping genes namely miR-24 and miR-26 were used to check the consistency of the tests. One positive control and one negative control were run in each plate. cDNA synthesized from from small human chorion RNA (sRNA) was used for positive control, while RNase free milli-Q water was used as negative control. The plate was centrifuged at 1200rpm for 1 minute before loading. PCR was carried out with 40 independent cycles of 95°C for 10 seconds, continued with 40 cycles of 60⁰ for 60 seconds each. Finally melting curve analysis was done.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
	Let-7b	miR18b	miR21	miR25	miR29c	miR106b	miR375	miR505	miR150	miR24	miR26b	spike in

Figure 4 Pattern of cDNA and miRNA primers used for PCR; first three row, A, B and C is triplicates of sample 1, D, E and F is triplicates of sample 2. G is positive control and H is negative control.

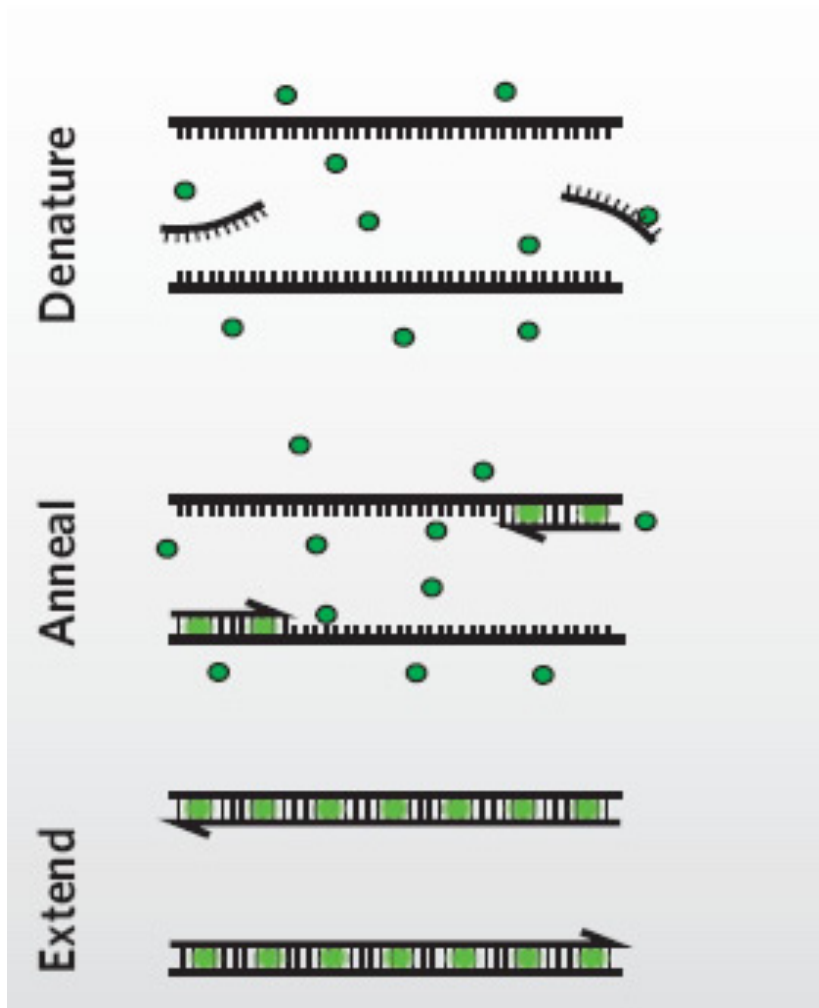


Figure 5. Mechanism of fluorescence from SYBR green dye during qPCR

Table 7. Volumes of reagents and cDNA used during the real time PCR.

Reagents	Vol. 1x
SYBR Green master mix	5
PCR primer mix	1
Diluted cDNA template	4
Total	10

TILs evaluation

H&E slides of FFPE tissues were observed under the microscope (Axio Imager A1 from Carl Zeiss Germany) Ocular x10 with objective x40, following the recommendation(46) TILs were reported as percentage of stromal area (= %stromal TILs), of which the denominator is the area

of stromal tissue not the stromal cells. All the slides were observed by two persons individually. The result was scored as a continuous variable and the results were compared. For those slides, with difference of more than five in two results, final conclusion was made in mutual consensus.

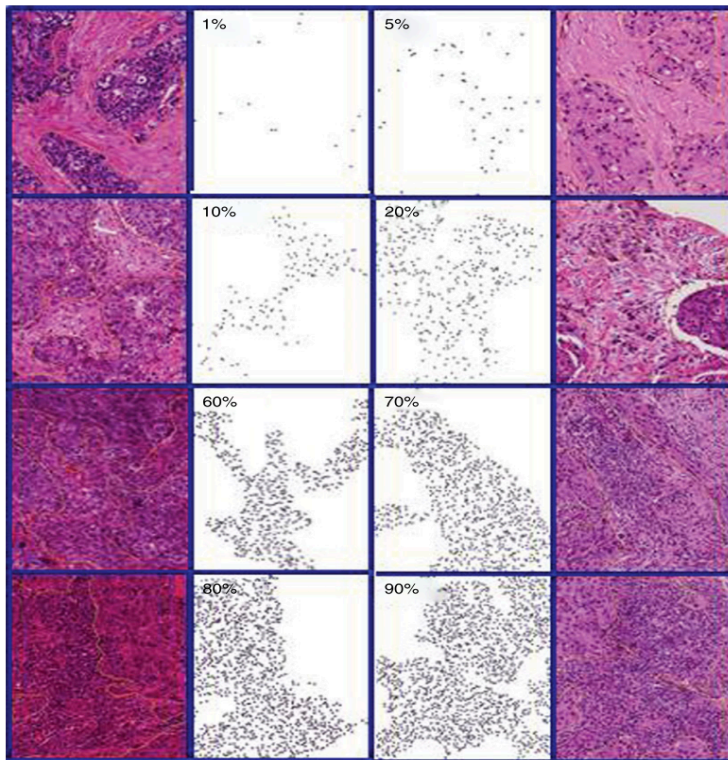


Figure 6: Standardization and guidelines for TILs evaluation (46)

Cell line experiment

MCF-7 and MDA-MB-231 cell lines were used for the experiment. The frozen stock culture of the cell line was used to proceed the experiments for which reanimation, growth and splitting to subsequent generations was done.

Reanimation of cells

Preheated medium (DMEM and RPMI 1640 for MDA-MB231 and MCF-7 respectively from the Thermo scientific) was poured into the T75 bottle. Both medium was prepared with 20%FBS, penicillin and L-Glutamine. The vial of cell from the nitrogen tank was taken out and quickly kept in water bath(37°C) to thaw until the ice crystal was dissolved. After thawing the content of the vial was poured onto the medium in complete sterile condition. The medium with cells was then incubated at 37°C incubator with 5% CO₂ for 2-3 days. The medium was replaced with fresh one the next day.

Splitting of cells

When the cells appeared in good shape and customizable confluent for splitting (70-80%) they were split. PBS, Trypsin EDTA and medium were preheated. Medium from the bottle was removed and cells were rinsed with 10ml PBS. 2ml of Trypsin EDTA was then added which

help to detach the adherent cells from the surface of cell culture vessel. After cells were released excess medium was used to deactivate trypsin. 5ml of medium was used to deactivate the trypsin. The cell suspension was centrifuged, the upper medium with trypsin was pipetted out and the cell were dissolved in new fresh medium then split into the bottle with medium on the basis of requirement or concentration of the confluent cells. 15ml of growth medium was used in each bottle. Generally, one T75 bottle was split into 4 or 5 bottles.

Counting of cells

Counting of cells is necessary for transfection experiments. For this an hemocytometer called Bürker counting chamber was used, which contains two chambers each with 9 major squares. (volume of $0,1\text{mm}^3$ or 1×10^{-4} ml each). After 2-3 days of culture, cells appear well grown and spread, then cells were scrapped with the help of cell scrapper. Cells were harvested in PBS. $50\mu\text{l}$ cell suspension was mixed with $50\mu\text{l}$ Trypan Blue (1:2 dilution) in Eppendorf tube and was mixed well. A clean cover glass was place over the counting chamber and one drop of mixed cell suspension was added onto it. Using x40 objective at least 200 cells were counted among nine squares. To prevent the counting of overlapping cells the cells touching the top and left boarder of the square were counted while those touching bottom and right were excluded, then cell concentration was calculated as:

Cells per ml = average number of cells per route x dilution factor x 10^4 .

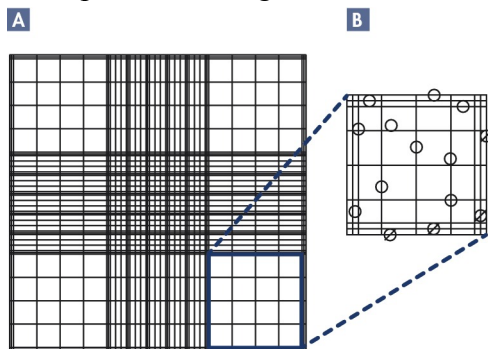


Figure 7. A chamber of hemocytometer with (B) Enlarged view of one major square

Transfection of cells with miRNA inhibitor

Background

Here the specific miRNA is inhibited with the miRNA inhibitor. miRNA Let-7b inhibitor from PNA Bio was used in our experiment. The miRNA inhibitor gets conjugated with cell penetrating peptide so it is simply mixed with the culture medium and the result is assessed after certain time.

Protocol

One day before transfection cells were counted and seeded as 1.5×10^5 cells in 1.5ml of growth medium without antibiotic in 6 well plates. Then the plate was incubated overnight. In the second day the medium was replaced with new medium without antibiotics. miRNA inhibitor was heated at 60°C for 10 minutes, and appropriate concentration was mixed with optimal medium and incubated for 15 minutes. We tested 5 different concentrations (0.05, 0.1, 0.5 and 1

and 2 μ M with 0.75,1.5,7.5, 15 and 30 μ l respectively) and obtained the minimum inhibiting concentrations as 0.1 μ M for MCF-7 and 0.01 μ M for MDA-MB 231. So 1.5 μ L per 1.5ml of total (growth medium plus optimal medium) of inhibitor was used in our experiment as the inhibiting concentration for both of the medium

RNA extraction from transfected cells

After 48 hours of incubation with inhibitor the cells were harvested removing the culture medium. Cells were lysed by suspending in 300 μ l of Binding buffer and vortex. Then isolation of miRNA and total RNA was done from the same cells in two fractions. We used PureLink isolation kit from Invitrogen and PureLink (Life Technologies) RNA mini kit from Ambion. to isolate miRNA and total RNA respectively. The complete protocol for the isolation of RNA provided by the manufacturer is attached in appendix II. Among two fractions obtained, miRNA fraction was used for the assessment of result with qPCR while the total RNA fraction was further used for RNA sequencing.

cDNA synthesis and real time PCR

The miRNA fraction isolated above was used to synthesize cDNA for further processing. For that, miRNA LNA[™] Universal RT microRNA PCR kit (Exiqon) was used. Same protocol as mentioned earlier in our experiment was followed for cDNA synthesis and real time PCR.

Wound healing assay

Background

Wound healing assay is done to study the cell migration and cell interaction. Here a scratch is made on the layer of confluent cells in a well after which cell will grow towards the gap. The growth of cells and the interaction between cells is observed. For that imaged were taken at subsequent interval of time and they are compared to see the change.

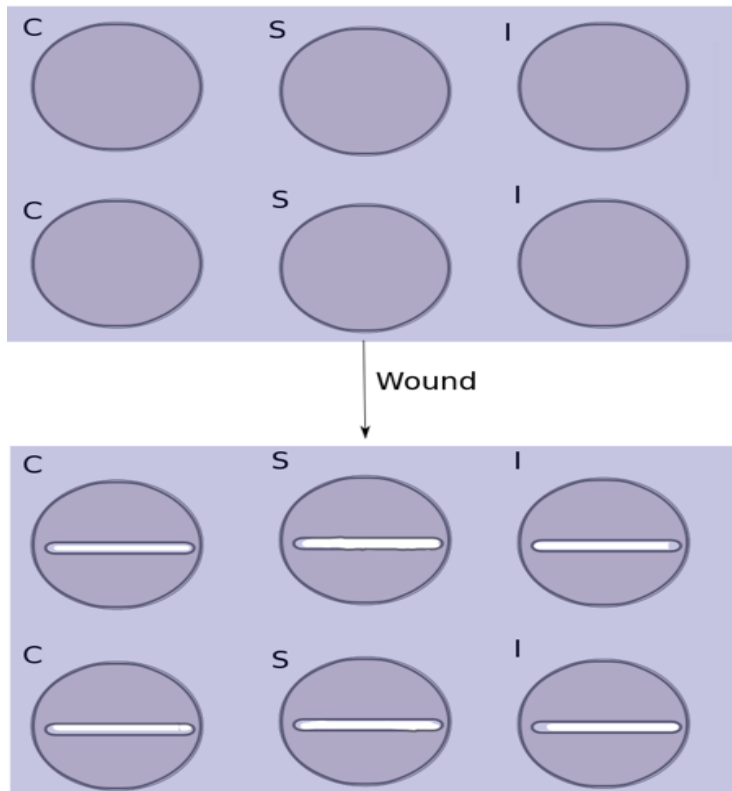


Figure 8. Wound healing assay; A-before making wound, B-after making wound, C=control SC=Scramble, and I=inhibitor

Protocol

Cells were cultured as 1.5×10^3 cells per well in 1.5ml of antibiotic free medium. After one-night incubation Let-7b inhibitor was added as mentioned in the transfection protocol above and incubated for 48 hours at 37°C and 5% CO_2 . Images were taken at the interval of 24 hours to see the growth of cells. When the confluent monolayer of cell was grown in the well, a scratch was made using a pipette tip of 10-100 μl in the area of cell growth. The cells were incubated and images were taken in 0, 6, 12, 24 and 48 hours of wound. All the images were taken at same objective with same resolution and at the same point of the wound each time to make the uniformity in the measurement.

The images taken at different time interval were compared and analyzed by using MicroimagerTM image analyzer from ViisioPharm (Hoersholm, Denmark). Here the decreased wound area due to cell growth and movements towards the wound from both sides with time described the growth rate, and difference of these areas from one another is compared to see if the inhibition of Let-7b does affect the proliferation rate or not.

Cell block preparation

Cell block was made for the immunohistochemical test. Cells were grown in medium without antibiotics in a T75 bottle for overnight. Both MCF-7 and MDA-MB-231 cells were cultured. In the second day both bottles of cells were transfected with Let-7b inhibitor. 0.5 μM concentration of inhibitor was used. The volume of growth medium and optimal medium was used as

mentioned in the table 8. Transfected cells were incubated at 37°C with 5%CO₂ for 48 hours. After incubation the medium was poured out and cells were scrapped and collected in PBS, centrifuged and PBS was replaced with 4% Formaldehyde and submitted for cell block formation. Cell block was made by experienced technicians.

Table 8: Volume of medium and inhibitors used for the cell block formation.

S.N	Samples	Final conc μM	Medium without PS	Optimal. Medium	Inh/scramble μL
1	control		9250	750	
2	Scramble	0,5	9200	750	50
3	Inhibitor	0,5	9200	750	50

Mitochondrial mito-stress test

This test was done to observe the cellular metabolism by measuring the oxygen consumption rate and extracellular acidic flux.

