Investigating Differential Expression of MicroRNAs Related to Recurrence in the Luminal Breast Cancer Subtype

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

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2014

Abstract

Breast cancer is a heterogeneous disease, and patients with the luminal subtype have a range of outcomes unable to be predicted by current clinical methods. My hypothesis was that microRNA (miRNA) expression profiling might provide a method of discriminating tumours of this subtype into good and poor prognosis groups, and reveal miRNAs involved in recurrence.

Expression profiling revealed nine miRNAs significantly differentially expressed in luminal tumours from patients with recurrence compared to patients without recurrence. Four, miR-135a-5p, miR-140-5p, miR-218-5p, and miR-200a-3p, selected for technical validation were confirmed as differentially expressed. Functional analysis in an *in vitro* breast cancer model revealed a reduction in cellular migration when miR-218-5p was ectopically overexpressed in two different assays, suggesting a possible association of miR-218-5p with metastasis, an important factor in recurrence. The expression of these miRNAs may allow improved prognostication in luminal tumours, and provide avenues of further research in breast cancer pathology.
Acknowledgments

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## Abbreviations

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<tr>
<td>ANN</td>
<td>Axillary node negative</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed, paraffin-embedded</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>ISH</td>
<td><em>In situ</em> hybridization</td>
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<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
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<tr>
<td>MEM-α</td>
<td>Modified essential medium, alpha</td>
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<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>Pre-miRNA</td>
<td>Precursor microRNA</td>
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<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
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<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>RNA-Seq</td>
<td>RNA sequencing</td>
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<td>RNU6-1</td>
<td>RNA, U6 small nuclear 1</td>
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<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SAM</td>
<td>Significance analysis of microarrays</td>
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<tr>
<td>SSP</td>
<td>Single sample predictor</td>
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<tr>
<td>TAMC</td>
<td>TaqMan array microRNA cards</td>
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<tr>
<td>TGFβ</td>
<td>Tumour growth factor beta</td>
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1 Introduction

1.1 Breast Cancer

Breast cancer is the most common cancer in Canadian women and the second most common cause of cancer related death, after lung cancer. Breast cancer incidence has remained relatively stable over the past decade, with the current lifetime probability of a woman developing breast cancer at 11.5%. However, mortality due to breast cancer has been declining for decades, and is the lowest it has been since 1950, with a five year overall survival rate of 88%\(^1\). Advances in early diagnosis and treatment have done much to reduce this mortality rate; however, a minority of breast cancer patients will eventually die from their disease, most often from complications of metastasis\(^2\). Patients can experience a recurrence of their cancer up to several decades after initial treatment, so it is important to increase the ability of clinicians to accurately diagnose and treat breast cancer patients, especially those greatest at risk of experiencing a recurrence\(^3\).

Surgery is the primary treatment for breast cancer, but non-excised tumour beyond the surgical margins or occult micrometastases elsewhere in the body can remain dormant and potentially lead to a recurrence of cancer\(^4\). To treat this, adjuvant chemotherapy and hormonal therapy has been shown to reduce recurrence and increase overall survival in patients. However, many individual patients either do not respond to certain types of therapy or do not require it, exposing them to harmful and occasionally serious side effects without benefit\(^5\). Breast cancer is a heterogeneous disease, and tumours vary widely in their pathological characteristics, including their potential for recurrence and response to treatment\(^6\). It is therefore important that when an individual presents with breast cancer, an accurate diagnosis of the specific type of tumour can be made, dictating a treatment personalized to the tumour and the patient’s prognosis.

1.1.1 Breast Cancer Development

The precise causes and mechanisms of breast cancer initiation and progression are poorly understood; however, much progress has been made in understanding how normal cells become cancer cells\(^7\). In general, cancer arises through a series of somatic mutations, deregulating normal cell functions, converting to a phenotype of aggressive and uncontrollable growth\(^8\). Inherited genetic predispositions, such as a mutation in the DNA repair gene BRCA1, or environmental
factors, such as carcinogens, can increase the chances of oncogenic somatic mutations through genomic instability\textsuperscript{7}. These can be dominant gain of function mutations in oncogenes, or recessive loss of function mutations in tumour suppressor genes\textsuperscript{8}.

Certain genes, such as \textit{TP53} or \textit{RB1}, encoding for proteins p53 and retinoblastoma protein (Rb) respectively, are considered “guardians” of the genome, as they coordinate monitoring of the integrity of the cell and its DNA, regulating repair or self-destruction if it is compromised. These are mutated or otherwise inhibited in many types of cancer, including breast cancer, preventing repair or destruction and allowing the mutated cells to propagate, and gain additional mutations\textsuperscript{8}. In addition to these two genes, a cell has a number of programs acting as a defense against uncontrolled growth and eventual development into a cancer. Cells must gain the ability to bypass each of these programs through mutation or epigenetic modification. These abilities were codified by Hanahan and Weinburg as the Hallmarks of Cancer, consisting of six required hallmarks and two potential hallmarks, all required for development of metastatic cancer\textsuperscript{4,8}.

The first hallmark is self-sufficiency in growth signals. Normal cells generally require external cues to begin proliferating, through endocrine or paracrine growth factors, or through cell-cell communication. Tumour cells can acquire their own growth program, through making their own signals, stimulating surrounding stromal cells to release signals, or by increasing the amount of signal receptors, so that normal low signal amounts are amplified\textsuperscript{8}. Disruption of negative feedback loops for growth signalling within the cell can also promote growth\textsuperscript{4}.

The second hallmark is insensitivity to growth inhibitory signals\textsuperscript{8}. Normally, cells have checkpoints in the cell cycle requiring proteins like Rb, monitoring for external inhibitory signals, and p53, monitoring the internal conditions of the cell, such as DNA damage, or nutrient availability, that would halt growth. Mutations in these genes or their pathways prevent these stop signals in cancer cells and growth can continue unabated. Additionally, normally inhibitory signals like tumour growth factor beta (TGFβ) can be converted to tumourigenic functions. Instead of halting growth in cancer cells, it promotes conversion of epithelial cells to a more invasive phenotype, through a process called the epithelial to mesenchymal transition (EMT), preventing growth inhibition through cell-cell contact\textsuperscript{4}.
The third hallmark is the evasion of programmed cell death. Cells normally monitor the cell environment, and if abnormal conditions such as major DNA damage occur, signalling through p53 and other proteins activate proteolysis to break down and eventually lyse the cell through apoptosis. Mutations disrupting the monitoring or effector proteins promote oncogenesis by allowing cells with compromising mutations to continue to thrive and divide.

The fourth hallmark is limitless replicative potential. Cancer cells avoid the normal limited lifespan of somatic cells by up regulating telomerase, an enzyme that restores telomeres, DNA segments at the end of chromosomes, which shorten every cell cycle. Normally, when the telomeres become too short, chromosomal integrity is threatened and cells enter a senescent state. If this is bypassed through disruption to p53 or other proteins, eventually chromothripsis occurs, causing major disruption in normal DNA replication, and major chromosomal rearrangements. Increased telomerase expression prevents this from being lethal, but the enzyme is often up regulated after major chromosomal rearrangements have already occurred, promoting increased mutagenesis leading to further DNA disruption.

The fifth hallmark is sustained angiogenesis. Cancer cells need to develop their own blood vessels to supply nutrients and remove wastes to grow into a full tumour. This happens relatively early, by mutations activating pathways expressing growth factors for blood vessel growth and expansion. Additionally, angiogenesis is promoted by immune cells inducing inflammation, as well as vascular progenitor cells recruited to the rapidly growing tumour.

The sixth hallmark is tissue invasion and metastasis, and what separates metastatic cancer from benign tumours. To grow beyond a certain size, tumour cells need to be able to advance through surrounding tissues and overcome natural cell-cell barriers to growth. Tumour cells lose the adherens junctions that connect them to other cells, and release enzymes such as matrix metalloproteinases (MMP) which break down the extracellular matrix. EMT is a major component in this process, as structured epithelial cells acquire a more flexible mesenchymal phenotype. Interactions with the microenvironment can also promote an invasive margin on the exterior of the tumour, and neighbouring mesenchymal stem cells and macrophages release invasive factors as well. The actual metastatic process requires a number of steps of its own, each requiring certain cellular abilities, including invasion out of the tissue and into circulation, and transitioning back into a new tissue environment to establish a secondary tumour.
There are two “emerging” hallmarks of cancer. One is reprogramming energy metabolism, as many cancer cells convert from oxidative phosphorylation to glycolysis with an associated increase in glucose transport, which is less energy efficient but requires less oxygen and allows glycolytic intermediates to be used for biosynthesis. The other is evading immune destruction, as some cancer cells are immunogenic and either need to hide from the innate immune system or inhibit it with secreted factors. Together, all these abilities allow tumours to grow, but the most damaging is the sixth hallmark of invasion and metastasis, allowing the cancer to spread, and eventually overwhelm the body’s organs, leading to death. However, not all tumours have the necessary abilities to metastasize. Research into the biological basis of breast cancer recurrence is important to be able to detect which tumours have the potential to recur, and how best to prevent that recurrence.