For seeding, both MCF-7 and MDA-MB-231 cells were transfected with appropriate concentration of inhibitor and scramble. The transfected cells were harvested after 48 hours and counted. XFp miniplate which contained 8 wells was used for further processing. 20,000 cells/80μl was poured in the wells B to G among the 8 wells, while 80μl of growth medium without any cells in the well A and H. The miniplate was then incubated for one night in cell culture incubator. To prevent the wells from getting dry the moat outside the well were filled with 400ul PBS in each moat.

Hydrating of sensor cartridge was done before the day of assay. Each utility plates were filled with 200μL of Seahorse XF celebrant, and moats around the well with 400μL per chamber. Then the cartridge was applied to the utility plate with celebrant and incubated overnight at 37°C without CO₂. On the day of FL assay media was prepared as per the guideline of manufacturer. Cells in Seahorse FL cell culture manipulates were washed gently with 200 μl of assay medium for two times and the medium was removed. 180 μl of new assay medium was added and incubated at 37°C incubator without CO₂ for one hour prior to the assay. Stock compounds for the injection were prepared prior to analysis. And loaded into the labelled wells in the cartridge. Assay template was created and the cartridge was loaded into the analyzer. After completion of the assay, the data were transferred to wave for analysis. Protein measurement was done using Quinter BCA Assay Kit from Sigma Aldrich.

Parameter	Rate Measurement Equation Used by Report Generator
Non-Mitochondrial Respiration	Minimum rate measurement after Rotenone/antimycin-A injection
Basal Respiration	(Last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate)
Maximal Respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration)
H+ (Proton) Leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration)
ATP production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare Respiratory Capacity	(Maximal Respiration) – (Basal Respiration)
Spare Respiratory Capacity as a %	(Maximal Respiration) / (Basal Respiration) × 100
Acute Response	(Last measurement rate before Oligomycin injection) – (Last rate measurement before acute injection)
Coupling Efficiency	(ATP Production Rate) / (Basal Respiration Rate) × 100

Figure 9. Different parameters and the rate measurement equations used by the report generator for mite-stress test by XF analyzer.

Data normalization and statistical analysis

The relative expression of miRNAs was normalized to the expression of two reference genes, miR-24 and miR-26b. Selection of these miRNAs as reference genes was done on the basis of former literature which had confirmed them as most stable genes by NormFinder analysis (76). Cycle threshold(Ct) values of each gene were measured by qPCR and normalized using $2^{-\Delta Ct}$ method relative to miR-24 and miR-26b. All statistical analysis was performed with SPSS 21.0 (SPSS Inc, Chicago, IL, USA). Independent t-test was used to observe the correlation between miRNA and clinical features of breast cancer. P values ≤ 0.05 were considered statistically significant. Kaplan–Meier survival curve were constructed and the difference between the two group were tested by log-rank test. The optimal expression threshold for ER was determined by Receiver Operating Characteristics (ROC) curve analysis (Med Calc statistical software v.9.3.7, MedCalc, Mariakerke, Belgium).

Results

The prognostic value of classical tumor characteristics for distant metastasis free survival, is illustrated in table 9. MAI and Nottingham grade were found to be statistically significant with log-rank P-value values of 0.003 and <0.001 respectively.

Table 9. Distant metastasis free survival in lymph node negative breast cancer with Kaplan-Meier analysis.

Characteristics	Distant metastasis	
	Event at risk(%)	Log rank p-value
MAI		
<10	5/49(89)	0.003
≥10	14/36(61)	
Tumor diameter		
>2cm	8/27(70)	0.352
<2cm	11/58(81)	
Age		
50 or more	2/16(87)	0.280
<50	17/69(75.4)	
Nottingham grade		
1	1/37(97.3)	0.001
2 and 3	18/48(62.5)	
ER		
Positive	9/47(80.9)	0.174
Negative/Dubious	9/26(65.4)	
TILS		
>10%	8/47(83)	0.227
10 % or more	5/14(64.3)	

Detection of TILs

TILs were scored using HE-section of the tumor. Independent t-test was performed to see the correlation between TILs and various clinical factors as illustrated in table 10. It was observed that tumor grade and ER gives significance association with p-values less than 0.05.

Table 10. Independent t-test between TILs and clinical features

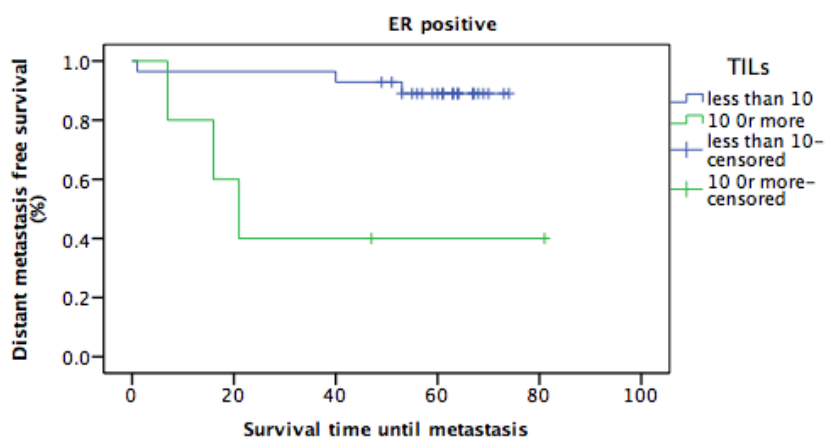
	MAI10	Grade	Tumor size	ER	DFS	Age (50)
TILs	0.161	0.024	0.07	0.045	0.724	0.313

Using the threshold of more or less than 10% (according to the TILs guidelines (46)) shows that it had no prognostic value for distant metastasis free survival, when evaluating all patients (Table 9). Furthermore, Kaplan Meier survival analysis shows that TILs can determine distant metastasis when looking only at the ER positive tumors, $P = 0.002$ (Table 11 and Figure 10A). Figure 10B, also showed that TILs could have a prognostic value for the patients with ER negative tumors, but that the cohort gets too small for statistical analysis.

Table 11. Kaplan-Meier survival analysis for ER and TILs.

Characteristics	Distance metastases	
	Event at risk (%)	Log Rank P-value
ER positive		
TILs<10	3/28(89.3)	0.002
TILs≥10	3/5(40)	
ER negative		
TILs<10	5/9(44.4)	0.062
TILs≥10	2/9(77.8)	

A



B

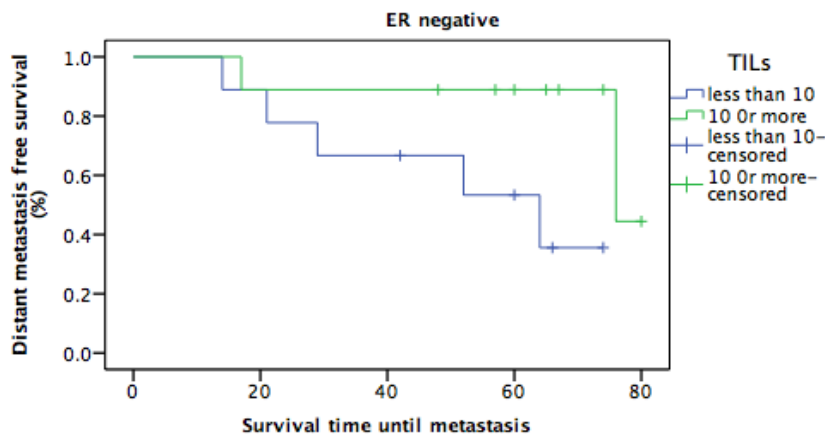


Figure 10. Distant metastasis free survival curve for ER expression and TILs.

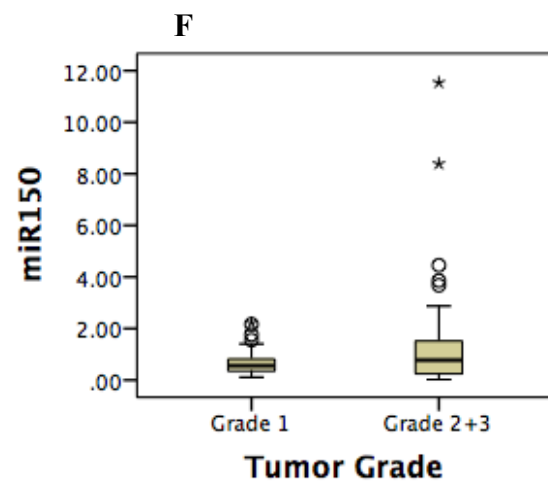
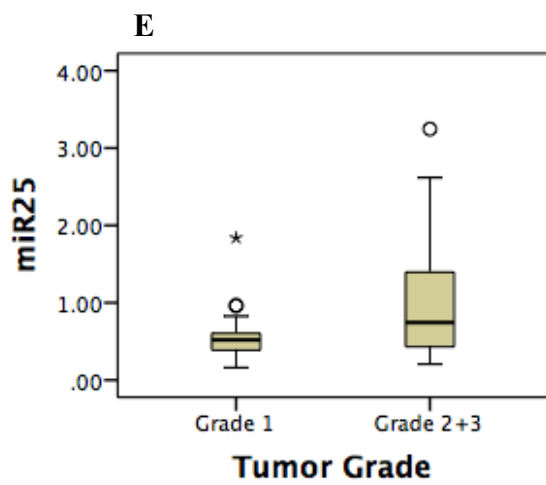
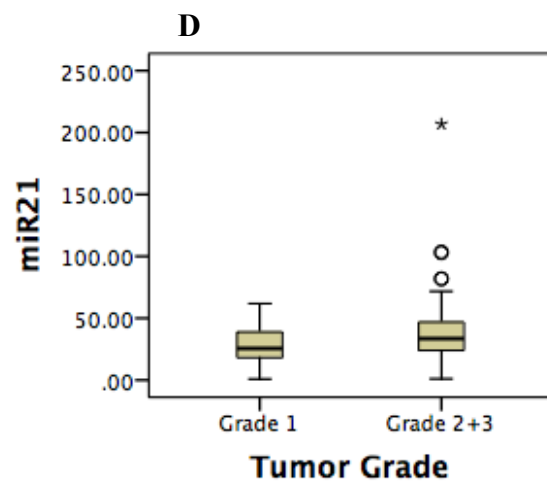
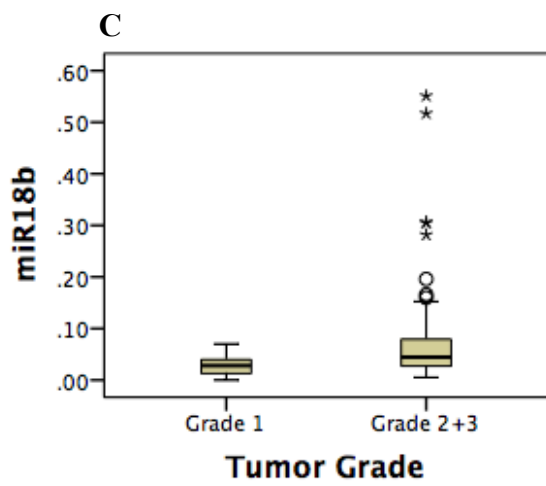
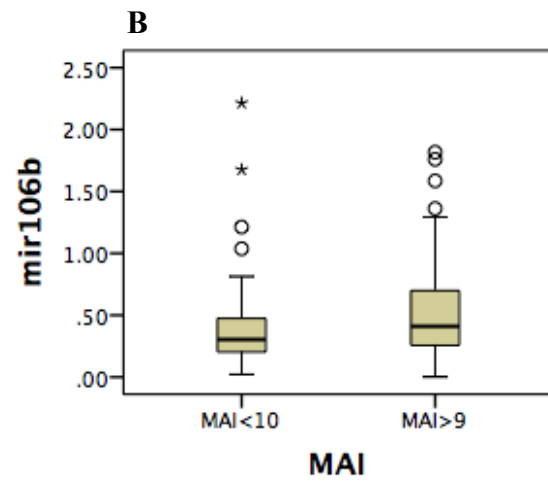
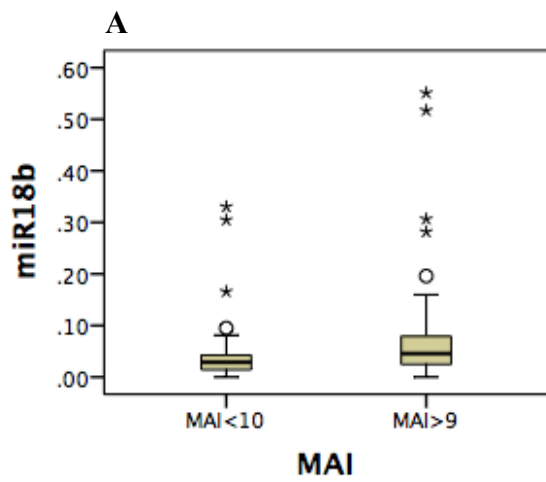
MicroRNA expression in tumor material

Independent t-test was done to see the association between different miRNAs and the clinical features of breast cancer. The results obtained from independent t-test is illustrated in table 12 with the significant result highlighted in grey. The most significant differences are illustrated by boxplots in figure 11. miR-18b (A) and miR-106b (B) both are upregulated in high MAI (MAI \geq 10). Four of the nine miRNAs, miR-18b (C), miR-21 (D), miR-25 (E) and miR-150 (F) are expressed higher in high tumor grade. Similarly, two miRNAs, miR let-7b (G) and miR-150 (H) showed significant association with higher TILs (TILs $>$ 10) while miRNAs let-7b (I), miR-375 (J) and miR-150 (K) shows significant association with age.