1.1.2 Breast Cancer Characterisation

The most important prognostic factor in breast cancer is the number of axillary lymph nodes containing cancer cells that have spread from the primary tumour. Patients with no affected nodes, known as axillary node negative (ANN), have a good prognosis, but around ~20% will still have a recurrence. To improve prognostication of ANN patients, the Toronto Breast Cancer Study group collected a cohort of breast tumour samples and associated patient clinical data from 1987 to 1993 for a prospective study on the association between human epidermal growth factor receptor 2 (HER2) amplification and recurrence. Since then, this cohort has been used for other studies into potential prognostic factors in ANN breast cancer, including p53 and HER2 combined, specific mutations in p53, and validation of prognostic factor analysis using tissue microarrays. The size of this cohort allows for studies within specific breast cancer patient subpopulations, and analyses of the prognostic ability of uncommon factors.

In addition to node status, a number of other tests are conducted in the clinical setting in order to characterise a breast tumour. Testing the protein expression of estrogen receptor (ER) and HER2 are important because there are specific treatments that target these factors, but these tests can also be used assist prognosis, as patients with tumours expressing ER (ER+), tend to have less chance of recurrence than those with ER- or HER2+ tumours. While these clinical methods are effective, individual prognostic factors may not be informative of the underlying biology responsible for the difference in prognosis. Improved classification methods would
benefit all breast cancer patients, as more discreet characterisation would improve prognostic accuracy and simplify treatment decisions. It is especially important for ANN patients, whose overall prognosis is good, but further methods of determining prognosis are required to identify those who will experience a recurrence of their cancer.

Much work has been done to investigate alternative and improved methods of stratifying breast cancer into discrete subtypes. The most prominent of these methods is messenger RNA (mRNA) molecular expression profiling. Based on the concept that the pathologic heterogeneity observed in breast tumours could be at least partially attributed to gene expression, this technique associates, or “clusters”, tumours and genes together based on similarity of expression patterns.

Perou et al. analyzed a cohort of breast tumours that had been sampled before and after treatment, measuring the expression of thousands of genes. By statistically identifying the genes that had greater expression variation between tumours than between samples of the same tumour, they identified an “intrinsic” set of 496 genes. Using this gene set, they were able to identify expression differences between ER+ and ER- tumours, as well as tentative subgroups within the ER groups. Sørlie et al. later analyzed the expression of this set of genes on a larger cohort of breast tumours, using unsupervised clustering to organise the tumours into subgroups based on expression similarity. This technique revealed a number of subgroups: basal, which correlated with ER-HER2- and expressed cytokeratins found in basal epithelial cells; HER2, which had high expression of HER2 associated genes and correlated with HER2+ tumours; normal, which had expression similar to non-cancerous cells; and multiple luminal groups, which correlate to ER+HER2- and express keratins 8 and 18 found in luminal epithelial cells.

These subtypes were additionally found to correlate with prognosis, with the basal and HER2 subtypes having a consistently worse overall survival, compared to the luminal groups, which varied in their survival rate. While the other subgroups were relatively homogenous in expression and consistent in characterisation and prognosis, tumours in the luminal subgroups were less consistent and had a range of prognosis outcomes. The luminal group with a good prognosis was defined as luminal A and the poor prognosis group luminal B. These early studies did not clearly distinguish between the luminal subgroups. Increasing the size of the cohort resulted in many samples changing their designation, suggesting that assignment to a specific luminal subgroup was not definitive.
1.1.3 The Luminal Breast Cancer Subtype

Clinically, molecular profiling subtypes can be approximated using histology without having to analyze expression on a microarray. “Luminal” tumours are defined as ER+ and HER2-. While the majority of patients with luminal tumours have a good prognosis, a subset will experience a recurrence of their tumour, and benefit from aggressive therapy. It has been shown that there is subset of around 30% of individuals with luminal tumours that show no response to hormone therapy, suggesting a conversion to alternative growth pathways. It is important to identify this poor prognosis luminal subset, so all patients with luminal tumours are not exposed to harmful side effects of therapy, potentially including serious conditions such as heart failure.

This challenge has motivated development of other methods of determining which patients are the most at risk for recurrence and would most benefit from aggressive treatment. A number of methods have been proposed to enhance or supplant the intrinsic luminal A or B molecular profiling subtyping. Sorlie et al. refined their subtypes into a single sample predictor (SSP) model, able to predict the subtype of an unknown tumour by comparing its expression profile to the averaged expression of each subtype’s tumours. In their SSP, poor prognosis luminal and good prognosis luminal were indistinct, especially compared to basal. They found that good prognosis luminal represented 50%-60% of total tumours and had a prognosis significantly better than all other subtypes, and even had a higher survival time if it did recur. Poor prognosis luminal represented 10-20% of all tumours and had a prognosis comparable to basal and HER2 cancers. These tumours had increased expression of proliferation genes and a worse response to anti-estrogen receptor therapy, but an increased response to chemotherapy compared to good prognosis luminal.

Other groups have developed SSPs for breast cancer with roughly the same subtypes and characteristics. An example is the PAM50 SSP, which uses the expression of 50 genes to make its designation, and designates good and poor subgroups for luminal tumours. In general, while the multiple SSPs have similar population distributions when applied to a cohort of tumours, the concordance rate for an individual tumour is only modest. Only basal tumours are highly consistent, and poor prognosis luminal tumours have an especially high variability, often being placed in the HER2 group and vice versa. The problems with current SSPs are with methodology
and variation between groups and platforms. Standardized guidelines could improve these issues, but currently SSPs are not yet sufficiently mature for general clinical use\textsuperscript{19}.

Gene expression has been used to develop other methods of characterisation that do not rely on subtyping directly. Analysis of a select gene signature can produce a quantitative result that can assist in clinical decision-making. Mammaprint was developed by analyzing genes in breast cancer that had expression associated with disease outcome, resulting in a 70-gene signature\textsuperscript{22}. This test is designed for luminal ANN breast cancer as another method to identify good and poor prognosis patients\textsuperscript{23}. While the results appear promising, this test requires a relatively large amount of tumour sample to be sent to Amsterdam for testing at a significant cost, and is not currently recommended for clinical use\textsuperscript{17,24,25}.

Oncotype DX is another signature comprised of 21 genes known to be important in breast cancer, designed for patients with ANN luminal cancer at an early age. This test results in a recurrence score out of 100, stratifying patients into high-risk poor prognosis, intermediate risk, or low risk good prognosis\textsuperscript{26}. This test is currently approved for use clinically to determine which patients with luminal tumours are candidates for chemotherapy.

A similar test is “genomic grade”, developed by analyzing genes that had expression that correlated with histological grade. Primarily measuring genes related to proliferation and cell cycle, it has also been suggested as a method to discriminate prognosis groups in luminal cancer\textsuperscript{27}. In general, the ability of gene signatures to predict prognosis in luminal cancer is largely based on measuring proliferation-related genes\textsuperscript{28}. These signatures also have few genes in common, suggesting that the genes being measured might not have a direct role in pathogenesis, but might instead have large expression changes that are statistically associated with common factors that are biologically more important, but are harder to detect\textsuperscript{25}.

Another factor preventing clinical use of microarray signatures is cost\textsuperscript{29}. A method of determining luminal prognosis groups using immunohistochemistry (IHC) would be preferable as it is already routinely in use to determine ER, progesterone receptor, and HER2 expression in the clinical setting. There is no current clinical marker in use, but the most promising is the protein Ki-67, a marker of cellular proliferation. In breast cancer, Ki-67 expression correlates with increased chance of recurrence\textsuperscript{29}. Recent recommendations on standardizing Ki-67 analysis have brought this marker closer to clinical use, but there continue to be a number of issues
preventing its routine analysis for luminal breast cancer\textsuperscript{17}. There is still debate on how the marker should be analyzed, what antibody should be used, and even with standardization there is at least a 25\% error rate\textsuperscript{21,29}. IHC in general is qualitative and subjective, and does not reveal information about the biology of luminal cancer\textsuperscript{29}.

DNA analysis is a relatively new avenue for subtyping breast cancer. Research using the PAM50 SSP demonstrated more somatic mutations and copy number variations in luminal B tumours compared to luminal A. The luminal B group also had almost twice the amount of loss of heterozygosity events in \textit{TP53} and \textit{RB1}, tumour suppressors important in breast cancer biology\textsuperscript{18}. Curtis \textit{et al.} combined DNA analysis with expression analysis to come up with 10 subtypes of breast cancer, eight of which correlated with luminal tumours using the PAM50 SSP\textsuperscript{30}. While this will help investigation into the biology of breast cancer, these subtypes displayed a range of outcomes, and significant work is needed before this DNA data could be used for clinical prognosis.

A common theme of all these methods is the significance of proliferation on the prognosis of patients with luminal tumours. Whether it is measured by gene expression or IHC marker, proliferation in luminal tumours appears to represent a continuous variable instead of discreet subgroups\textsuperscript{19,28}. Continuous variables are a problem clinically, as the question becomes what counts as good or poor within a range, and where to make a cut off\textsuperscript{21}. Clearly more information on the biology of luminal tumours is needed to complement the information gained from these methods. In the last decade a specific type of RNA, MicroRNA (miRNA) has been investigated to provide this new information and opportunity for improving luminal prognosis.