Table 12. Independent t-test between miRNAs and different clinical features of the breast cancer.

miRNAs	Grade	MAI 10	DFS	Tumor size	ER	TILs 10	Age (50)
let-7b	0.227	0.164	0.584	0.260	0.531	0.000	0.002
miR-18b	0.001	0.020	0.133	0.053	0.190	0.060	0.108
miR-21	0.013	0.135	0.708	0.627	0.915	0.552	0.207
miR-25	0.001	0.240	0.420	0.293	0.126	0.326	0.454
miR29c	0.890	0.859	0.428	0.963	0.598	0.957	0.193
miR-106b	0.078	0.036	0.738	0.580	0.382	0.096	0.135
miR-375	0.482	0.239	0.478	0.862	0.674	0.549	0.021
miR-505	0.142	0.375	0.867	0.905	0.111	0.426	0.354
miR-150	0.033	0.753	0.684	0.943	0.019	0.007	0.014

Abbreviations: MAI, Mitotic activity index; DFS, distant metastasis free survival



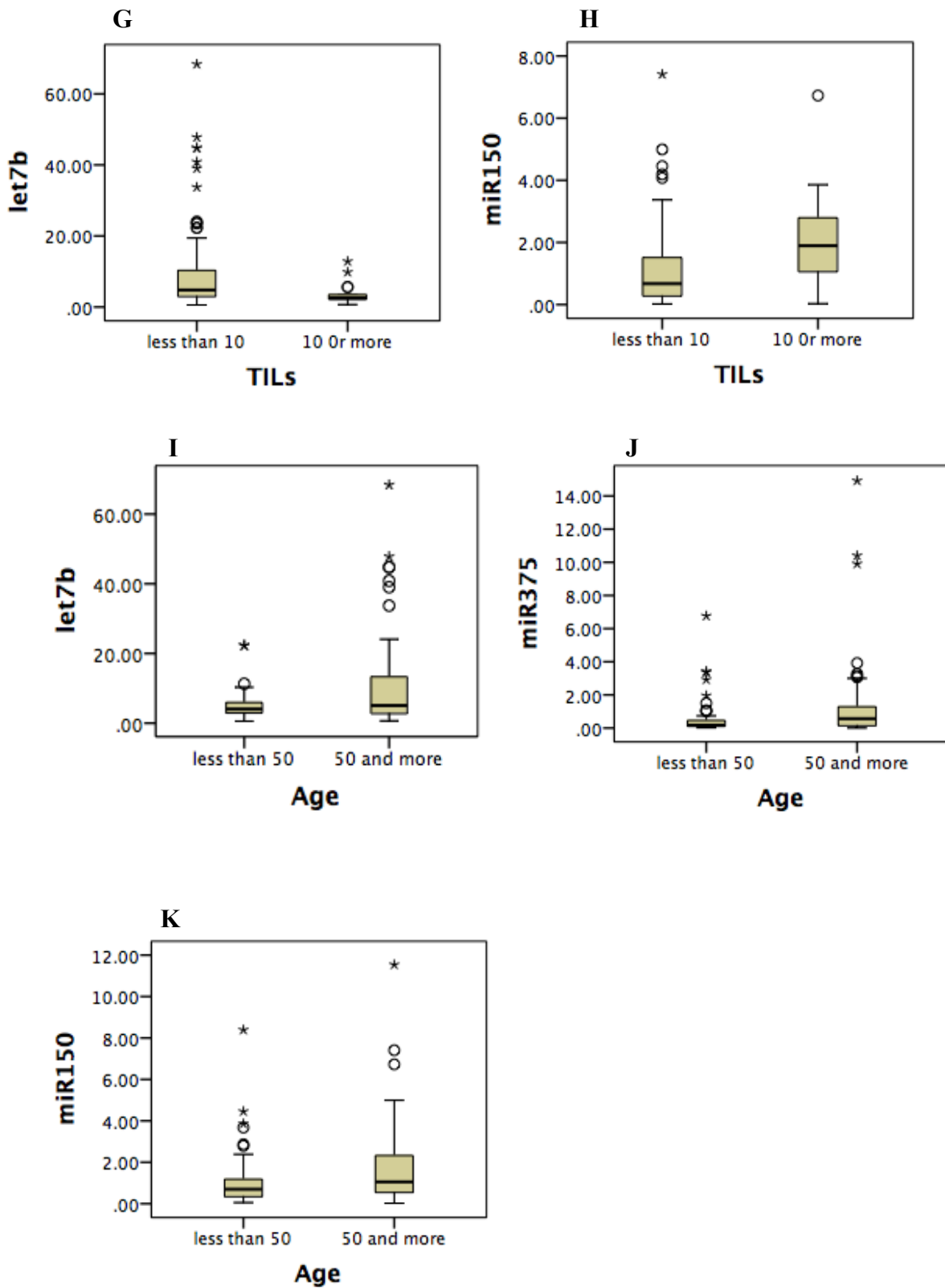


Figure 11. Expression level of miRNAs for different prognostic features. Significant relationship was determined by using Independent t-test. Expression level of both miR18b (A) and miR-106b (B) is higher in MAI \geq 10. The expression of all four miRNAs, miR18b(C), miR-21 (D), miR-25 (E) and miR-106b(F) are seen to be upregulated with high grade. Similarly,

miR let-7b (G) and miR-150 (H) are expressed higher in TILs more than 10 while miRNAs let-7b (I), miR-375 (J) and miR-150 (K) shows significant association with age.

Inhibition of miRNA let-7b in breast cancer cell lines

To observe the role of let-7b in breast cancer cells, first the optimal concentration of the inhibitor that can knock out the expression of let-7b was determined. Breast cancer cell lines MCF-7 and MDA-MB-231 were transfected with different concentrations ranging from 0.05 μM to 1 μM (0.05, 0.1, 0.5, 1 μM) of PNATM miRNA inhibitor for let-7b. Positive control cells that were not transfected and negative control cells transfected with scramble were used to compare the expressions level. Finally, the expression level was determined by qRT-PCR. During qRT-PCR miR-24 was used as control gene. Among different concentrations used, 0.5 μM concentration of let-7b inhibitor was found to be sufficient to knock out the expression of let-7b in MCF-7 and 0.1 μM of inhibitor concentration in MDA-MB-231 cell. So we choose 0.5 μM concentration for both of the cells for further processing.

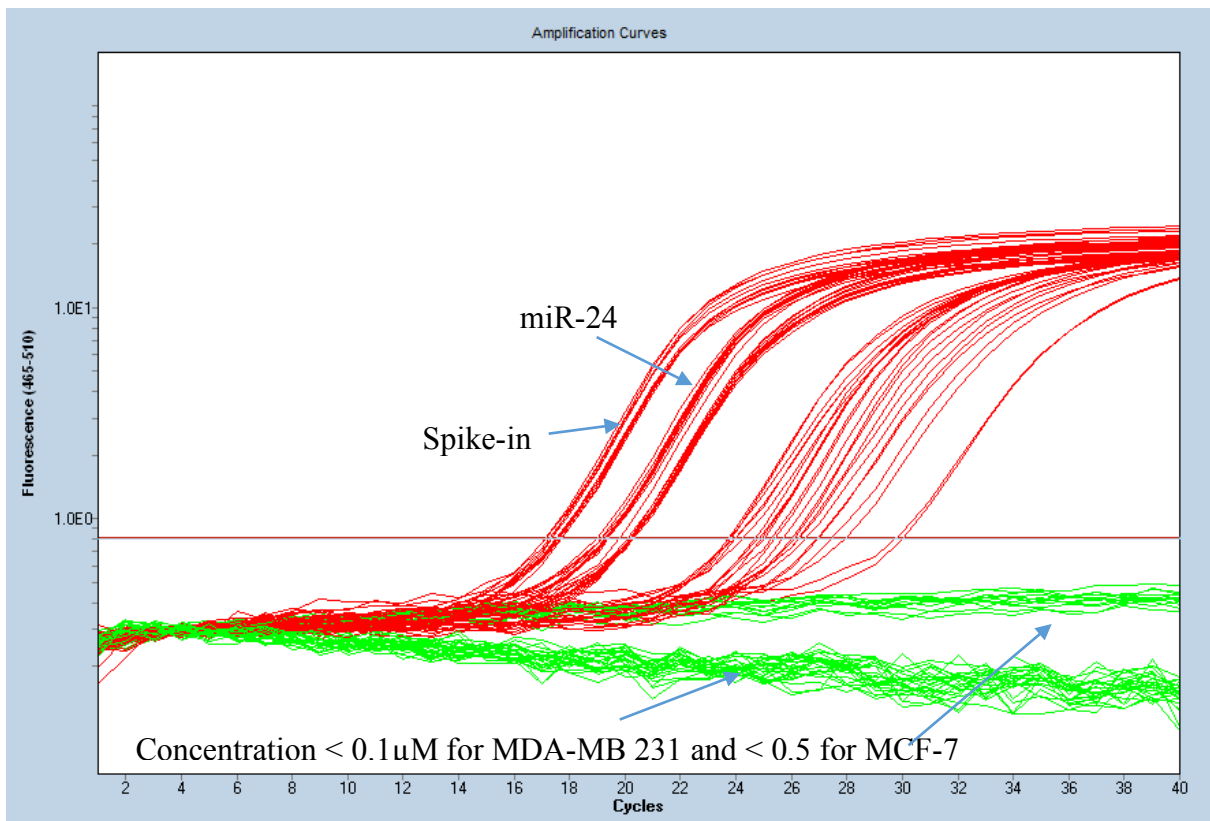
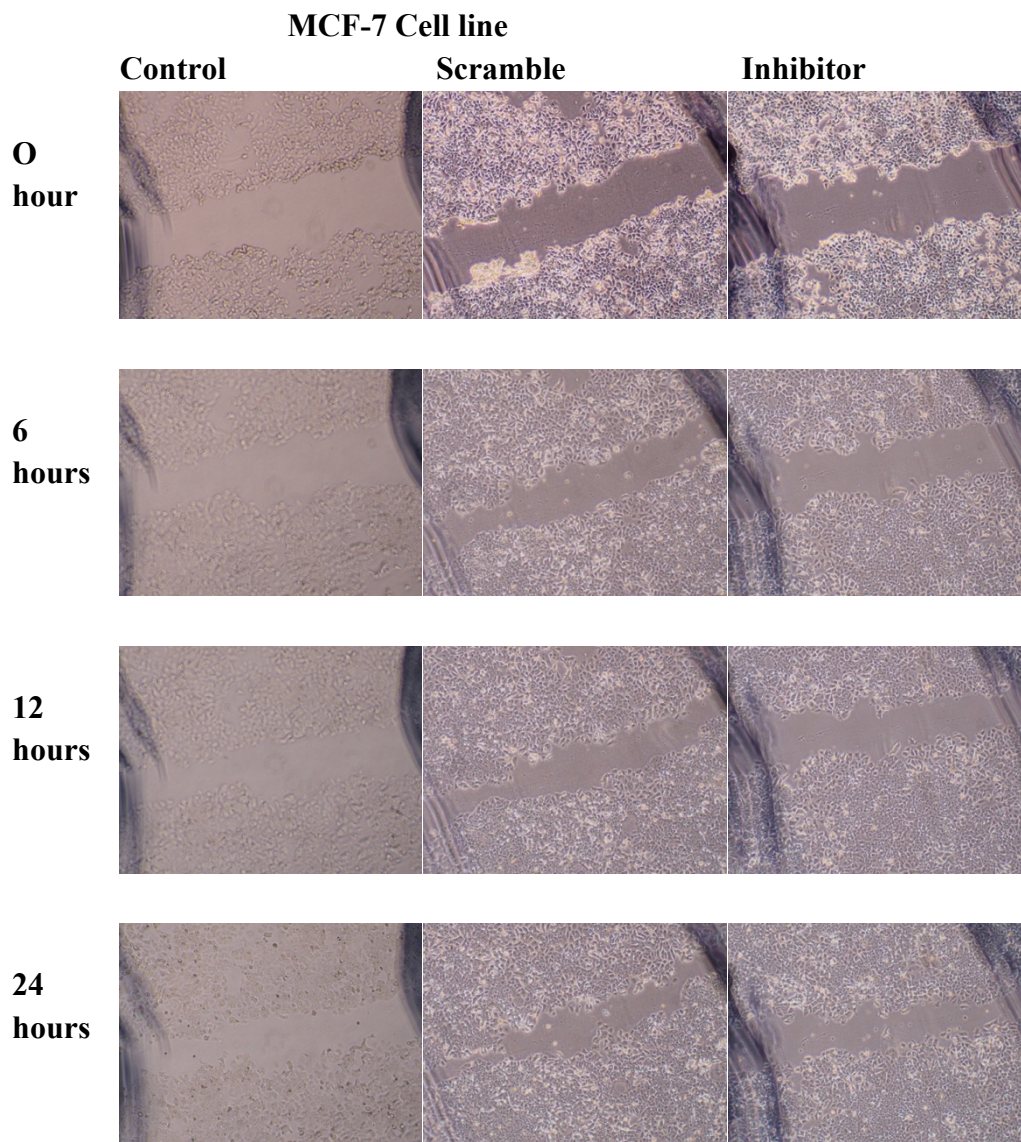


Figure 12. Amplification curve obtained in qPCR of MCF-7 and MDA-MB-231 cells after transfection with let-7b inhibitor. No amplification curve was obtained for 0.5 μM and higher concentration of let-7b inhibitor concentration for MCF-7 cells and, 0.1 μM and higher concentration in MDA-MB-231 cells.

Wound healing assay

In attempt to observe the involvement of let-7b in proliferation and to examine the long term effect of the inhibition, wound healing assay was performed. Wound was made on the each well with cells after 48 hours of incubation with inhibitor, scramble and control. Pictures were taken at time interval of 0, 6, 12, 24 and 48 hours as illustrates in figure 13 and figure 14

The pictures where analyzed using the Microimager™ image analyzer from Visiopharm (Hoersholm, Denmark) by differentiating cell area and wound area and comparing the growth rate.



**48
hours**

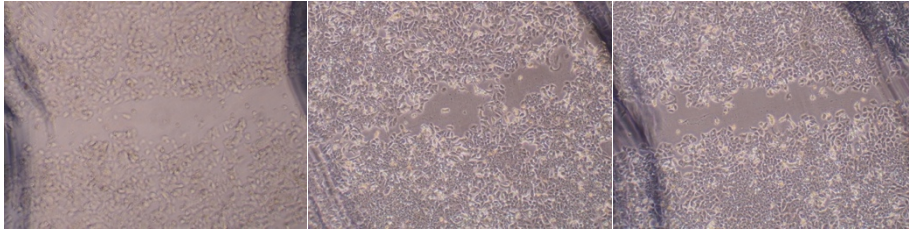


Figure 13. Images of wound healing assay on MCF-7 cell line at different interval of time. Images were taken at 0, 6, 12, 24 and 48 h after confluent cells had been scratched

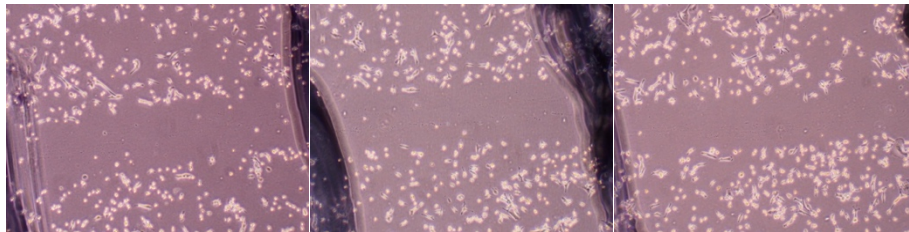
MDA-MB-231 cell line

Control

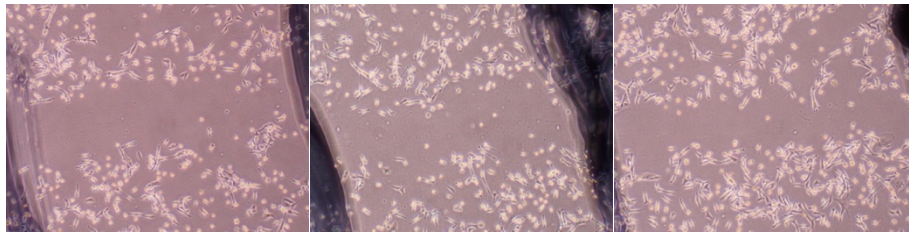
Scramble

Inhibitor

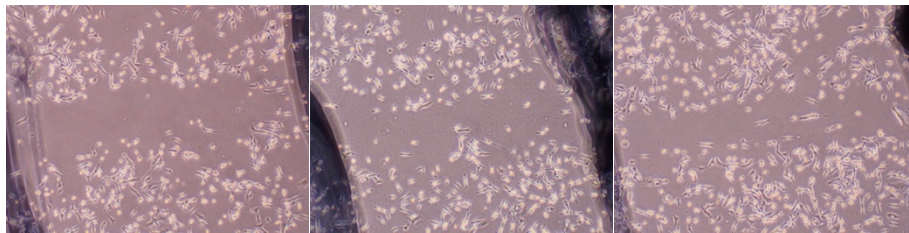
**0
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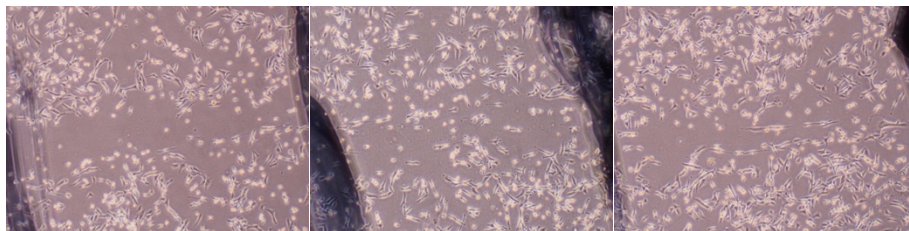
**6
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48
hours

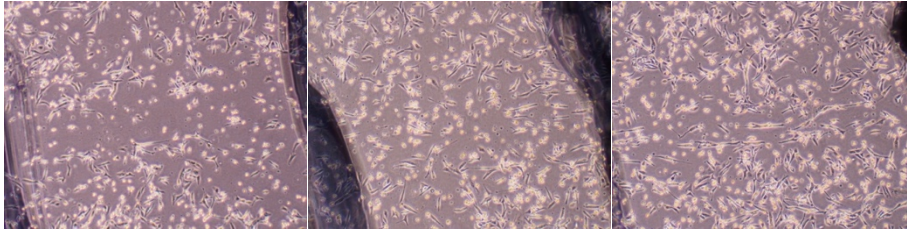
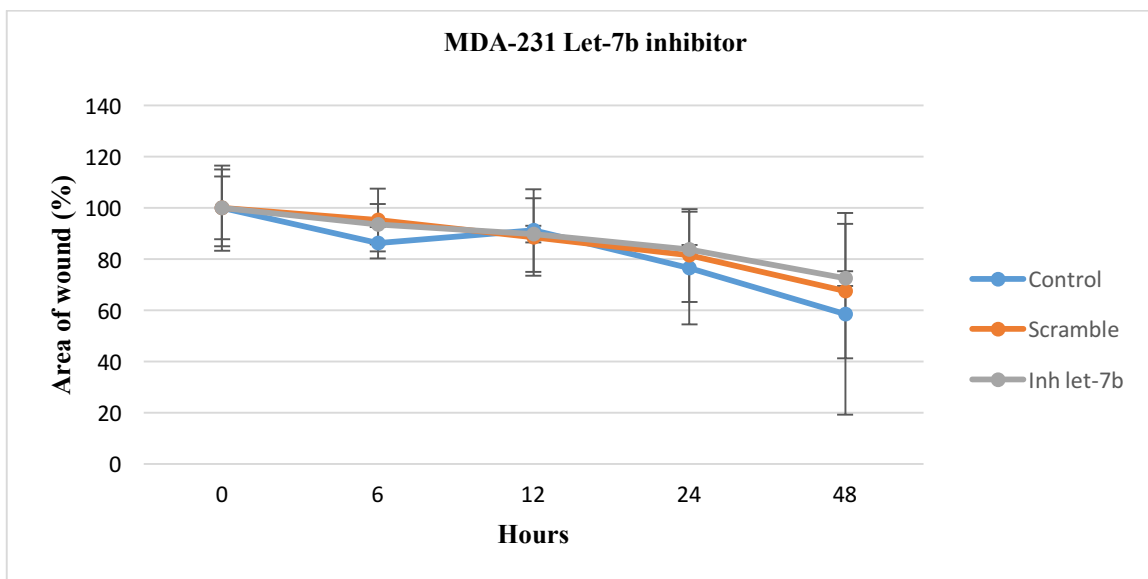
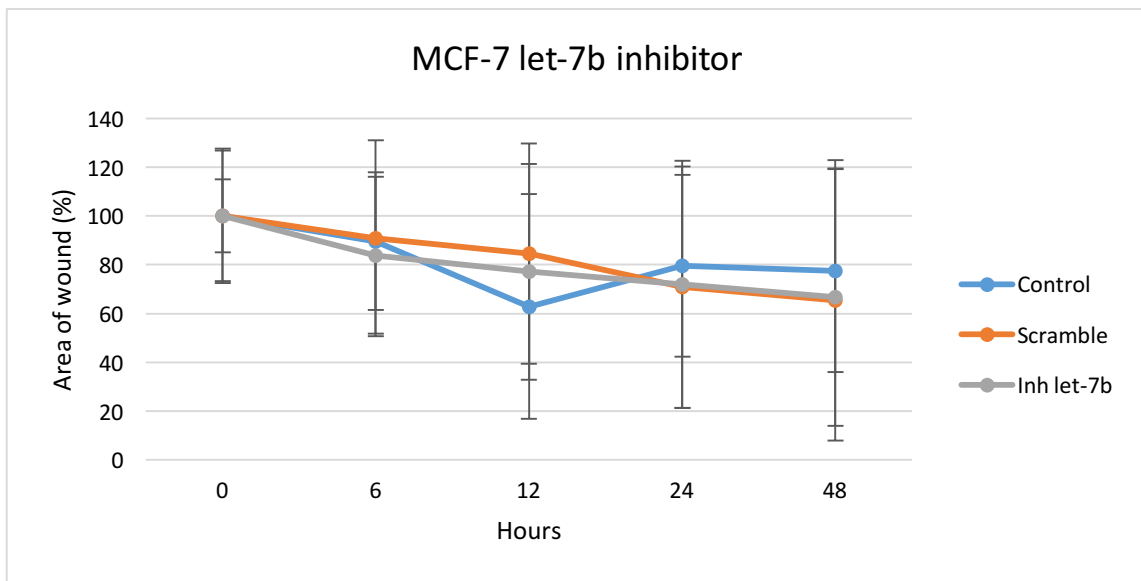


Figure 14. Images of wound healing assay on MDA-MB-231 cell line at different interval of time. Images were taken at 0, 6, 12, 24 and 48 h after confluent cells had been scratched



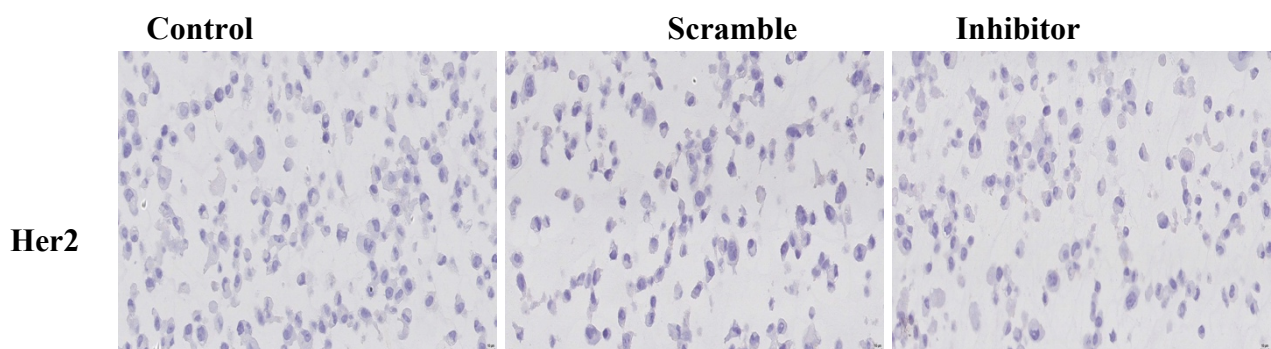
B

Figure 15. Wound healing assay of MCF-7 cells. (A) and MDA-MB-231 (B) cells with let-7b inhibitor. The figures illustrate the changes in the wound area in percent due to cell growth over time. The data is mean value from three parallels.

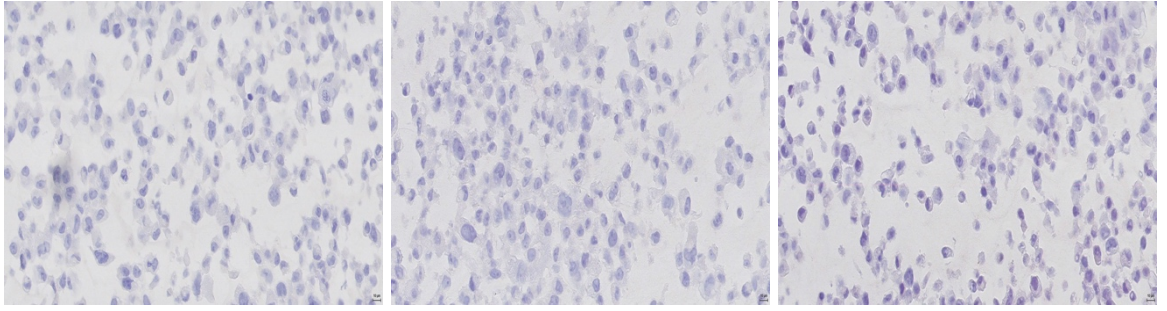
The progression of cell growth and thus healing of the wound over the time span of 48 hours is illustrated in figure 15. It was observed that there was steady decrease in the wound area in the cells with inhibitor for let-7b in MCF-7 initially which shows higher proliferation in compare to control and scramble. While after 12 hours the growth rate is almost constant. In MDA-MB-231 cells there is no any difference in the growth rate among inhibitor and scramble.

Immunohistochemistry

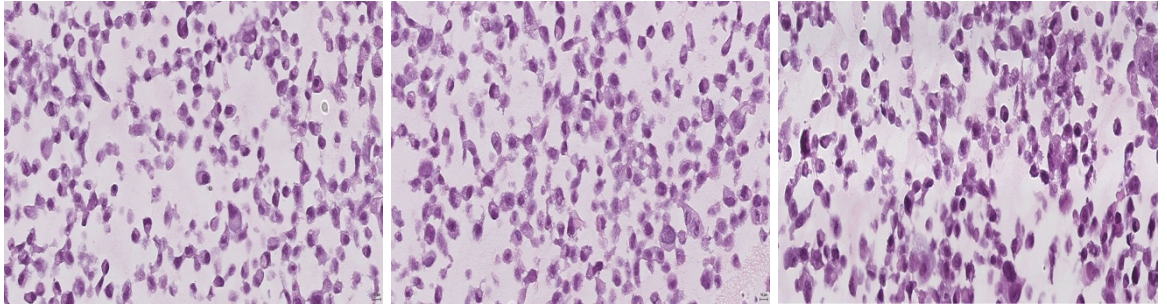
Cell blocks from both MCF-7 and MDA-MB-231 cell lines were sectioned and stained. The slides were observed under microscope to analyze different prognostic markers including ER, PR, HER2, KI67, MAI, PPH3 and Cyclin D1. The results obtained are illustrated in figure 15 and table 13. The sections from MCF-7 cells block contained few cells which made it difficult to scan. So only images of MDA-MB-231 are presented here. The data showed that MAI in MCF-7 cells with inhibitor was seen to be higher than in control cells, but it was below 10 which is considered a low count. MDA-MB-231 cells was negative for ER, PR and HER2 while MCF-7 was positive for ER, and negative in the cells with inhibitor in remaining two markers. Although ER expression was slightly low in cells with inhibitor, there was no any significant change in ER compared to control and scramble in MCF-7 cells. High level of Ki67 expression was seen in both cell lines, but the expression level was almost the same within control, scramble and inhibitor for both cell lines. Cyclin D1 was seen in the range of 12-21% in MDA-MB-231 cells while in MCF-7 cells the expression of cyclin D1 is significantly higher in the inhibitor (19%) in comparison to scramble (4%) and control. (9.6%). No big difference was seen in the three samples for the immunohistochemical staining results, so the effect of inhibitor cannot be described on the basis of these results. However, presence of high Cyclin D in the let-7b inhibited cells of both type showed the involvement of let-7b in proliferation. The image for Ki67 control appears to be unclear due to difficulty in scanning.



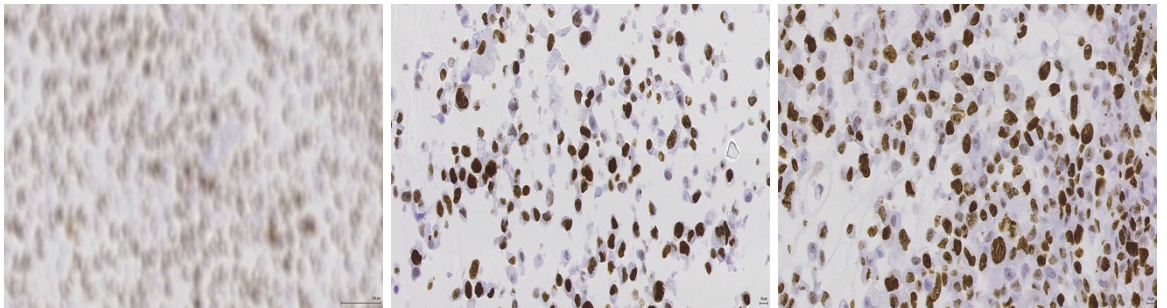
ER



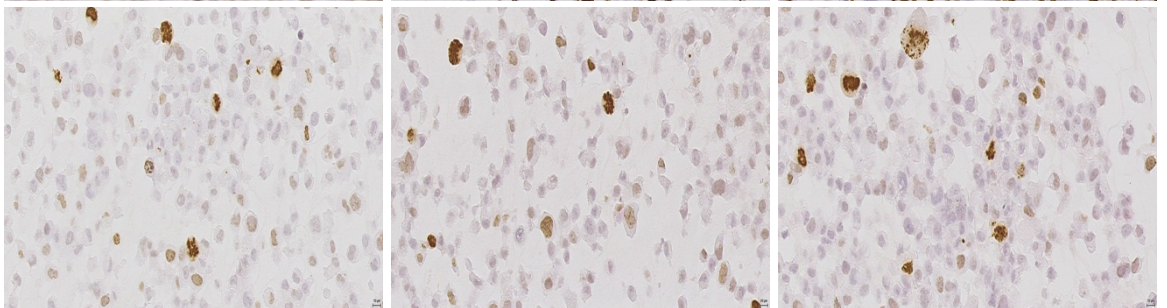
**HE/
MAI**



KI67



PPH3



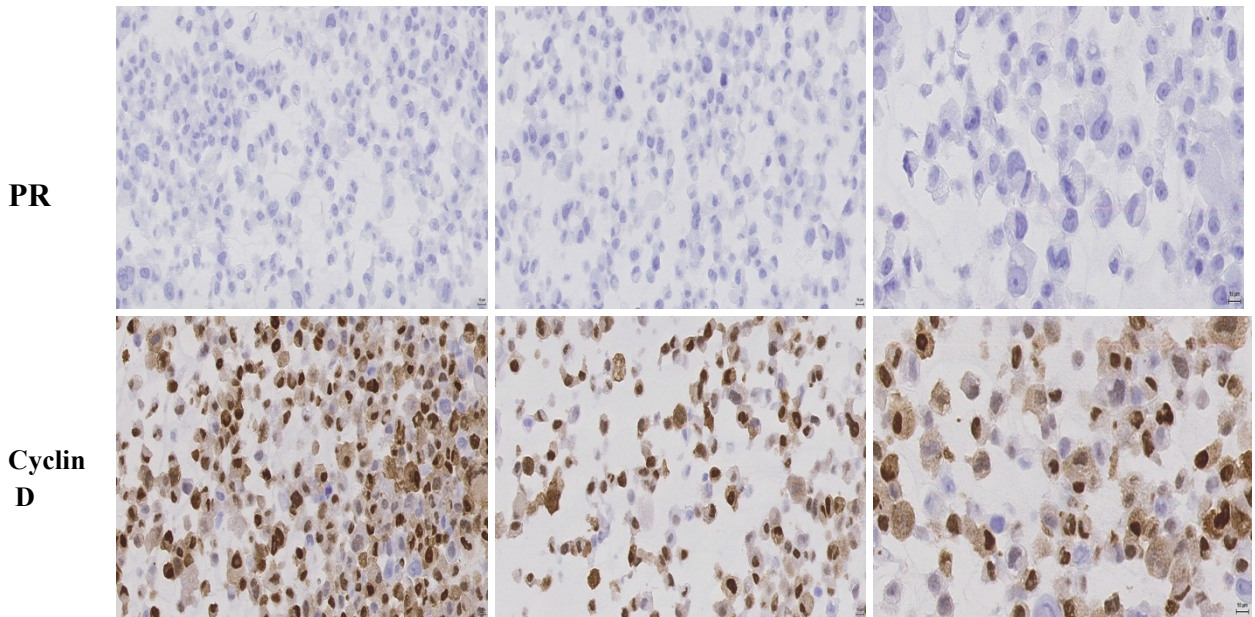


Figure 16. Images from immunohistochemical staining of different markers on FFPE MDA-MB-231 cell block