\section{1.2 MicroRNA}

MiRNAs are a class of short noncoding RNA molecules that serve as post-transcriptional regulators of mRNA expression. Discovered in \textit{Drosophila melanogaster} relatively recently, they are generally highly conserved among species lineages. Often tissue or developmentally specific, they are transcribed by RNA polymerase II as a primary miRNA transcript, and undergo a series of processing steps becoming a precursor miRNA (pre-miRNA) and then a mature miRNA as outlined in Figure 1\textsuperscript{31}. There are currently more than 2500 published mature human miRNAs and new RNA analysis technologies are rapidly revealing more\textsuperscript{32}. 
Figure 1: miRNA biogenesis pathway from DNA to a mature miRNA.
1.2.1 MicroRNA Function

MiRNAs function by targeting a sequence complementary site on the 3’ untranslated region (UTR) of its target mRNA. MiRNAs can target and regulate multiple mRNAs, and each mRNA can have multiple, even complementary, regulating miRNAs\textsuperscript{31,33,34}. While it is clear that the function of miRNAs is to reduce the amount of translated protein of its target mRNA, how it achieves this function is not entirely clear. MiRNA activity is carried out by the RNA induced silencing complex (RISC). A mature miRNA is loaded into the RISC, which binds to the target mRNA. If the miRNA and target are completely complementary, the target mRNA is cleaved, analogous to the function of small interfering RNA. However, if there is a slight mismatch between the miRNA and target, as is almost universally the case in humans, a different function results\textsuperscript{31}.

When the miRNA is not completely complementary to the target sequence, instead of cleavage, the mRNA is prevented from transcribing a protein and is eventually degraded, through a still ambiguous process\textsuperscript{31}. Inhibition of translation is through either the initiation or elongation steps, or combination of the two, potentially depending on the mRNA promoter, the cellular context, or external factors. Regardless of the mechanism, inhibited mRNAs are sequestered into P-bodies, areas of the cell rich in mRNA processing proteins\textsuperscript{35,36}. There they are deadenylated and the 5’ cap is removed, followed by digestion\textsuperscript{36}. This degradation of the mRNA transcript allows analysis of miRNA function by examining the expression of potential targets.

1.2.2 MicroRNA in Breast Cancer

MiRNAs were originally discovered in \textit{D. melanogaster} as important regulators of developmental timing gene expression\textsuperscript{37}. Their ability to control many genes simultaneously suggested a role as high-level regulators of cellular growth and differentiation, the deregulation of which is an important hallmark of cancer\textsuperscript{38}. The first direct link of miRNAs to cancer was the discovery that miRNAs are statistically overrepresented at fragile sites and other chromosomal variations frequently seen in cancer\textsuperscript{39}.

Lu \textit{et al.} analyzed a number of miRNAs in a series of samples from different types of cancer, and found that miRNAs were widely deregulated\textsuperscript{40}. Clustering tumours, based on similarity of miRNA expression, associated them together based on their developmental origin, a
trend not observed in mRNA profiling. MiRNA expression correlated with cellular differentiation, and less differentiated tumours had lower overall expression. Compared to mRNA expression profiling, it appeared that fewer miRNAs needed to be examined to achieve equivalent profiling significance.

Later work demonstrated that miRNA profiling could discriminate between breast cancer and normal breast tissue, and that the expression of certain miRNAs correlated with patient clinical characteristics. Tumours do not cluster into subgroups similar to mRNA expression, but instead represent a unique set of associations, adding an additional layer of information. Breast cancer cell lines were also shown to have clear deregulation of miRNAs.

Research that is more recent has demonstrated the importance of miRNAs in breast cancer biology. The expression of many miRNAs is under control of important transcription factors such as p53 and c-Myc. ER and HER2 have been shown to be targeted and regulated by miRNAs, and in turn, the ER growth pathway has been shown to regulate some miRNAs. MiRNAs can act as either tumour suppressors or oncogenes, depending on the function of their target genes. Tumour suppressive miRNAs target and regulate growth factors and other components of proliferation or invasion pathways, and inactivation can contribute to the cancer hallmarks of self-sufficient growth and invasion. Oncogenic miRNAs target genes that regulate homeostasis or other genes involved in cell maintenance pathways, such as Rb. Increased expression of these miRNAs inhibit the vital cellular functions of their targets, potentially contributing to the inhibitory signal insensitivity and evasion of apoptosis cancer hallmarks.

MiRNA expression is associated with the response to breast cancer therapy and may be responsible for a large portion of heterogeneity.

While miRNA expression has been profiled in breast cancer demonstrating correlation with subtypes, there is little gene overlap in proposed signatures, similar to mRNA profiles. More focused analysis within a specific subtype, such as luminal, may improve the quality of results. Analysis of luminal miRNA expression profiles may provide an alternative or even superior method of determining prognosis for patients.

An additional benefit of exploring miRNA expression within the luminal subtype is to reveal which individual miRNAs might be associated with the biology of breast cancer, and help prevent or contribute to the pathology of the disease. When originally discovered n D.
*melanogaster*, miRNAs were recognised as vital controllers of growth and differentiation\(^ {37}\). They play a similar role in humans, and many miRNAs have been shown to target and regulate genes with these important functions. MiRNAs have been associated with all the hallmarks of cancer, and are especially important in evading growth suppressors, sustaining proliferative signalling, avoiding apoptosis, and activating invasion and metastasis\(^ {35,38}\). MiRNA deregulation has been recognised as playing a significant role in breast cancer\(^ {41}\).

As an example, miR-21-5p has shown increased expression in most human cancers compared to matched normal tissue\(^ {49}\). In breast cancer, it has been found to be one of the most significantly up regulated miRNA compared to matched normal tissue in multiple studies\(^ {41,50-52}\). Additionally, its expression also correlated with advanced tumour stage, axillary lymph node metastasis, increased Ki-67 expression, and reduced survival\(^ {51,52}\). The consistency with which miR-21-5p has been found with high expression in cancers has driven much investigation into its role in tumourigenicity and potential target pathways, confirming its role as an oncogene.

*In vitro* and *in vivo* studies have shown miR-21-5p involved with multiple hallmarks of cancer. Inhibition of this miRNA resulted in reduced growth of breast cancer cells, increased apoptosis, and reduced tumour growth in mice inoculated with breast tumour cells\(^ {50}\). Additionally, ectopic overexpression of miR-21-5p induced EMT in epithelial breast cancer cells with associated increases in migration and invasion, and conversely, inhibited miR-21-5p reduced cellular migration and invasion\(^ {53,54}\). Increased levels of miR-21-5p expression is associated with drug resistance in multiple types of cancer, and there is evidence that inhibiting the miRNA sensitises cells to chemotherapy, and is a potential target for therapy\(^ {49}\).

Targets of note include *TPM1*, an actin binding protein involved in microfilament stabilization and growth regulation. Breast cancer cells with exogenously increased *TPM1* regulation by miR-21-5p demonstrated increased colony formation ability\(^ {55}\). Additionally, *PDCD4*, a tumour suppressor and transformation inhibitor, is a target. *PDCD4* regulates the transcription initiation factor eIF4A, and promotes expression of p21, a regulator of the cell cycle to maintain normal growth rates\(^ {56}\). MiR-21-5p also contributes to cellular migration and invasion by promoting the expression of two types of MMP, through targeting and down regulating MMP inhibitors *TIMP3, RECK*, and *PTEN*\(^ {57}\). PTEN also inhibits the cellular signalling protein AKT,
and miR-21-5p inhibition of PTEN reduces apoptosis and increases cell survival through the PI3K-AKT pathway.

MiRNAs that have expression correlating with prognosis in luminal breast cancer may be associated with any or all of the hallmarks of cancer. Determining what functional aspects of tumourigenicity they are involved in might provide insight into the causes of recurrence, as well as opportunities for further avenues of research towards improved diagnosis, prognosis, and treatment.

1.2.3 MicroRNA Analysis

Working with miRNA presents a number of challenges unique to their tiny molecules. Their small size, similarity of mature species with precursors, and need for a detection range over a wide order of magnitudes means that analysis technology has to be specifically adapted for miRNA. While there are a number of microarray platforms available, reverse transcription quantitative polymerase chain reaction (RT-qPCR) is considered the “gold standard” of miRNA expression analysis. The TaqMan system uses proprietary stem-loop primers to measure only the mature miRNA as well as improve the sensitivity and efficiency of detection (Applied Biosystems, CA, USA).

Mature miRNAs are cleaved from a pre-miRNA during biogenesis, and standard PCR primers bind to both species, preventing accurate measurement of the functional form. By including a loop on the end of the RT primer, it will only bind the cleaved mature miRNA. Additionally, adding extra sequence to the resulting complementary DNA (cDNA) increases specificity, important with many miRNAs that only differ by a single nucleotide, and the increased length improves the template for the real time reaction. The double stranded primer also increases binding stability and reaction efficiency.

MiRNA can be analyzed using TaqMan RT-qPCR in a medium throughput manner using TaqMan Array MicroRNA Cards (TAMC). With high technical reproducibility and controls, TAMCs are the preferred method of miRNA analysis. Data from the TAMCs can be analyzed like any other microarray, allowing for straightforward analysis using standard microarray statistics.
Recently, a technology has been developed allowing for analysis of miRNA expression in formalin-fixed, paraffin-embedded (FFPE) samples \textit{in situ}. As opposed to extracting RNA from tumour samples, this enables localization of miRNA expression within the sample, and determines which cells are actually expressing each miRNA. Breast tumours have a heterogeneous cellular population, and the microenvironment is known to play an important role in the biology of tumour development\textsuperscript{4}. \textit{In situ} hybridization (ISH) analysis would reveal which cells were expressing a given miRNA. In addition to the malignant tumour cells themselves, miRNA expression could be altered in the involved stroma, as well as other cell types such as invading lymphocytes. ISH also allows determination if expression levels are due to altered cell type heterogeneity within a sample, or if the cells themselves are each expressing more or less miRNA\textsuperscript{62}. A robust ISH technique could also allow for simultaneous analysis of miRNA expression and cellular grade, and any potential association between them. Other markers could be examined along with miRNA, such as Ki-67 for proliferation, or even potential targets to determine if there is an inverse correlation of expression\textsuperscript{63}.