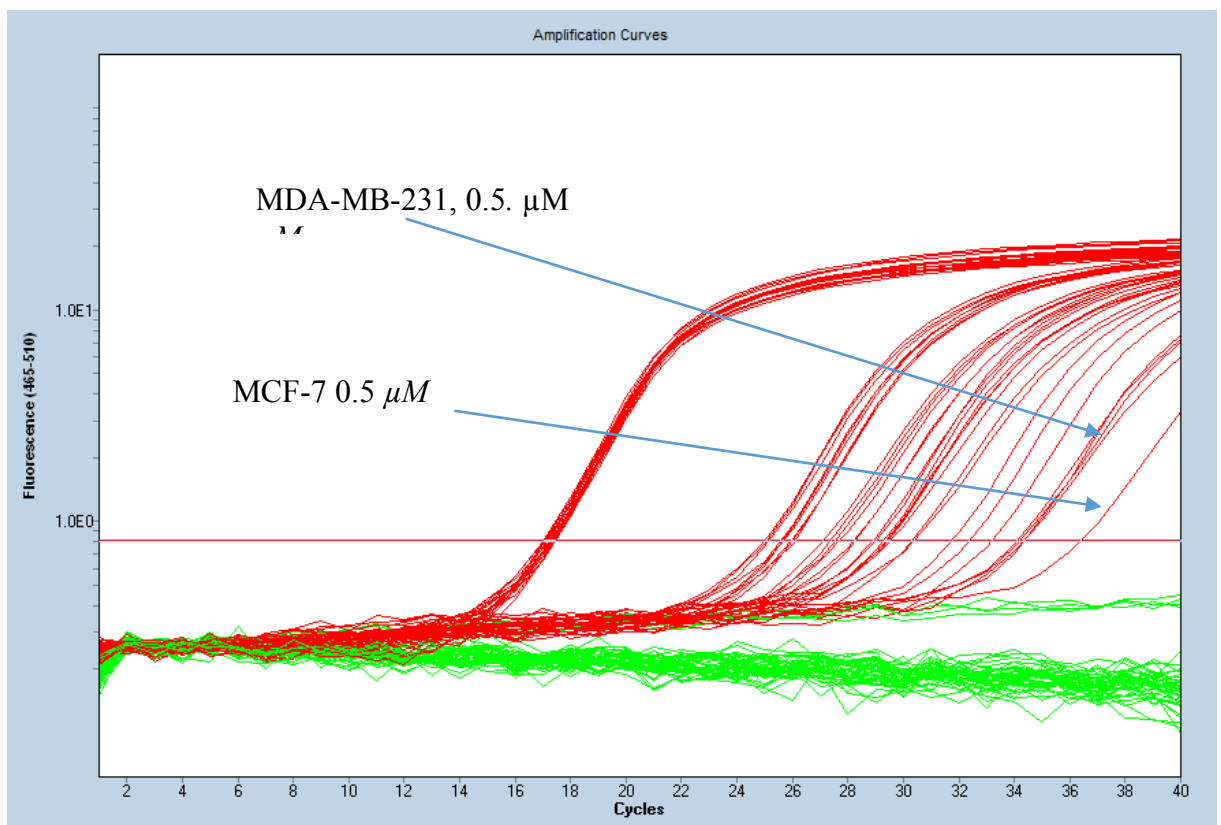


Figure 17. Amplification curve obtained in qPCR of MCF-7 and MDA-MB-231 from the cell block material. MCF-7 gave one amplification curve among the triplicate while all MDA-MB-231 gave all amplification curve for all of triplicates in 0.5 μ M concentration of inhibitor. This showed that there was no effect of inhibitor of selected concentration.

Table 13. Proliferation markers in FFPE cell blocks of MCF-7 and MDA-MB-231.

Markers	MCF-7			MDA-MB-231		
	Control	Scramble	Inhibitor	Control	Scramble	Inhibitor
MAI	2	9	9	0	0	0
KI67	99%	95%	95%	98%	95%	97%
PPH3	4%	3.5%	2.5%	2.5%	2%	2%
ER	95	99	95	Negative	Negative	Negative
PR	2	1	Negative	Negative	Negative	Negative
HER2	Negative	1	Negative	Negative	Negative	Negative
Cyclin D1	9.7%	4%	19%	12.5%	16%	21%

Mitochondrial mito-stress test

Key parameters of the mitochondrial function were measured by Seahorse XF Cell Mito Stress Test. To reveal those key parameters different modulators of respiration which target components of electron transport chain (ETC) in the mitochondria were used. The compounds (oligomycin, FCCP, and a mix of rotenone and antimycin A) were serially injected to measure ATP production, maximal respiration, and non-mitochondrial respiration, respectively. Proton leak and spare respiratory capacity were then calculated. Figure 18 reveals the mitochondrial respiration of MCF-7 cell lines measured by XF cell Mito Stress in Seahorse XF analyzer. All the others results obtained after serial injection of modulators compounds during mitochondrial respiration in MCF-7 cell lines are illustrated in figure 19. The extracellular acidification rate (ECAR) is presented in figure 20. Similarly Figure 21 reveals the mitochondrial respiration of MDA-MB-231 cell lines measured by XF cell Mito Stress in Seahorse XF analyzer. All the others results obtained after serial injection of modulators compounds during mitochondrial respiration in MDA-MB-231 cells are illustrated in figure 22. The extracellular acidification rate(ECAR) is presented in figure 23.

It is observed that the all parameters of mitochondrial respirations are significantly lower in the MCF-7 cells transfected with let-7b inhibitor, leading to the decrease in ATP production. While in case of MDA-MB-231 cells no significant difference is observed in any of the parameters between the cells transfected with inhibitor and scramble.

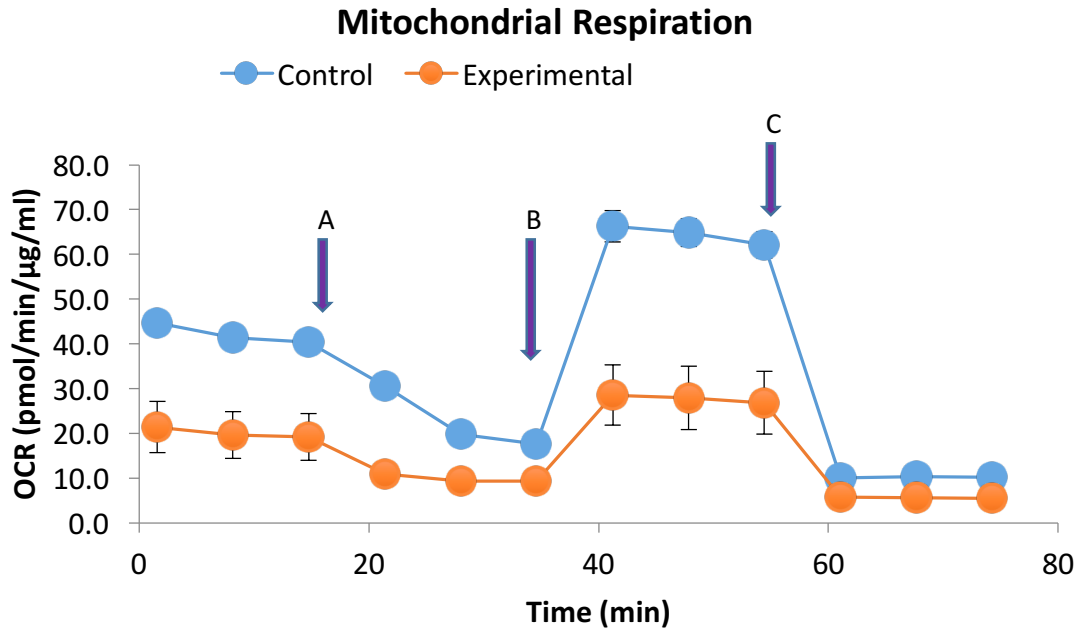
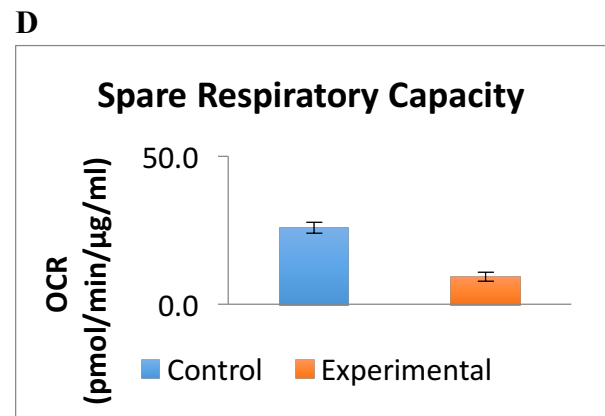
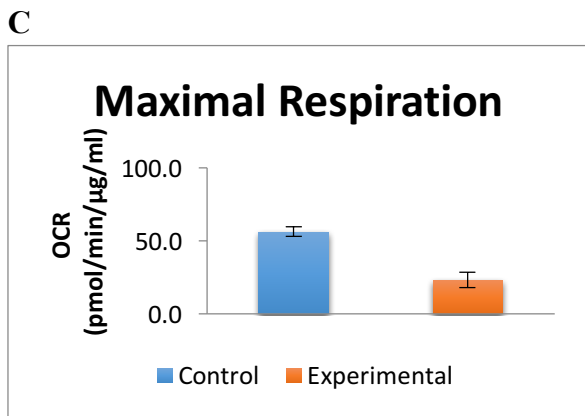
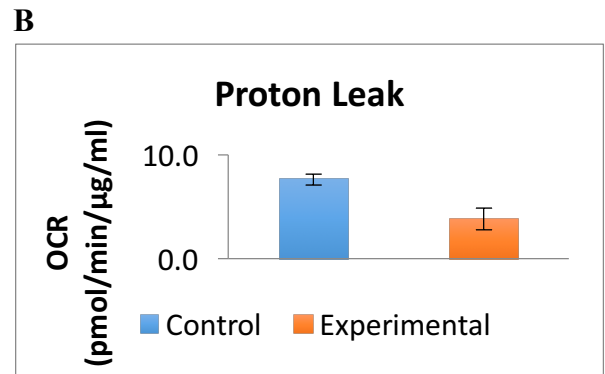
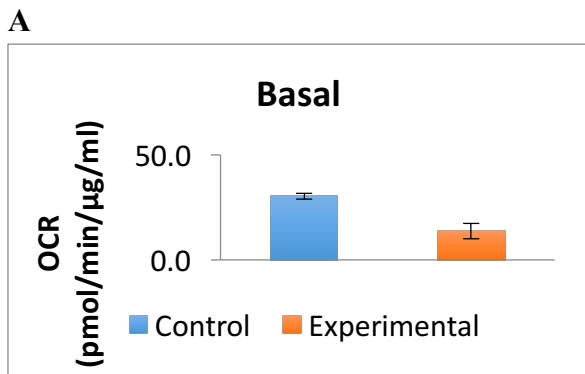


Figure 18. Differences in OCR response to mitochondria stress test between control and inhibitor in MCF-7 cells. Control was transfected with scramble, while experimental was transfected with let-7b inhibitor. Different modulators were injected sequentially at the indicated time point. to measure different parameters including basal and maximal respiration, ATP production, spare respiratory capacity. A=oligomycin, B=FCCP, and C=Antimycin and Retenone



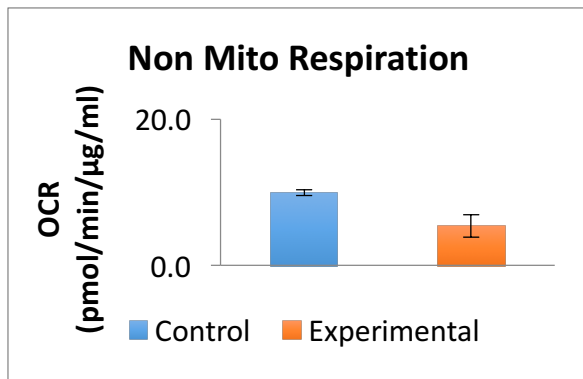
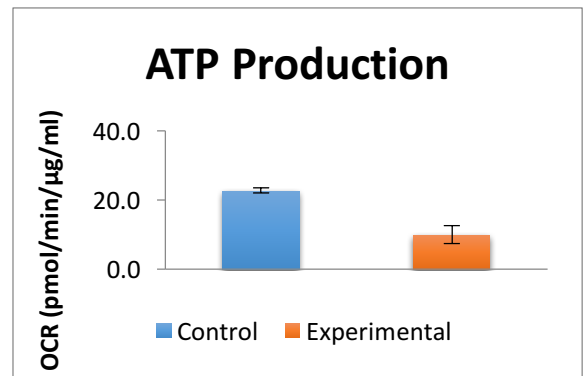
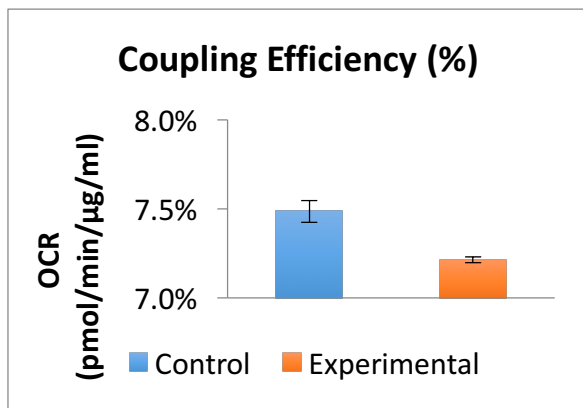
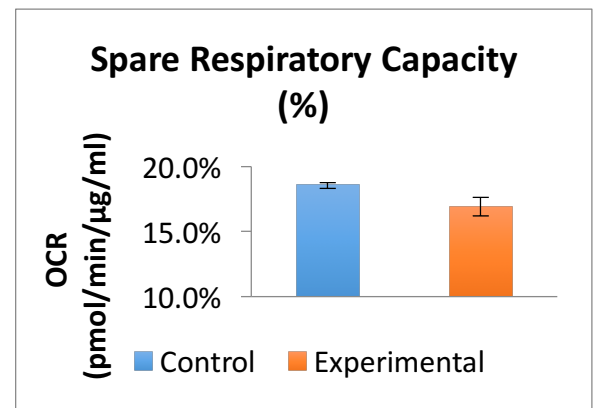
E**F****G****H**

Figure 19. Different parameters measured in Seahorse XF Cell Mito Stress Test using different modulators that target component of electron transport chain in MCF-7 cells. basal respiration (A); proton leak (B); maximal respiration (C); Spare respiratory capacity (D); non-mito respiration (E); ATP production (F); coupling efficiency (G); spare respiratory capacity (H).

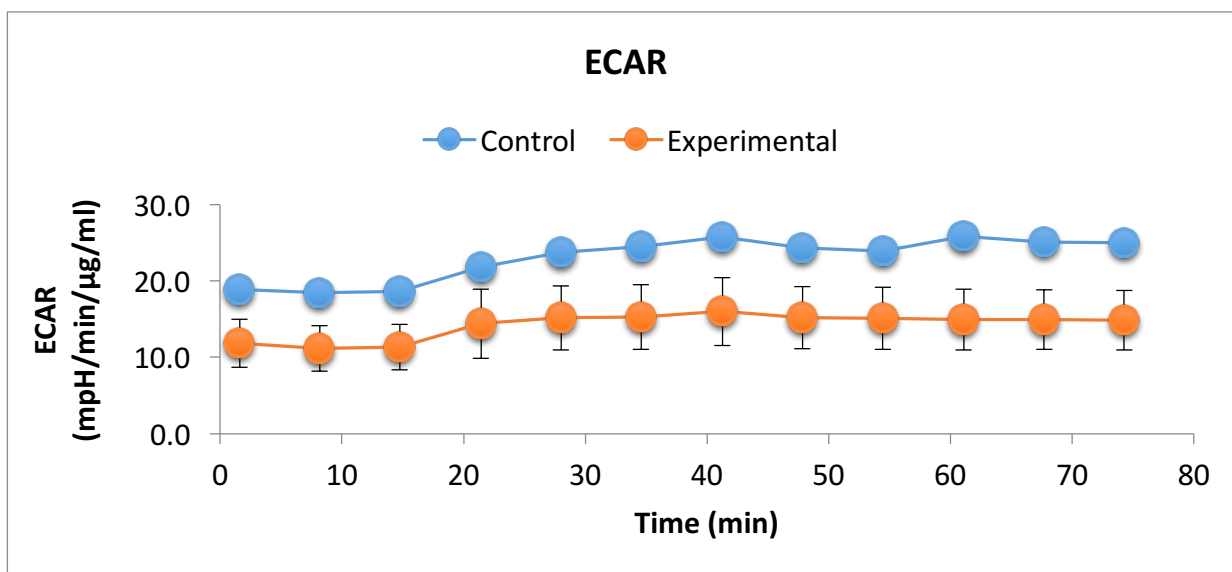


Figure 20. Extracellular acidification rate (ECAR, mpH/min) measured by XF analyzer in MCF-7 cells.

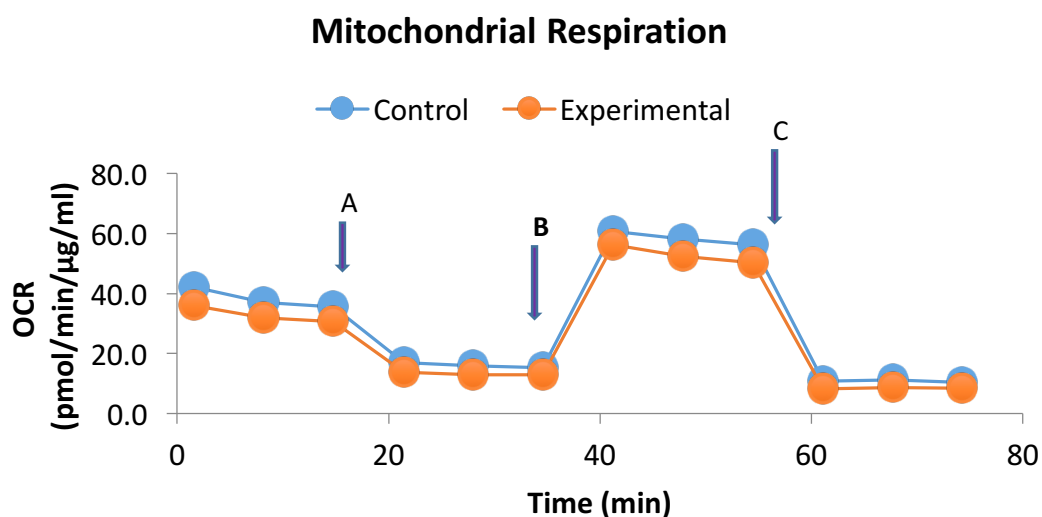
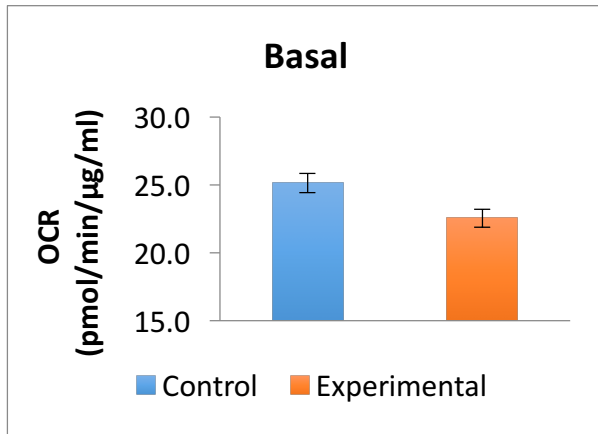
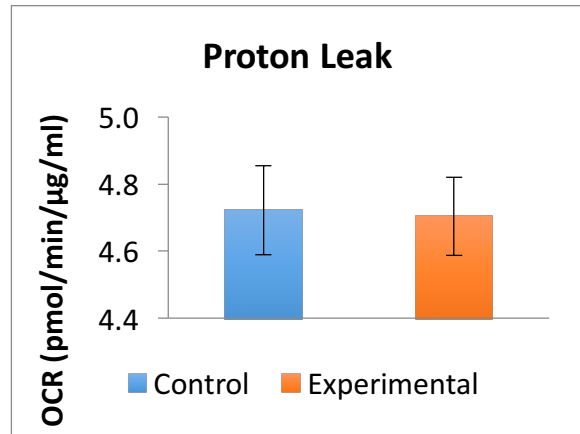
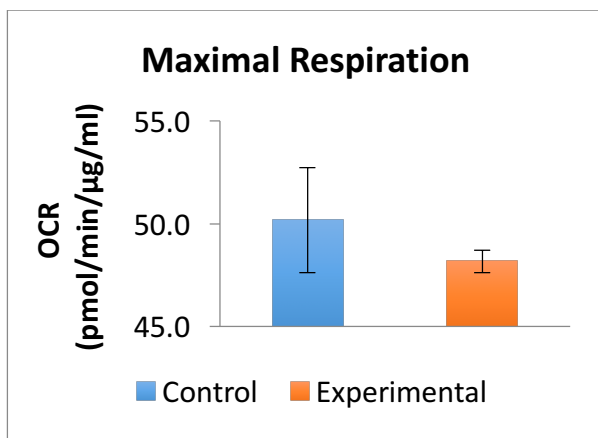
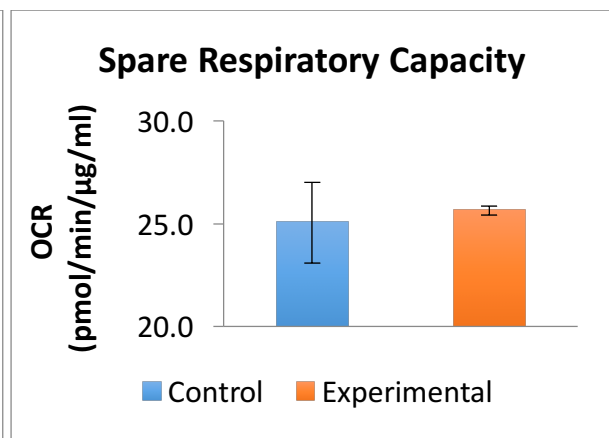
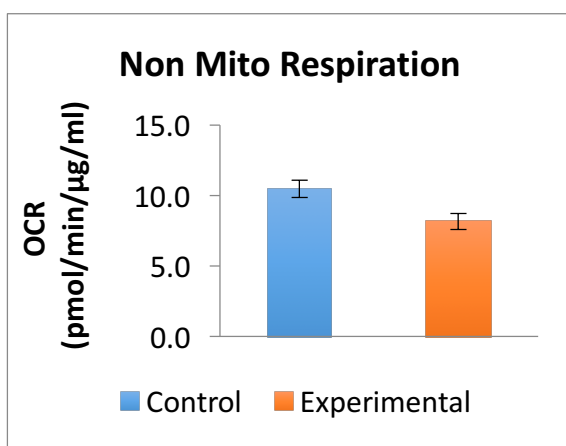
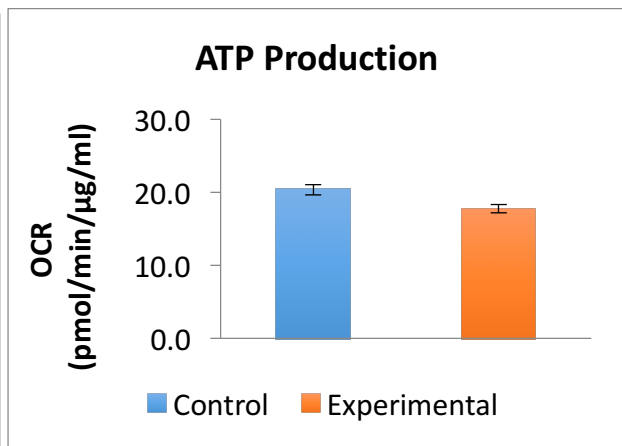


Figure 21. Differences in OCR response to mitochondria stress test between control and inhibitor in MDA-MB-231 cells. Control was transfected with scramble, while experimental was transfected with let-7b inhibitor. Different modulators were injected sequentially at the indicated time point. to measure different parameters including basal and maximal respiration, ATP production, spare respiratory capacity. A=oligomycin, B=FCCP, and C=Antimycin and Retenone

A**B****C****D****E****F**

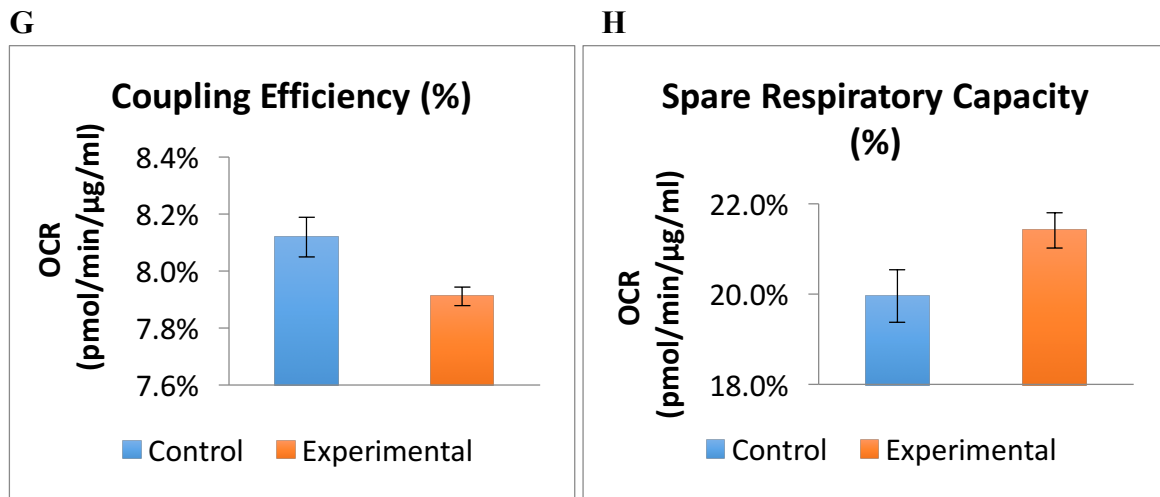


Figure 22. Different parameters measured in Seahorse XF Cell Mito Stress Test using different modulators that target component of electron transport chain in MDA-MB-231 cells. basal respiration (A); proton leak (B); maximal respiration (C); Spare respiratory capacity (D); non-mito respiration (E); ATP production (F); coupling efficiency (G); spare respiratory capacity (H).

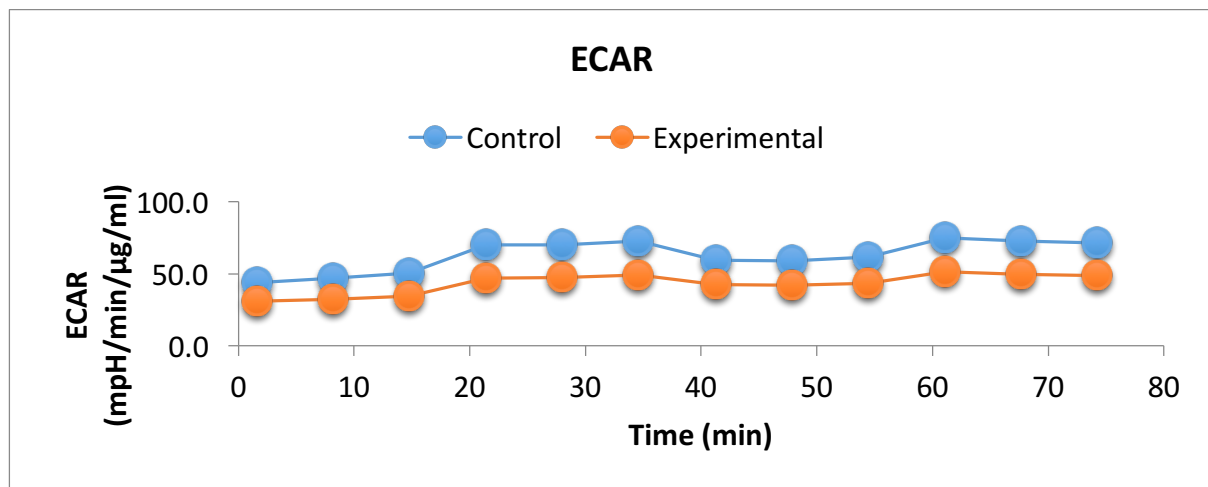
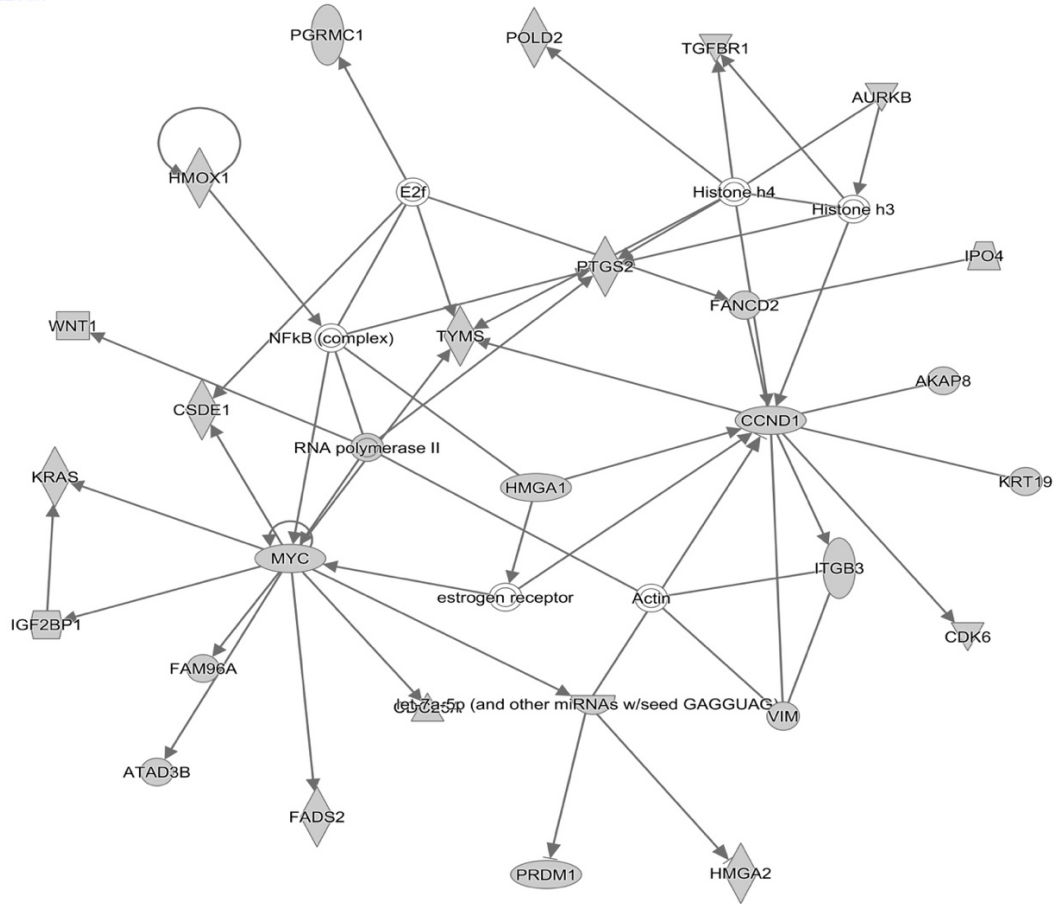


Figure 23. Extracellular acidification rate (ECAR) measured by XF analyzer in MDA-MB-231 cells.

Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) is a web based software application which is used to search targets information in genes and to build interacting models for experimental system. MicroRNA target filter in IPA showed that at the time of the analysis there are 132 experimentally observed targets of let-7b. Core analysis of these 132 let-7b targets showed that MYC (Write the name out) and cyclin D1 are essential in the top network (Figure) for these then targets of let-7b. When comparing these 132 experimentally observed target with predicted target for let-7b using miRtarbase and Exiqons miRSearch V3.0, 12 target genes shown in table 14 were found in all lists.



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Figure 24. Top target network for experimentally observed targets of let-7b obtained from IPA.

Table 14. list of experimentally observed targets of miR-let-7b common in IPA, miRtarbase and exiqon.

CCND1	CDC25A	CDK6	HMGA1	HMGA2	IGF2BP1
IGF2BP2	LIN28A	MTPN	NRAS	RDH10	TLR4

Discussion

Correlation between TILs and clinical features of breast cancer

The relationship between TILs and various clinical features of breast cancer shows that, TILs are associated with high histological grade ($P=0.024$), and ER positivity ($P=0.045$). It was also observed that TILs could have a prognostic value for patients with ER negative tumors but the cohort goes too small in our study for statistical analysis. Similar results were demonstrated previously, in which different subset of TILs showed strong association with high histological grade and ER negativity (45). TILs are consistently observed in TNBC and HER2 positive breast cancer (84) which is further supported by another paper which showed high TILs in triple negative (OR=2.49, 95% CI: 1.61-3.83) and HER2 positive (OR=5.05, 95% CI: 2.86-8.92) breast cancer (85). A study accessing TILs in more than 1300 TNBC and more than 3500 hormone receptor positive breast cancers found TILs to be a positive prognostic biomarker in TNBC patients but not in luminal subtypes. They conclude that TILs is associated with excellent prognosis in node-positive, ER-negative/Her2-negative breast cancer regardless of chemotherapy. In HER2 positive patients, TILs were significantly correlated with a beneficial effect of chemotherapy they evaluated (i.e. Higher dose of anthracycline vs combination anthracycline-doxetaxel therapy) (86). All of these findings show that TILs could be potential biomarkers in ER-negative and HER2 breast cancer.

Correlation between miRNAs and clinical features of breast cancer

Different miRNAs showed association with various clinical features of breast cancer.

The role of let-7b in human cancer can be discussed on the basis that it regulates the expression of RAS-oncogene family (87), along with MYC (88) and HMGA2 (89). Its expression is negatively associated with poor prognosis in human lung cancer (90). Overexpression of let-7 reduces the cell division by altering the cell cycle progression (91). Let -7b in our experiment showed correlation with TILs less than 10 and age more than 50. As higher TILs and lower age is associated with high histological grade tumors (45). Association of let-7b with low TILs shows its prognostic value as a tumor suppressor miRNA. miR-18b is shown to be associated with high proliferation which resembles with previous results (76,92). A study in hepatocellular carcinoma also shows that miR-18b is an important marker of cell proliferation (93). miR-18b along with miR-18a is associated with features of basal-like breast cancer (50). miR-21 is associated with cell migration and invasion in breast cancer and help in tumor progression and metastasis (94). Another study reported that miR-21 is involved in all phases of breast cancer (66). Iorio *et al* describes the expression of miR-21 to be upregulated in breast cancer suggesting it as an potential oncogene (87) which supports our results as we found the association of miR-21 with high grade (>2). miR-21 helps in oncogenesis by targeting a cytokine, TGFB which functions in cell growth, proliferation, differentiation and apoptosis (68). miR-25 is located in the same cluster with miR-106b and is found to be upregulated in different types of human tumors of stomach, liver and prostate. miR-25, in those tumors, seems to be an oncogene as it

exerts potential proliferative and anti-apoptotic effects (77). miR-25, in our results, showed correlation with high grade which is in accordance with these findings. As miR-25 and miR-106b are located as same cluster in the intron of minichromosome maintenance protein 7 (Mcm7) they were thought to have common expression pattern but such correlation was not noticed in our results. miR-29c did not show a correlation with any of the clinical features in our experiment. miRNA-29c is a tumor suppressor gene and its expression is associated with good prognosis (76). It is downregulated in ER α - negative, cytokeratin 5 and 6 positive and high proliferating tumors (92). Similar findings were seen in another article by Jonsdottir *et al* ., which suggests miR-29c as tumor suppressor gene because of its low expression in TNP, ER α negative and cytokeratin 5 and 6 positive cancers (76).

miR-106b promotes proliferation and invasion in laryngeal carcinoma cells (95), invasion in gastric cancer by inhibiting PTEN (96). Gong *et al.*, describes the paradoxical effect of TGF- β in breast cancer cells and its relation with miR-106b, showing the involvement of miR-106b in tumor cell proliferation (97) which supports our finding that miR-106b shows significant relation with proliferation marker MAI (P=0.036). miR-375 did not show any significant correlation with any of the clinical features except age more than 50, however many studies found the downregulation of this gene in different cancers including colorectal cancer, squamous cervical cancer and esophageal cancer. This suggest that miR-375 is a tumor suppressor miRNA which could target a tumor promoter gene(76). miR-505 did not show correlation with any of the clinical features however former research showed the correlation of highly expressed miR-505 with CK5/6 positive, ER negative and highly proliferative cells (76). Very few literatures are available on the involvement of miR-150 in breast cancer. One research in ovarian cancer showed that decreased expression of miR-150 results in decreased sensitivity to a drug pertuzumab and apoptosis. Pertuzumab is a drug approved for treating breast cancer which exert its effect by binding to the extracellular region of HER2/ErbB2. miR150 is associated with microenvironment in breast cancer and associated with better prognosis due to linkage with gene for immune response (78). Our result showed that miR-150 is correlated with high grade and TILs more than 10. Although 4 miRNAs were correlated with the grade, none of the miRNAs showed correlation with distance free metastasis (DFS). One reason behind this could be due to large number of patients for DFS missing in the database.

One of the main aim of this study was to evaluate and compare miRNA expression from material described in this thesis with a previously published article (76). The differences and similarity between the two different sample cohorts is illustrated in the supplementary table S1, while expression level of miRNAs is illustrated in figure S1. Isolation of total RNA and miRNA detection from both cohorts in supplementary table S1 were performed using the same protocol. Despite that, comparing the Ct value from the real time PCR experiment showed that the MMMCP material have significant higher Ct values for let-7b, miR-18b, miR-25, miR-29c, miR-375, miR505, miR-24 and miR-26b in comparison to materials from Jonsdottir *et al* (76) with independent t-test P<0.001 for all miRNAs. Although this does not apply for miRNA-106b expression, (independent t-test, P=0.492).

Formalin fixation process provides an easy way for the preservation of the tissues in optimal histological condition, however the yield quality and integrity of miRNA may be compromised through degradation, various chemical modifications and cross-linking with proteins (64). Difference in the results between this thesis and Jonsdottir *et al.* (76) could be due to the higher

age and poor quality of the MMMCP tissues those we used for the isolation of total RNA . One positive part of this study in very old tissues samples is, it is confirmed that formalin fixed tissues after a very long stand without any special preservation could also be used to analyze small RNAs. Despite of many positive insights obtained from the study about the miRNAs and their correlations with different clinical features of breast cancer, there are some limitations of the study. While comparing the sample nature of our thesis with the former article (76) There are some differences in the sample nature which could be the reason behind difference in the results. The RNA was isolated from the tissues with at least 50% tumor in former experiment, but there was no any known amount of tumor in MMMCP materials. Whole section of the formalin fixed tissue roll was used. So presence of more mixed cells could dilute the tumor cells. Moreover, MMMCP samples were taken from different country (Netherlands) and also from different hospitals while all samples for former research were from the same hospital (Stavanger University Hospital, Norway). In addition to that, storage condition of the sample and the time of sample stored could have played some role in the fluctuations of the results. Patients for former research were treated with chemotherapeutics while all the patients enrolled in MMMCP are untreated. Despite these differences in sample cohorts, some observations could be confirmed in this study.

Inhibition of let-7b in breast cancer cell lines

Among different miRNAs related to human cancer, the let-7 family is one of the most significantly studied miRNAs because of its expression in different cancers such as lung, breast and colon carcinoma (98). In breast cancer reduced expression of let-7b is associated with poor prognosis (55). An experiment on let-7a in cancer cells and adjacent normal tissues showed that downregulation of let-7a was associated with breast cancer development, describing the tumor suppressor function of let-7a. Let-7a also has the same target (HMGA1) as let-7b (98) This assay was done to observe activity of let-7b as a tumor suppressor gene. qPCR was performed to find the optimal concentration of inhibitor that could knock out the let-7b expression. We used inhibitor concentrations of 0.01 μ M, 0.1 μ M, 0.5 μ M and 1 μ M. Among these concentrations 0.5 μ M concentration of let-7b inhibitor knock out the expression of let-7b in both MCF-7 and MDA-MB-231 cells. So inhibitor concentration of 0.5 μ M was used in both wound healing assay and in making the cell blocks. Three consecutive wound healing assays were done with same inhibitor and scramble concentrations and mean of the all three were used to analyze the results. However higher standard deviation was obtained than expected.

The wound healing assay analysis showed that the tumor suppressor activity of let-7b was inhibited initially as expected. In MCF-7 cells high proliferation was observed in the cells transfected with the inhibitor in comparison to negative scramble and control cells. However, the inhibitory effect of the let-7b inhibitor was not found to have satisfactory long term effect on both of the cell types. So it did not provide any conclusive result. Some difficulties raised during the analysis of the wound area, because of the picture quality and contrast. Many factors such as the floating cells, change in the medium transparency and color in long incubation time and difficulty in maintaining the exact position of the wound at different time intervals could be the reason behind it. In addition to that maintaining the position of camera and the light from

outside could also have some effect in the picture contrast.

Although there are different other targets for let-7b we have selected MAI, ER, PR, Ki67, PPH3, HER2 and CyclinD1 for this study. Since we are also comparing the results with one former research from our group as mentioned earlier had used the same markers. Besides this, the availability of the immunohistochemical experiments already established in the Stavanger University Hospital also limits our choice. None of the immunohistochemical analysis provides the significant results except cyclin D1. Higher expression of cyclin D1 in let-7b inhibited cells showed involvement of let-7b in cell proliferation in addition to that the the qPCR results of the miRNA from the cell blocks also gave negative results for MDA-MB-231 cells and only two amplification curve among the triplicate rises in MCF-7 It shows that the inhibitory effect of the let-7b inhibitor did not persist for long time. During cell block preparation, the MCF-cell population was very low which could have resulted in low count and difficulty in immunohistochemistry. Besides this, use of large T75 flask for cell growth could have some role in the effect of the inhibitor because of large volume of medium and cells. Low cell number in the plate during the time of wound making could be confusing during assay, so it would be better to let the cells to grow until 100% confluency. The use of manual camera also limits the picture quality in wound healing assay. Furthermore, the changing of medium each day during assay could improve the results by removing the floating cells that gives false positive results during analysis.

Mitochondrial mito stress test was done to observe the metabolic activity of the cells using seahorse XF analyzer. This test is a standard and comprehensive method to analyze the key parameters of the mitochondrial functions in cells. It measures Basal respiration, H⁺ (proton leak), ATP-linked respiration, maximal respiration, non-mitochondrial respiration and spare respiratory capacity. For this different modulators of the cellular respirations were used that targets the components of electron transport chain (ETC). Oligomycin was injected to measure ATP-linked respiration while FCCP and mix of rotenone and antimycin were injected to measure maximal respiration and mitochondrial respiration. Finally, these parameters along with basal respiration are used to calculate the proton leak and spare respiratory capacity (82). In our experiment both MCF-7 and MDA-MB-231 cells were used to analyze the mitochondrial activity in Let-7b inhibited and negative scramble control. Negligible or no effect was seen in the MDA-MB-231 cells in any of the parameters, while in MCF 7 cells all the mitochondrial activities including basal respiration, proton leak, maximal respiration and spare respiratory capacity were seen to be lowered significantly in the cells treated with let-7b inhibitor in comparison to control leading to lowered ATP production. However, the same concentration of inhibitor used for wound healing assay, cell block and immunohistochemistry did not give any significant results. This signifies that there could be some unknown mechanism other than mitochondrial respiration by which the proliferating cells generates ATP to compensate the requirement. Since this test was done with single concentration of inhibitor, more experiment using different concentrations should be done to confirm results.

Future directions

Regarding the patients and sample our study was a retrospective so we don't have any control over our study, we had to analyze the materials that were available to us. Although such study has many advantages some of the disadvantages still persist. Many of the samples that would add important information for example many of the database for DFS was missing. So similar nature of prospective study would provide much more information. Although formalin fixed tissues are stable and provide tissue preservation in optimal preservation condition, very long storage without any specific preservation may bring change in the sample tissues due to chemical modifications and cross linking of proteins. In addition, the amount of tumor cells in the tumor tissue was not specified for the MMMCP material. This could influence the expression level of the microRNA since tumor microenvironment could have another expression pattern. This could be the reason for difference in RNA expression. So any techniques like laser microdissection that could isolate only the tumor tissues could be used to obtain the specific results. We used two reference genes, miR-24 and miR-26b on the basis of previous literatures, but with the change in nature of sample the expression level of genes could change. Genes confirmed to have stable expression in one sample type may give different results in another. So for a study in large cohort validation of reference gene should be done in each experiment.

While performing wound healing assay, different factors like growth of cells, inconsistency in the images due to manual camera and floating cells played important role for inconclusive results. Better results could be generated if the confluency of the cells allowed being 100% before making wound. Changing the medium containing same concentration of inhibitor or scramble with slight rinse before taking pictures could help to get rid of the floating cells which provide false positive results. Different type of proliferation assay such as Methylthiazole Tetrazolium assay (MTT) could be used to obtain more specific results. Most importantly images taken with manual camera are not consistent due to difficulty in maintaining the same light condition and position so it would be better to use microscope attached with camera. Use of samples cells with different concentration of inhibitors could provide more information in the mitochondrial mito-stress test.