In terms of functional analysis of miRNAs, there are methods available to ectopically overexpress or inhibit the function of individual miRNAs. This can be done transiently in cell lines, and the resulting altered phenotype, if any, analyzed through appropriate tumourigenicity assays. Functional analysis of the effects of altering miRNA function through either overexpression or inhibition will potentially reveal associations that miRNA has on breast cancer pathology\textsuperscript{64}. Individual tumourigenicity assays test for hallmark abilities such as increased proliferation, reduced apoptosis, and increased migration and invasion, as described in the Materials and Methods chapter. Care must be taken because the cellular environment plays a significant role in miRNA expression, and therefore cell line conditions \textit{in vitro} may not necessarily be extendible to clinical tumours\textsuperscript{24}.

While determining if miRNA function modulation has an effect on cellular phenotype is relatively straightforward, determining the molecular pathways by which they occur is more difficult. MiRNAs recognise specific “seed” sequences on the 3’ UTR of the target mRNA. However, unlike other types of transcriptional regulators, the seed sequences are not fully complementary to the miRNA, preventing determination of targets by genome alignment. Instead, multiple target prediction algorithms have been developed to determine potential targets with varying levels of accuracy\textsuperscript{65}. 
These algorithms generally work by searching a database of mRNA sequence for semi-complementary seed sequences to find potential hits. The thermodynamic properties of the predicted duplex is then analyzed to determine if it is too weak or strong, and finally the target site is analyzed in other species to see if it is evolutionarily conserved. Once a target is predicted, it requires experimental validation, usually through a reporter gene assay where the target site is cloned into a reporter gene, such as luciferase, where inhibition of the target by miRNA can be easily quantified\textsuperscript{65}. In the years after miRNAs were discovered, many targets were validated for the most common miRNAs, and now there is a large library of validated targets available for most miRNAs. However, there are many miRNA-target interactions yet to be discovered for even the most intensely studied miRNAs\textsuperscript{32}. 
2 Rationale and Objectives

2.1 Rationale

Clinical characterisation of breast cancer is important to deliver the ideal treatment regimen, personalised to the specific type of tumour, with the least amount of side effects. The decision to use adjuvant chemotherapy is guided by prognostic factors that indicate the likelihood that the patient will experience a recurrence of their tumour. Node status and the presence or absence of histology markers such as ER and HER2 are sufficient information for some types of breast cancer. However, determining which luminal tumours will respond to chemotherapy is ambiguous with current clinical methods, and further characterisation is required to avoid treatment side effects in those patients who do not need it.

One potential method of determining which patients with luminal tumours have a good or poor prognosis is through miRNA. By characterising the expression of miRNA in luminal tumours and determining which miRNAs have expression that correlates with recurrence, it is possible that in the future these genes could be used as a marker indicating the potential for recurrence in a newly diagnosed luminal tumour. Additionally, characterisation of individual differentially expressed miRNAs may reveal information about the biology of luminal tumours, the pathology of recurrence, and potential avenues for further investigation and treatment target development.

2.2 Hypothesis

MicroRNA expression profiling will allow improved discrimination of the luminal subtype of ANN breast cancer into distinct subtypes with differing prognoses.

2.3 Specific Aims

1. Find differences in miRNA expression in luminal tumours between ANN patients who experience recurrence and those who do not experience recurrence.

2. Characterize significantly differentially expressed miRNAs and explore their role in luminal breast cancer.
3 Materials and Methods

I performed all of the miRNA expression experiments as well as the *in vitro* functional analysis. Dr. Dushanthi Pinnaduwage conducted the statistical analysis of the results of the miRNA array expression profiling, as well as the validation expression profiling results. Lucine Collins performed the ISH experiments under my direction.

3.1 Tumour Samples

The Toronto Breast Cancer Study Group has collected a cohort of fresh frozen ANN tumour samples with matched patient data. It was assembled from September 1987 to March 1993 with the objective of analyzing the prognostic value of molecular alteration in ANN breast cancer\(^9\). From the 998 breast tumours available, luminal samples were selected by choosing from those that expressed ER, but not HER2, as HER2 is an independent prognostic factor\(^9\). Of the 666 qualifying tumours, a sample set consisting of 19 tumours from patients who experienced a recurrence of their tumour, and a matched set of 20 tumours from patients who did not experience recurrence was compiled, for 39 tumours total. This number was based on a statistical prediction of the number of samples required for significant results and sample availability. Only distant relapse was considered a recurrence\(^9\). In the recurrence group, median follow-up time was 36.11 months with a minimum of 11.04 months and maximum of 135.89 months. For the no-recurrence group, median follow-up time was 114.74 months with a minimum of 81.02 months and maximum of 157.54 months. The samples had their RNA previously extracted using trizol and quantified.

3.2 MicroRNA Array

MiRNA expression profiling of the tumour samples was done using the TaqMan array microRNA cards system (Life Technologies, Burlington, ON). The TaqMan Human MicrRNA Array A contains 377 miRNAs, three potential endogenous controls, and one negative control. Following the manufacturer’s protocol, the Megaplex RT Human Pool A primer pool, containing proprietary reverse transcription primers for every miRNA on the array, was used to reverse transcribe the RNA samples, using 45 ng of total RNA per sample, into cDNA. A quantitative pre-amplification followed, using the TaqMan PreAmp Master Mix Kit and Megaplex PreAmp Primers, Human Pool A, to increase the overall amount of cDNA template, minimising the initial
amount of tumour RNA required. Real-time analysis on the TAMCs was performed using the PRISM 7900HT Sequence Detection System (Life Technologies).

Every amplification reaction in each sample was reviewed, and failed reactions were censored. The Ct results for each sample were normalized using the $2^{-\Delta\Delta Ct}$ method to the endogenous control RNA, U6 small nuclear 1 (RNU6-1), averaged from 4 wells on each card. The normalized expression of each sample’s miRNAs was then submitted to Dr. Dushanthi Pinnaduwage who conducted the statistical analysis. After removing miRNAs which were expressed in less than 5 in each group as required for effective statistical analysis, she used significance analysis of microarrays (SAM) to rank significantly differentially expressed miRNAs between recurrence groups, as well as provided a t-test p-value for each miRNA’s difference in expression. From this analysis, those miRNAs in the top 20 ranked by SAM that had a p-value less than 0.05 were designated as significantly differentially expressed to reduce the potential for false positive results.

3.3 MicroRNA RT-qPCR

To validate the array results, TaqMan singleplex miRNA analysis (Life Technologies) was used. In a protocol analogous to the array, 15 ng of RNA from each tumour sample was used to make cDNA using pooled proprietary TaqMan miRNA singleplex primers for four miRNAs and one endogenous control as shown in Table 1. From that cDNA, three individual pre-amplification steps were conducted to ensure that amplification was consistent and properly quantitative, using the TaqMan PreAmp Master Mix Kit and pooled real-time miRNA primers for the four miRNAs and control.

Real-time analysis for each of the three pre-amplified cDNA replicates was run on the 7500 Fast Real-Time PCR System (Life Technologies). The real time results were reviewed to censor failed reactions and the $2^{-\Delta\Delta Ct}$ method used to normalize the results to RNU6-1. The results of the three replicates for each miRNA in each sample were then averaged. Dr. Dushanthi Pinnaduwage statistically analyzed the validation results in the same manner as the initial array, resulting in a t-test p-value for the difference in expression.
<table>
<thead>
<tr>
<th>MirBase ID</th>
<th>MirBase Accession</th>
<th>Assay Name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-135a-5p</td>
<td>MIMAT0000428</td>
<td>hsa-miR-135a</td>
<td>000460</td>
</tr>
<tr>
<td>hsa-miR-140-5p</td>
<td>MIMAT0000431</td>
<td>mmu-miR-140</td>
<td>001187</td>
</tr>
<tr>
<td>hsa-miR-200a-3p</td>
<td>MIMAT0000682</td>
<td>hsa-miR-200a</td>
<td>000502</td>
</tr>
<tr>
<td>hsa-miR-218-5p</td>
<td>MIMAT0000275</td>
<td>hsa-miR-218</td>
<td>000521</td>
</tr>
<tr>
<td>-</td>
<td>NR_004394*</td>
<td>U6 snRNA</td>
<td>001973</td>
</tr>
</tbody>
</table>

*NCBI Accession
3.4 Messenger RNA RT-qPCR

Analysis of the expression of mRNA targets of the miRNAs was conducted using the Power SYBR Green system (Life Technologies). Five ng of extracted RNA was used per µg of cDNA reaction mixture. Using the High Capacity cDNA Reverse Transcription Kit, the RNA was reverse transcribed into cDNA followed by real-time analysis using custom primers and Power SYBR Green Master Mix on the 7500 Fast Real-Time PCR System. The results were normalised using the $2\Delta\Delta C_t$ method to endogenous control gene HPRT1. Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)67. Primers for mRNA expression analysis are outlined in Table 2.