Conclusion

The main purpose of this study was to validate the prognostic role of different miRNAs. We observed the correlation of miRNA expression with different clinical features of breast cancer. We found that miR-18b, miR-21, miR-25 and miR-150 are associated with high grade while miR-18b and miR-106b are associated with higher proliferation in breast tumor cells. TILs was assessed from the samples and analyzed to see its prognostic value. TILs was seen to determine distant metastasis when looking only at ER positive tumors. Similarly, TILs showed association with tumor grade. Along with that, we explored the effect of let-7b in proliferation on breast cancer using two different cell lines by performing wound healing assay. Increase in proliferation rate was observed in the cells in which let-7b was inhibited, however the results are not conclusive. Immunohistochemical staining did not provide any significance results on let-7b with selected biological markers. Mitochondrial mito-stress test was done to observe the basic cellular metabolism by measuring ATP turnover, proton leak, maximal respiration, and spare respiratory capacity of the cells. In MCF-7 cells transfected with let-7b inhibitor, all of these parameters were found to be lowered in comparison to the control transfected with scramble leading to the significant decrease in ATP production. In conclusion our results confirm the role of Let7b as a tumor suppressor gene in breast cancer. Furthermore, we confirm the strong prognostic value of proliferation in lymph node negative breast cancer, also the prognostic value of TILs is confirmed and different correlations with different microRNAs have been observed. Some of these observations needs further validation studies in larger patient cohorts.

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Appendices

I. Isolation of total RNA from FFPE Tissues using MiRNeasy kit Quiagen, (Germany)

1. Add 1 ml of xylene and vortex vigorously for 10s, and centrifuge at full speed for 2min.
2. Remove the supernatant without disturbing the pellet.
3. Add 1ml of 100% ethanol, mix by vortexing. Centrifuge at full speed for 2 mins. Remove the supernatant by pipetting, do not disturb any pellet.
4. Repeat step 3.
5. Incubate at 37°C for 10 min/ until the residual ethanol has evaporated.
6. Add 240µl Buffer PKD, and mix Immediately place the sections in a 2 ml micro centrifuge tube and close the lid.
7. Add 10µl proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
8. Incubate at 56°C for 15 min, then at 80°C for 15 min. Transfer the lower, uncolored phase into a new 2 ml microcentrifuge tube.
9. Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 x g (13,500 rpm).
10. Transfer the supernatant to a new microcentrifuge tube taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA.
11. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately 20µl) and 10µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.
12. Incubate at room temperature for 15 min.
13. Add 500µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.
14. Add 1750ul ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 17.
15. Transfer 700µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through. Reuse the collection tube in step 18.
16. Repeat step 17 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 19.
17. Add 500µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
18. Add 500µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.
19. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow- through.
20. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add

10µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

II. Isolation of miRNA and total RNA from the same cells but in two fractions, using Purelink® miRNA Isolation kit, Invitrogen, Life Technologies and PureLink® RNA Mini Kit Thermo Fisher Scientific

Purelink® miRNA Isolation kit,

1. Remove the growth medium from the cultured bottle and harvest the cells in 300ul of binding buffer. Vortex to assure the cells are lysed.
2. Add 300ul of 70% ethanol to the lysate and mix well by vortex.
3. Add 600ul of the Lysate into the spin cartridge and centrifuge at 1200xg for 1 minute at room temperature.
4. Keep the column for RNA isolation with Purelink RNA isolation Kit. And proceed from step 5 with flow throw.
5. Add 96-100% ethanol to the flow through to make final concentration 75% of ethanol.
6. Transfer sample to the spin cartridge and centrifuge at 1200xg for 1 minute at room temperature.
7. Discard the flow throw and proceed with the spin cartridge kept in a collection tube for washing steps.
8. Add 500ul of wash buffer (W5) with ethanol to spin cartridge.
9. Centrifuge 12000xg for 1 minutes at room temperature and repeat the step 8.
10. Discard the flow through and keep the spin cartridge into new wash tube
11. Centrifuge at maximum speed for 2-3 minutes at room temperature to remove any residual wash buffer, discard wash tube and proceed with spin cartridge for elution step.
12. Place the spin cartridge into the recovery tube.
13. Add 50-100(75ul was used) of sterile RNase free water to the center of spin cartridge.
14. Incubate at room temperature for 1 minute then centrifuge at maximum speed for 1 minute at room temperature.
15. The recovery tube contains purified small RNA molecules.
16. Store at -80°C.

PureLink® RNA Mini Kit

1. Add 700ul of wash buffer I to the spin cartridge obtained from step 4 of the above isolation procedure with miRNA isolation kit. Centrifuge at 12000xg for 15 seconds at room temperature. Discard the flow through and place the spin cartridge into new collection tube.
2. Add 500ul Wash buffer II with ethanol to the spin cartridge
3. Centrifuge at 12000xg for 15 seconds at room temperature. Discard the flow through and reinsert the spin cartridge into same collection tube and repeat the step 2 and 3 once.
4. Centrifuge the spin cartridge at 12000xg for 1-2 minutes too dry the membrane. Discard the collection tube and insert a new recovery tube and proceed for elution steps.

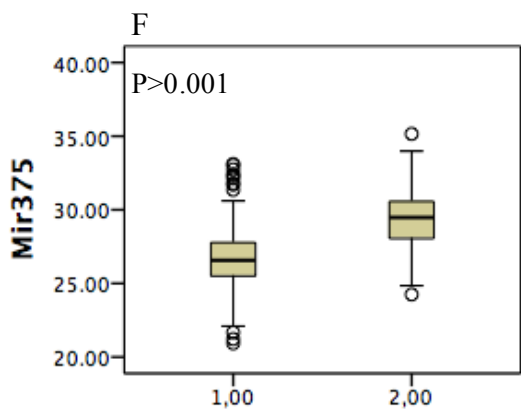
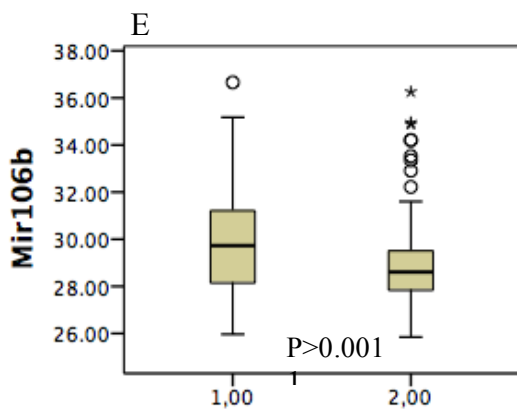
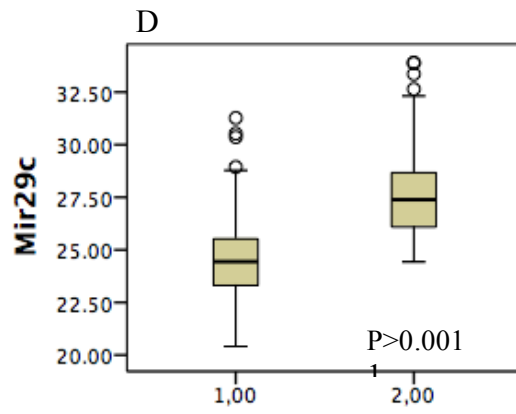
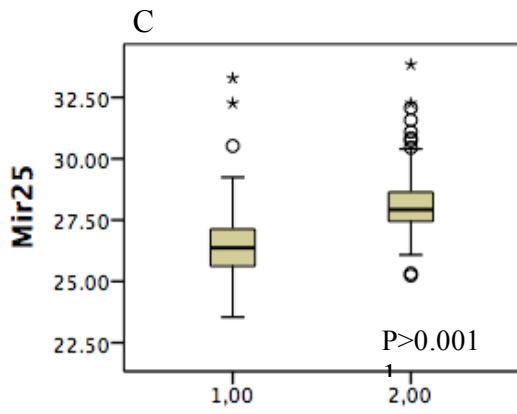
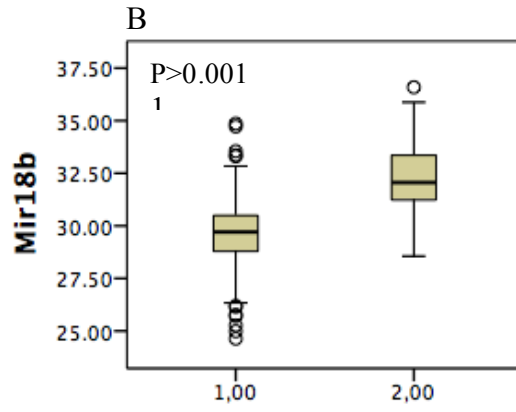
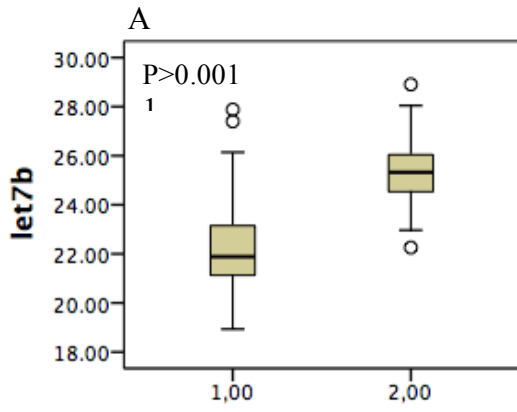
5. Add 30ul x3 RNase free water to the center of the spin cartridge and incubate at room temperature for one minute.
6. Centrifuge at $\geq 12000 \times g$ at room temperature to elute the RNA from membrane into recovery tube.
7. Store the eluted RNA T -80°C .

III. Transfection of cell lines with PNATM miRNA inhibitor

1. Harvest and count the cells.
2. Transfer 150,000 cells per well in medium without antibiotics in 6 well plates and incubate for 24 hours at 37°C and 5% CO₂.
3. After 24 hours, keep appropriate volume of PNATM inhibitor and scramble in Eppendorf tubes and incubate at 70°C water bath for 10 minutes.
4. Mix the scramble and inhibitor with 150µl optimal medium and incubate at room temperature for 15 minutes.
5. Replace the medium in the wells with new medium without antibiotics so that the final volume with inhibitor and optimal medium becomes 1000µl.
6. Incubate the plate at 37 °C and 5% CO₂ for 48 hours

IV. Table 15. Data for quantification of wound area in MCF-7 and MDA-MB-231 cells:
C, Control; S: Scramble; I: Inhibitor

	Mean area of wound	Standard deviation
MCF-7 c 0hour	2.666020314	0.397913928
MCF-7 c 6hour	2.391195218	0.675804445
MCF-7 c 12hour	1.676876694	0.772319732
MCF-7 c 24hour	2.121393402	0.790041107
MCF-7 c 48hour	2.068989515	0.860695089
MCF-7 s 0hour	1.929588914	0.53127487
MCF-7 s 6hour	1.753234347	0.70595092
MCF-7 s 12hour	1.632421851	0.736185789
MCF-7 s 24hour	1.366469085	0.675784769
MCF-7 s 48hour	1.263913691	0.726037845
MCF-7 i 0hour	2.083836238	0.557814394
MCF-7 i 6hour	1.74734509	0.562600564
MCF-7 i 12hour	1.607155999	0.712143188
MCF-7 i 24hour	1.501480659	0.761614528
MCF-7 i 48hour	1.391837517	0.734125581
MDA231 c 0hour	2.915086428	0.436341399
MDA231 c 6hour	2.518758694	0.153695965
MDA231 c 12hour	2.659542203	0.428849086
MDA231 c 24hour	2.232822021	0.492669911
MDA231 c 48hour	1.708793441	0.673542827
MDA231 s 0hour	2.759171168	0.457608118
MDA231 s 6hour	2.628631751	0.321550482
MDA231 s 12hour	2.446439902	0.368679629
MDA231 s 24hour	2.247263153	0.407747336
MDA231 s 48hour	1.865307609	0.489414384
MDA231 i 0hour	2.782085021	0.339662278
MDA231 i 6hour	2.605218331	0.203919882
MDA231 i 12hour	2.496558905	0.080414042
MDA231 i 24hour	2.329114199	0.04454041
MDA231 i 48hour	2.014931917	0.058030372



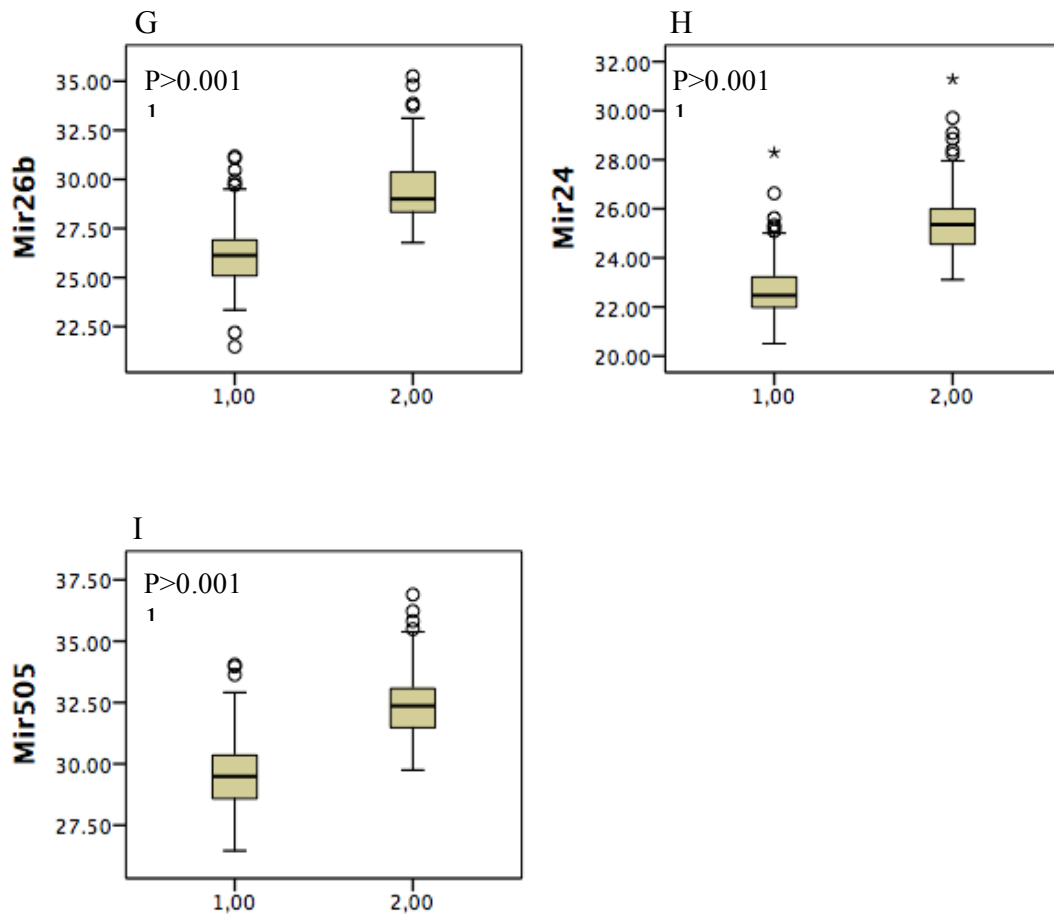


Figure S1. Comparison of expression level of miRNAs between present experiment and former experiment (1). 1=former experiment;2=present experiment.

Table S1. comparison of nature of samples between two experiments.

	Jonsdottir et al. 2012	MMMCP
Material type	FFPE	FFPE
Collected	1996-98	1987-89
Preserved	Block in cool room	Rom temperature, and in roll of section
Isolation	Only tumor area	Hole section