3.5 In Situ Hybridization of miRNAs

MiRNA ISH experiments were conducted by Lucine Collins. To determine miRNA expression in situ, the miRCURY LNA platform was used (Exiqon, Woburn, MA). This technology uses locked nucleic acid (LNA) probes, which have a higher melting temperature than RNA or DNA probes, increasing the specificity and sensitivity of hybridization68. The microRNA ISH Optimization Kit 2 and microRNA ISH Buffer and Controls Kit were used to develop a protocol, and LNA probes, double tagged with digoxigenin, specific for each miRNA used for analysis, as shown in Table 3. FFPE slides of the breast cancer cell lines BT474, MDA-MB-231, and SKBR3, and FFPE slides of normal breast tissue and lymph nodes were used during optimization. MiR-21-5p and RNU6-1 were used as positive controls. FFPE slides were hybridized with either a probe for a miRNA of interest, a positive control, or a scrambled probe negative control, stained, and photographed using an optical microscope.

3.6 Cell Culture

MCF7 cells, purchased from ATCC, (Manassas, VA) were grown in modified essential medium, alpha (MEM-α, Life Technologies), supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies), 0.01 mg/ml bovine insulin (Sigma-Aldrich, Oakville, ON), and 1:100 dilution penicillin/streptomycin (Sigma-Aldrich) at 37°C at 5% CO₂.
### Table 2: Primer sequences for mRNA expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>5' → 3' Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTEN</strong></td>
<td>Forward</td>
<td>CTTTGAGTTCCCTCAGCCGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTGAGGTTTCCCTCTGCTCT</td>
</tr>
<tr>
<td><strong>PDCD4</strong></td>
<td>Forward</td>
<td>GTGACGCCCTTAGAAGTGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTACCCAGACACCTTTGC</td>
</tr>
<tr>
<td><strong>BIRC5</strong></td>
<td>Forward</td>
<td>GGTTGCCTTTCCCCTTTCTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCACTTTCTCCGCAGTTTCC</td>
</tr>
<tr>
<td><strong>LASP1</strong></td>
<td>Forward</td>
<td>ATGCTTCCATTGCGAGACCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAACCTCCTCTTGTAGCGCA</td>
</tr>
<tr>
<td><strong>HPRT1</strong></td>
<td>Forward</td>
<td>ATGCTGAGGATTTGGAAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTGAGCACACAGGGGCTA</td>
</tr>
</tbody>
</table>

### Table 3: MiRCURY LNA detection probe product numbers

<table>
<thead>
<tr>
<th>Target</th>
<th>Product Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21-5p</td>
<td>90002 (Optimization Kit 2)</td>
</tr>
<tr>
<td>miR-140-5p</td>
<td>21309-15</td>
</tr>
<tr>
<td>miR-200a-3p</td>
<td>18094-15</td>
</tr>
<tr>
<td>RNU6-1</td>
<td>90010 (Buffer and Controls Kit)</td>
</tr>
</tbody>
</table>
3.7 Cell Line RNA Extraction

Total RNA was extracted from cells using the MiRNeasy Micro Kit (Qiagen, Toronto, ON), according to the manufacturer’s instructions, with the addition of an RNase-Free DNase Set (Qiagen), to prevent DNA contamination. Extracted RNA was quantified using a Nanodrop 1000 Spectrophotometer (Fisher Scientific, Ottawa, ON).

3.8 Transfection

MCF7 cells were transfected using a reverse transfection protocol according to the manufacturer’s instructions (Life Technologies). For each set of assays, the transfection process occurred in 15 ml centrifuge tubes and then aliquots of appropriate cell count and volume for each type of assay plated with a final concentration of 150,000 cells per millilitre. Cells were transfected with a mirVana miRNA inhibitor or mimic (Life Technologies), specific for an individual miRNA or scrambled as a negative control, as indicated in Table 4. Lipofectamine RNAiMAX (Life Technologies) was used as the transfection reagent. Media was changed after 24 hours, and RNA extracted 72 hours after transfection.

3.9 XTT Cell Quantification Assay

To test changes in cellular proliferation and survival, the XTT assay was used. XTT is a tetrazolium salt that is cleaved by dehydrogenase enzymes in active cells when added in vitro. The result of this cleavage is a coloured product in an amount relative to the number and viability of cells in solution. The amount of coloured product can be quantified by measuring the absorbance, and compared against cells under different conditions.

To quantify cellular proliferation and survival, the XTT Cell Proliferation Kit II (Roche Diagnostics, Laval, QC) was used according to the manufacturer’s instructions. Transfected MCF7 cells in complete media were plated into 16 wells per transfection condition on a 96-well flat bottom plate with 1500 cells per well. The well locations for each condition were randomized for every repeat to prevent well location from affecting the results. Media was changed 24 hours after transfection.
<table>
<thead>
<tr>
<th></th>
<th>Mimic</th>
<th></th>
<th>Inhibitor</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Product Number</td>
<td>Assay ID</td>
<td>Product Number</td>
<td>Assay ID</td>
</tr>
<tr>
<td>miR-200a-3p</td>
<td>4464066</td>
<td>MC10991</td>
<td>4464084</td>
<td>MH10991</td>
</tr>
<tr>
<td>miR-21-5p</td>
<td>4464066</td>
<td>MC10206</td>
<td>4464084</td>
<td>MH10206</td>
</tr>
<tr>
<td>Negative Control</td>
<td>4464058</td>
<td>-</td>
<td>4464076</td>
<td>-</td>
</tr>
</tbody>
</table>
72 hours after transfection, the XTT reagent was added to each well. 4 hours after addition, absorbance of each well was measured on a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm with a reference wavelength of 650 nm. The peripheral wells of each plate were omitted due to variations from evaporation, and the absorbances of the remaining wells of each transfection condition were averaged.

3.10 Scratch Wound Assay

For analysis of migration, there are a number of available assays. Migration ability can be measured by scratching a confluent monolayer of cells and measuring their rate of movement to fill the wound over time. This is known as the scratch wound assay, and can be quantified either as the distance or area travelled by cells at a certain time, or by time taken to close the wound.

To quantify the cellular migratory ability, the amount of closure of a scratch wound in a confluent monolayer under starvation conditions after 24 hours was measured. Transfected MCF7 cells in complete media were plated into six-well plates at $3.75 \times 10^5$ cells per well. Media was changed at 24 hours. Seventy-two hours after transfection, the media was changed to low FBS media (MEM-α, with 0.01 mg/ml bovine insulin and 1% FBS). Twenty-four hours later each well was scratched with a p1000 pipette tip introducing a 1 mm wound to the cellular monolayer. Cells were washed with phosphate buffered saline and low FBS media re-added.

Designated wound areas were imaged using a computerised phase contrast microscope. 24 hours after wounding, the same areas previously imaged were imaged again to record the changes in cellular location after a 24-hour period. Quantification was done using TScratch image analysis software, which resulted in a measurement of scratch area as a percentage of image field$^{70}$. The area designation for each image was reviewed to ensure proper measurement. Migration for each area was quantified as the difference in scratch area after 24 hours compared to initial scratch area. Three representative areas were quantified for each transfection condition, and the area differences averaged together.

3.11 Transwell Assays

A transwell chamber can be used to measure migration. In this assay, cells are added to the top chamber of a two chambered well with a porous membrane separating them. Adding a
chemoattractant induces the cells to move through the pores into the lower chamber. To measure ameboid motility, a synthetic matrix that emulates the extracellular matrix is added to the membrane requiring cells to move through it before entering the lower chamber. This assay is quantified by measuring the number of cells in the lower chamber after a certain amount of time.71

To measure the cellular migration and invasive abilities, their ability to cross a porous membrane both with and without a coating of simulated extracellular matrix was measured. 6.5 mm transwell inserts with 8 µm pores (Corning Life Sciences, Tewksbury, MA) were used. Transfected MCF7 cells in complete media were plated in 6-well plates to proliferate, and the media changed after 24 hours. 48 hours after transfection, the cells were isolated and 1500 cells in low FBS media were added in the upper chamber of the insert. The insert was placed in a 24-well plate and complete media added to the lower chamber. To test ameboid motility, the protocol was the same, with a 25 µg layer of Matrigel (Corning Life Sciences) added to each insert before plating.

Forty-eight hours after transwell plating, cells were trypsinized from the underside of the insert and the lower chamber. The number of cells was quantified using the CyQUANT NF Cell Proliferation Assay Kit (Life Technologies) according to the manufacturer’s instructions. Cells were collected in a 96-well plate along with CyQUANT NF reagents. After 45 minutes, the fluorescence was measured using a FlexStation 3 Microplate Reader.
4 Results

4.1 MiRNA Expression Profiling of Luminal Tumours

I analyzed a set of 39 luminal tumours using the TaqMan system to determine differences in miRNA expression between tumours from patients who subsequently experienced cancer recurrence and those that did not. Of the 377 miRNAs analyzed, 198 were expressed in five or more samples per group. Overall, the majority of miRNAs had less expression in the recurrence group compared to the no-recurrence group, with 76% (151 of 198) miRNAs having higher expression in the no-recurrence group. Total expression of all miRNAs expressed in both groups was higher in the no recurrence group as well.

Nine significantly differentially expressed miRNAs were found, as shown in Table 5 and Figure 2. All nine had lower expression in the recurrence group compared to the no-recurrence group, following the trend of the miRNAs as a whole. The expression levels of the differentially expressed miRNAs were low, all on the scale of 1000 times less expressed than endogenous control gene RNU6-1. RNU6-1 was selected due to having multiple wells on the TAMC, its significance as a normalization control for RT-qPCR, and it not being associated with any breast cancer clinical factors. The magnitude of the differences in expression varied for each miRNA, ranging from miR-135a-5p, which was expressed 13.96 times more in the no-recurrence group than in the recurrence group, to miR-199b-5p, which was expressed only 2.85 times more. MiR-218-5p had the most significant difference in expression based on SAM, followed by miR-135a-5p, with p-values less than 0.01.

4.2 Technical Validation of Differentially Expressed MiRNAs

To validate the results of the miRNA TAMC, I selected four miRNAs for technical validation in the same samples using singleplex TaqMan RT-qPCR. I chose miR-135a-5p, miR-140-5p, miR-200a-3p, and miR-218-5p based on significance and overall expression levels. The results are shown in Table 6 and Figure 3. All four had expression that remained significantly greater in the no-recurrence tumour group compared to the recurrence group.
Table 5: Significantly differentially expressed miRNAs between breast tumours from patients with recurrence of their cancer and patients without recurrence as analyzed on the TAMC

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>T-test p-value</th>
<th>SAM Rank</th>
<th>Average Expression Relative to RNU6-1 (10^-3)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No Recurrence</td>
<td>Recurrence</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>95% CI</td>
<td>Mean</td>
<td>95% CI</td>
<td></td>
</tr>
<tr>
<td>miR-218-5p</td>
<td>0.006</td>
<td>1</td>
<td>7.55</td>
<td>5.33-10.69</td>
<td>2.44</td>
</tr>
<tr>
<td>miR-200a-3p</td>
<td>0.022</td>
<td>2</td>
<td>3.77</td>
<td>1.54-9.22</td>
<td>0.73</td>
</tr>
<tr>
<td>miR-135a-5p</td>
<td>0.003</td>
<td>3</td>
<td>1.19</td>
<td>0.54-2.65</td>
<td>0.09</td>
</tr>
<tr>
<td>miR-198</td>
<td>0.028</td>
<td>5</td>
<td>0.60</td>
<td>0.22-1.65</td>
<td>0.14</td>
</tr>
<tr>
<td>miR-511-5p</td>
<td>0.014</td>
<td>6</td>
<td>0.42</td>
<td>0.25-0.69</td>
<td>0.12</td>
</tr>
<tr>
<td>miR-140-5p</td>
<td>0.034</td>
<td>8</td>
<td>1.98</td>
<td>1.03-3.82</td>
<td>0.63</td>
</tr>
<tr>
<td>miR-495-3p</td>
<td>0.015</td>
<td>13</td>
<td>0.32</td>
<td>0.15-0.70</td>
<td>0.08</td>
</tr>
<tr>
<td>miR-199b-5p</td>
<td>0.018</td>
<td>15</td>
<td>0.70</td>
<td>0.44-1.13</td>
<td>0.25</td>
</tr>
<tr>
<td>miR-455-5p</td>
<td>0.020</td>
<td>16</td>
<td>0.40</td>
<td>0.22-0.73</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Figure 2: Statistically significant differentially expressed miRNAs between breast tumours from patients with recurrence of their cancer and patients without recurrence as analyzed on the TAMC. Bars represent average expression relative to RNU6-1 on an order of $10^{-3}$. A $^*$ denotes a p-value <0.05 and $^{**}$ denotes a p-value <0.01 by standard non-paired 2-tailed t-test.
Table 6: MiRNAs validated as differentially expressed between breast tumours from patients with recurrence of their cancer and patients without recurrence using singleplex TaqMan RT-qPCR.

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>T-test p-value</th>
<th>Average Expression Relative to RNU6-1 (10^3)</th>
<th>No Recurrence</th>
<th>Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>95% CI</td>
<td>Mean</td>
</tr>
<tr>
<td>miR-135a-5p</td>
<td>0.017</td>
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<tr>
<td>miR-140-5p</td>
<td>0.014</td>
<td>6.46</td>
<td>4.20-9.95</td>
<td>2.87</td>
</tr>
<tr>
<td>miR-200a-3p</td>
<td>0.004</td>
<td>16.47</td>
<td>11.93-22.73</td>
<td>6.50</td>
</tr>
<tr>
<td>miR-218-5p</td>
<td>0.006</td>
<td>3.97</td>
<td>2.58-6.10</td>
<td>1.44</td>
</tr>
</tbody>
</table>
Figure 3: Differential expression of four miRNAs selected for technical validation conducted by singleplex TaqMan RT-qPCR on luminal tumours from patients with breast cancer recurrence and patients without recurrence. Bars represent average expression relative to RNU6-1 on an order of $10^{-3}$. P-values are by standard non-paired 2-tailed t-test.
MiR-135a-5p was expressed 8.05 times greater in the no-recurrence group than the recurrence group with a p-value of 0.017. MiR-140-5p was 2.25 times more expressed with a p-value of 0.14, miR-200a-3p was 2.54 times more expressed with a p-value of 0.004, and miR-218-5p was 2.76 times more expressed with a p-value of 0.006. These results confirm the array results, indicating these four miRNAs are differentially expressed in my luminal tumour cohort.

4.3 **In Situ Hybridization (ISH) of miRNAs**

To examine the localization of miRNA expression within FFPE tumour samples, Lucine Collins optimized a miRNA ISH protocol using LNA probes. ISH of two of the differentially expressed miRNAs, miR-140-5p and miR-200a-3p, as well as RNU6-1 as a positive control and a scrambled negative control on a normal breast tissue FFPE sample can be seen in Figure 4. While RNU6-1 expression was clearly visible, the miR-140-5p and miR-200a-3p probes resulted in staining indistinguishable from the scrambled negative control. Due to the apparent success of the procedure, but no visible expression of the differentially expressed miRNAs, further ISH analysis was not completed.

4.4 **Altering MiRNA Function in vitro**

To determine if any of the differentially expressed miRNAs played a functional role in the biology of breast cancer recurrence, I conducted functional analyses in an *in vitro* model of breast cancer. To focus my research, I chose to investigate miR-218-5p because it is well characterised, and potentially linked to other types of cancer in the literature. I also decided to use miR-21-5p in my functional experiments as a positive control, as this miRNA has been well studied in breast cancer. To obtain results most relevant to luminal breast cancer, I chose a cell line model with the same subtype.
Figure 4: ISH using LNA probes for miR-140-5p, miR-200a-3p, the positive control RNU6-1, and a scrambled negative control probe on a normal breast tissue sample. Arrows highlight purple staining representing expression.
The breast cancer cell lines MCF7, T47D, and ZR751 are all ER+, HER2-, so I measured their endogenous expression of miR-218-5p to see if expression was similar to my tumour cohort, as shown in Figure 5. The expression of all three cell lines was less than in the no-recurrence group, but the expression in ZR751 was about the same as in the recurrence group. The expression in MCF7 was lower than both recurrence groups, and expression of miR-218-5p was undetectable in T47D. Though the expression of miR-218-5p in ZR751 is most similar to my tumour cohort, I chose MCF7 for my functional assays as it is easier to culture and more commonly used in the literature, allowing me to use established protocols and compare my results with previously published studies.

To determine the effect miR-218-5p function has on cell phenotype in MCF7 cells, I had to alter the function and observe any resulting change in phenotype relative to unaltered control cells. I accomplished this by transiently transfecting miRNA mimics or inhibitors specific to a single miRNA. MiRNA mimics simulate increased expression by introducing a large number of synthetic copies of the gene into the cell that are functionally the same as the endogenous miRNA, potentially resulting in increased regulation and lower expression of target mRNAs. MiRNA inhibitors bind to the target miRNA and prevent it from regulating its target, potentially resulting in an increase of target expression.

To confirm the success of the transfection protocol and ensure the miRNA function was altered, I analyzed the expression of genes confirmed as targets of each miRNA in the literature (Figure 6). For miRNA-21-5p, I measured the expression of PTEN and PDCD4\textsuperscript{54,56}. MiRNA-218-5p has a number of validated targets; I selected BIRC5, a caspase inhibitor, and LASP1, involved in movement of actin filaments, for expression analysis\textsuperscript{73,74}. 
Figure 5: Expression of miR-218-5p relative to endogenous control RNU6-1. A) Average expression of miR-218-5p in the tumour recurrence groups. B) Average expression of miR-218-5p in ER+, HER2- breast cancer cell lines.
Figure 6: Diagram of validated miR-218-5p oncogenic targets and pathways from the literature\textsuperscript{73-86}. 
The expression of each target in each transfection condition is shown in Figure 7. For \textit{PTEN}, the expression was consistent between both controls and in cells transfected with an inhibitor for miR-21-5p, suggesting no effects of inhibition on \textit{PTEN} expression. The expression of \textit{PTEN} in cells transfected with the miR-21-5p mimic was slightly less than the controls, at around 80% of expression in cells transfected with the mimic negative control (p=0.033), demonstrating an increase in regulatory activity. \textit{PDCD4} had consistent expression between its negative controls, but showed a marked reduction of expression in cells transfected with the mimic at around 55% (p=0.004) compared to cells transfected with the scrambled mimic control. \textit{PDCD4} also had an increase in expression in cells transfected with the inhibitor at around 180% (p = 0.010) of cells transfected with the inhibitor negative control. This demonstrated a successful transfection and alteration of miR-21-5p function in these cells.

For the targets of miR-218-5p, \textit{BIRC5} and \textit{LASP1} both showed similar changes in function. They both had consistent levels of expression in the negative controls, but also in the cells transfected with an inhibitor to miR-218-5p, showing little effect of the inhibitor in these cells. Cells transfected with the mimic, however, showed expression of approximately 55% of the expression in the scrambled mimic control for both \textit{BIRC5} (p = 0.001) and \textit{LASP1} (p = 0.002). These observed changes in miRNA target expression demonstrate the success of the mimic and inhibitor transfection process, and confirms miRNA function is being altered in these cells.

4.5 Phenotype Analysis of Altered MiRNA Function

To investigate if the altered miRNA function from transfection of mimics or inhibitors would have an effect on cellular phenotype, I carried out four types of assays to measure both cell growth and cell motility, both important aspects of breast cancer recurrence. I analyzed the effects of altered miR-218-5p function on cell growth using the XTT cell quantification assay, and on migration using the scratch wound assay and transwell migration assay.
Figure 7: MiRNA target expression relative to endogenous control HPRT1 in cells transfected with miRNA mimics or inhibitors. A. Expression of miR-21-5p targets. B. Expression of miR-218-5p targets. C. Expression of miR-21 targets relative to scrambled negative control. D. Expression of miR-218-5p targets relative to scrambled negative control. A ✱ denotes a p-value <0.05 and ✱✱ denotes a p-value <0.01 by standard non-paired 2-tailed t-test relative to scrambled control.
4.5.1 XTT Cell Quantification Assay

To determine the effects of altered miR-218-5p function on the ability of cells to grow and divide, I used the XTT assay to quantify the number of living cells 72 hours after transfection with either a miRNA mimic or inhibitor, in triplicate. The amount of absorbance correlates to the number of viable cells for each transfection condition. For miR-21-5p, no change in cell number was seen in the mock-transfected cells, demonstrating that the scrambled controls did not have an effect on growth, as seen in Figure 8. No change in cell number was seen in cells transfected with a mimic or inhibitor of miR-21-5p.

For miR-218-5p, also shown in Figure 8, the scrambled controls also did not affect cell number, with the mock-transfected cells showing similar levels of absorption to cells transfected with the scrambled mimic or inhibitor. Similar to the results for miR-21-5p, cells transfected with either a mimic or inhibitor for miR-218-5p activity did not show a change compared to the negative control, suggesting miR-218-5p activity does not affect cell number.

4.5.2 Scratch Wound Assay

I used a scratch wound healing assay to assess the effects of altering miR-218-5p function on the ability of cells to move and travel across a surface. By making a scratch in a confluent monolayer of cells, I could measure the distance cells had moved after 24 hours to fill the gap as an indication of the ability of cells to move. Overall, there was a low amount of movement observed in MCF7 cells, with an average reduction in scratch field as a proportion of the total image of 4%.

As seen in Figure 9, variation was high for mimics and inhibitors of both miRNAs. The cells transfected with the scrambled inhibitor had the same migration distance as mock-transfected cells, but cells transfected with the scrambled mimic had a lower average migration distance than mock-transfected cells, suggesting a possible cellular effect from the transfection process. For miR-21-5p, both the cells transfected with the mimic and the cells transfected with the inhibitor had high variation and average migration distances that were similar to mock-transfected cells.
Figure 8: XTT cell quantification assay absorbance relative to scrambled negative controls. Cells were incubated with XTT reagent for four hours, beginning 72 hours after transfection. A) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p. B) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-218-5p. Bars represent average absorbance relative to scrambled control ± standard deviation. N=3
Figure 9: Reduction of scratch wound image area relative to cells transfected with negative controls. Cells were imaged 24 hours after scratching and movement measured. A) Representative phase contrast photographs of transfected cells showing migration immediately after wounding and 24 hours later. B) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p. C) Cells that underwent mock-transfection or transfection with and inhibitors for miR-218-5p. Bars represent average percent reduction relative to scrambled control ± standard deviation. A ** denotes a p-value <0.01 by standard non-paired 2-tailed t-test relative to scrambled control. N=4
For miR-218-5p, the inhibitor had a large variation and similar average migration distance to the mock transfection as well. The cells transfected with the miR-218-5p mimic, however, showed a notable reduction in migration distance relative to cells transfected with the mimic negative control, averaging no movement at all after 24 hours \((p=0.007)\). This suggests a role for miR-218-5p in cellular migration, with increased activity correlating with reduced cell movement.

### 4.5.3 Transwell Assays

The transwell migration assay is another method of measuring cellular mobility potential. By plating cells in low FBS media on a porous surface with full FBS media on the other side, cells are attracted towards the FBS gradient and slowly travel to the other side of the membrane. I then measure the number of traversed cells using reagents that allow quantification of cells by fluorescence. The ameboid motility assay is identical except that there is a layer of matrigel emulating the extracellular matrix that requires the cell to migrate it through to the other side.

The results of the transwell assays were highly variable, reflecting the very low number of migrating cells overall. As seen in Figure 10, migration in the mock-transfected cells was the same as that of cells transfected with the scrambled mimic, but cells transfected with the scrambled inhibitor had slightly higher migration, as demonstrated by the relatively lower migration of the mock-transfected cells. Cells transfected with either a mimic or inhibitor miR-21-5p showed no difference in migration relative to the negative controls.

Cells transfected with a mimic for miR-218-5p had an average amount of migration that was slightly less than that of the negative control. Likewise, cells transfected with the miR-218-5p inhibitor had slightly more migration than the negative control. Neither of these results was statistically significant however, as high variability in both the negative control cells and experimental cells prevented obtaining conclusive results.
Figure 10: Transwell migration assay of migrated cells relative to cells transfected with a negative control. Migrated cells were collected after 48 hours and quantified using fluorescence. A) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p and miR-218-5p. B) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p and miR-218-5p. Bars represent average fluorescence relative to scrambled control ± standard deviation. N=4
The Matrigel assays also had high variability, as seen in Figure 11. Average migration through the Matrigel for all cells was consistently similar. No change was seen between the mock-transfected cells, control cells, or cells transfected with a mimic and inhibitor for miR-21-5p. Likewise, cells transfected with a mimic or inhibitor for miR-218-5p had the same amount of migration through Matrigel as mock-transfected cells and control cells. These results suggest little direct effect of miR-21-5p or miR-218-5p on the ability of MCF7 cells to move through a Matrigel layer.
Figure 11: Transwell assay of cells invading through a Matrigel layer relative to cells transfected with a negative control. Migrated cells were collected after 48 hours and quantified using fluorescence. A) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p and miR-218-5p. B) Cells that underwent mock-transfection or transfection with mimics and inhibitors for miR-21-5p and miR-218-5p. Bars represent average fluorescence relative to scrambled control ± standard deviation. N=4
5 Discussion

Patients with the luminal subtype of breast cancer generally have a good prognosis; however, a minority will experience a reoccurrence of their cancer, making them good candidates for effective chemotherapy. Chemotherapy has undesirable side effects so ideally this treatment would be given only to those most likely to benefit from it, sparing the majority of luminal patients who would receive no benefit. Effective methods of determining which patients with luminal breast cancer fall into the high risk or poor prognosis group in the clinical setting are limited. I proposed profiling the miRNA expression in a set of luminal tumours from patients who had a recurrence of their cancer and from patients who did not have recurrence to try to determine if any miRNAs were expressed differently between these groups. These differentially expressed miRNAs could represent prognostic factors to predict the prognosis for future luminal breast cancer patients, as well as provide insight to the biology of luminal breast cancer.

I profiled the expression of 377 miRNAs in luminal breast tumours from 20 patients with recurrent cancer and 19 matched patients who were recurrence free. The results of this analysis revealed that 76% of expressed miRNAs had average expression levels lower in the recurrence group compared to the no-recurrence group. These findings are intriguing, as similar trends have been seen in other comparisons of miRNAs; tumour cells in general have less miRNA expression compared to normal tissues of their respective origin. Since miRNAs are important in controlling cellular differentiation, it is possible that lower overall miRNA levels reflect a loss of differentiation, characteristic of high-grade aggressive tumours. The lower miRNA expression seen in the recurrence group could therefore reflect their more aggressive nature.

Of the 198 miRNAs with expression in both tumour groups, I found nine that were significantly differentially expressed. In order of significance based on SAM, miR-218-5p, miR-200a-3p, miR-135a-5p, miR-198, miR-511-5p, miR-140-5p, miR-495-3p, miR-199b-5p, and miR-455-5p were less expressed in tumours from patients without recurrence. To validate these results, I chose miR-135a-5p, miR-140-5p miR-200a-3p, and miR-218-5p for follow up, due to their significance and overall expression. I validated all four of these miRNAs as differentially expressed within the tumours samples with very high significance. These results confirm the differential expression of these miRNAs in my samples, and, along with the other five that were not selected for validation, may represent a group that has expression associated with recurrence
in luminal breast cancer in general, although much more investigation into this potential is required. The four validated miRNAs have been described in the literature, and have been previously implicated in cancers of various types.

I was partially involved in supervising a student in our lab, James Lee, who profiled the expression of the four validated miRNAs in an independent luminal tumour set selected from the Toronto Breast Cancer Study Group cohort, with 23 tumours from patients with recurrence and a matched set of 23 tumours from patients without recurrence, for 46 tumours. These tumours had the same distribution of clinical characteristics as shown in Table 7. He performed the RT-qPCR experiments and I analyzed and compiled the results, for statistical analysis by Dr. Dushanthi Pinnaduwage. As shown in Table 8 and Figure 12, results were similar to the initial cohort, with miR-135a-5p, miR-140-3p, and 218-5p, having lower expression in the recurrence group compared to the no recurrence group. miR-140-5p and miR-218-5p maintained their significance, with p-values of 0.04 and 0.01, respectively, while miR-135a-5p was close to significant with a p-value of 0.08. Notably, miR-200a-3p did not follow the trend from my cohort, and showed no significant difference in expression. These results suggest that miR-140-5p, miR-218-5p, and possibly miR-135a-5p may represent significantly differentially expressed miRNAs in luminal breast cancer in general, and could be candidates for prognostic indicators.

To determine the localization of miRNA expression within FFPE samples, ISH analysis was attempted but ultimately proved inconclusive. Visible expression of the positive control confirmed that the experiment was successful in normal breast tissue, but the cellular staining of differentially expressed miRNAs was indistinguishable from the scrambled negative control probe. The positive control, RNU6-1, is around 1000 times more expressed than these miRNAs, suggesting the lack of visible expression is due to their extremely low expression and an insufficiently sensitive ISH method. Additional positive controls of samples with known high expression of these miRNAs would be required to confirm this. The inability to detect miRNA expression negated further ISH analysis in tumour samples.
**Table 7:** Comparison of clinical characteristics between study tumour cohort and validation tumour cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study cohort (n=39)</th>
<th>Validation Cohort (n=42)</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td><strong>Number of Recurrences</strong></td>
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<td>22</td>
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<td><strong>Menopausal status</strong></td>
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<tr>
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<td>Peri</td>
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<td><strong>Tumour Size</strong></td>
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<tr>
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<td>5</td>
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<tr>
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<td>41.0</td>
<td>15</td>
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<tr>
<td>&gt; 2.0 to 5.0 cm</td>
<td>16</td>
<td>41.0</td>
<td>20</td>
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<td>&gt; 5.0 cm</td>
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<td><strong>Histological grade</strong></td>
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<td>1*</td>
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<tr>
<td>2</td>
<td>19</td>
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<tr>
<td>3</td>
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<tr>
<td>ND‡</td>
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<td><strong>Lymphatic invasion</strong></td>
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<tr>
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<tr>
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<td>Maximum</td>
<td>75.14</td>
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</tr>
</tbody>
</table>

‡Unknown, not done or missing; *Includes mucinous, lobular and tubular subtypes; **by Chi-square test or Fisher's exact test; ***by standard t-test
Table 8: Differential expression of MiRNAs between breast tumours from patients with recurrence of their cancer and patients without recurrence in an independent tumour cohort using singleplex TaqMan RT-qPCR.

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>T-test p-value</th>
<th>Average Expression Relative to RNU6-1 (10^{-3})</th>
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<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No Recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>95% CI</td>
<td>Mean</td>
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<tr>
<td>miR-135a-5p</td>
<td>0.076</td>
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<tr>
<td>miR-140-5p</td>
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<td>miR-200a-3p</td>
<td>0.420</td>
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<td>15.95-50.68</td>
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<td>miR-218-5p</td>
<td>0.013</td>
<td>8.71</td>
<td>5.67-13.36</td>
<td>4.24</td>
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</table>
Figure 12: Differential expression of four miRNAs conducted by singleplex TaqMan RT-qPCR on an independent cohort of 46 luminal tumours from patients with breast cancer recurrence and patients without recurrence. Bars represent average expression relative to RNU6-1 on an order of $10^{-3}$. P-values are by standard non-paired 2-tailed t-test.
The role of miR-135a-5p in cancer is controversial, as it has been found to be both suppressive and contributory to different types of cancers. It was found to have higher expression in colorectal cancer compared to normal colon tissue, and increased expression resulted in increased proliferation and migration in colorectal cancer cell lines. Two validated targets involved in this oncogenic potential were found, *MTSS1*, a tumour suppressor, as well as *APC*, a component of the WNT pathway, responsible for regulating β-catenin<sup>87,88</sup>. Another validated target in the WNT pathway is *SIAH1*, which also regulates β-catenin, implicated in malignant cervical squamous cell carcinoma where miR-135a-5p expression is up regulated<sup>89</sup>.

In these models, miR-135a-5p acts as an oncogene, targeting tumour suppressors and contributing to tumour progression. However, in other cell types and models the reverse has been demonstrated. Expression has been shown to be reduced in classic Hodgkin lymphoma, gastric cancer, and malignant glioma compared to normal tissues<sup>90-92</sup>. This activity is oriented around the JAK-STAT pathway, with *JAK2*, *STAT6*, and *SMAD5* as validated targets. MiR-135a-5p is also down regulated in renal cell carcinoma, where *MYC*, an oncogene, is a validated target as well<sup>93</sup>.

*HOXA10* is also a target of miR-135a-5p, but there is disagreement on whether this results in activating p53, resulting in tumour suppression, as seen in epithelial ovarian cancer where miR-135a-5p expression was down regulated, or suppressing p53, resulting in support of tumour progression, seen in metastatic breast tumours where miR-135a-5p was up regulated<sup>94,95</sup>. Another study looked for prognostic miRNAs in breast cancer, and found that miR-135a-5p expression correlated with good prognosis in ER+ breast cancer, as well as inversely correlated with proliferation signature, supporting the findings in my analysis<sup>96</sup>. Outside breast cancer, this miRNA’s apparently opposite effects on tumourigenicity could be due to tissue or cell type specific function. Although expression of miR-135a-5p was low in my samples, and only found in a minority (13 of 39) of tumours, another member of its family, miR-135b-5p, had a significant t-test p-value of 0.01 on my array results, but was not in the top 20 SAM ranking. Future exploration of these miRNAs in luminal breast cancer may prove informative.

Unlike miR-135a-5p, research into miR-140-5p has consistently implicated it in tumour suppressive functions. It is down regulated in non-small cell lung cancer, where it regulates the growth factor receptor *IGF1R*<sup>97</sup>. In osteosarcoma and colon cancer cell lines, forced expression
of miR-140-5p inhibited proliferation in a p53 dependant manner, through directly targeting the histone deacetylase $HDAC4$, inducing cell cycle arrest$^{98}$. The two primary areas where miR-140-5p has been studied are the TGFβ pathway and cellular self-renewal systems. This miRNA is down-regulated in hepatocellular carcinoma, with low expression correlating with a worse prognosis. It directly targets the TGFβ receptor $TGFBR1$, growth factor $FGF9$, and $SMAD3$, all members of the TGFβ pathway$^{99,100}$. TGFβ is involved in EMT, and frequently deregulated during metastasis.

In breast cancer, the miR-140 gene is regulated by ER binding to an estrogen response element. On binding, miR-140-5p expression is down-regulated, and through this method, ER is thought to prevent cellular differentiation and promote a “cancer stem cell” (CSC) phenotype capable of self-renewal and pluripotency$^{101}$. As part of this system, miR-140-5p was found to directly target $SOX2$, $SOX9$, as well as $ALDH1$, all genes responsible for maintenance of the “stemness” important for pluripotency$^{101,102}$. MiR-200a-3p has been extensively studied, primarily in breast cancer. It is a member of the miR-200 family, consisting of five extremely similar miRNAs with overlapping target sequences; they are generally studied as a group, and properties attributed to them as a whole, which makes determining the specific properties and function of miR-200a-3p a challenge. Low expression of the miR-200 family has been found to be associated with aggressive and metastatic breast cancer$^{103}$. MiR-200 family expression inversely correlates with invasiveness, and lower expression is found in metastases compared to primary tumours$^{104}$. Heavily implicated in the metastatic process, the miR-200 family has been shown to be integral to EMT. When induced by TGFβ, epithelial cells undergo the EMT process, and the miR-200 family is significantly down regulated. It appears that miR-200a-3p and miR-200b-3p are integral to this process, and in epithelial cells are responsible for regulating a number of pathways supressing EMT$^{104}$. Most important are $ZEB1$ and $ZEB2$, both of which are direct targets of miR-200a-3p$^{103}$. In addition, β-catenin ($CTNNB1$) is also a direct target, and interacts with E-cadherin to maintain the epithelial phenotype$^{38}$. Demonstrate the pivotal role of miR-200a-3p in EMT, inhibiting miR-200a-3p and miR-200b-3p’s activity in vivo was enough to induce the transition, and overexpression of these
miRNAs in mesenchymal cell lines induces the reverse mesenchymal to epithelial transition (MET)\textsuperscript{103}. Unlike its role in breast cancer, miR-200a is up-regulated in ovarian cancers, and expression is associated with reduced survival. This may be because of MET being advantageous in the late stages of advanced cancers, as some micrometastases have the potential to transition back to epithelial phenotypes and form secondary tumours. This was demonstrated in a mouse model of breast cancer, as increased miR-200 family expression resulted in enhanced ability to form distant metastases\textsuperscript{103}. Low expression of the miR-200 family is also associated with maintenance of self-regulation in stem cells. Mammary stem cells have low expression of these miRNAs, as well as cells with breast CSC phenotypes, possibly reflecting an association with differentiation\textsuperscript{104}.

Like miR-200a-3p, miR-218-5p has also been extensively studied in cancer; however, its functions seem to be varied and not focused on one specific aspect of cancer. It is heavily implicated as a tumour suppressor, and is suppressed in a range of tumours. It is under expressed in cervical cancer\textsuperscript{84}, gastric cancer\textsuperscript{75}, glioma\textsuperscript{76}, nasopharyngeal carcinoma\textsuperscript{73}, metastatic prostate cancer\textsuperscript{105}, oral squamous cell carcinoma\textsuperscript{78}, colorectal cancer\textsuperscript{80}, medulloblastoma\textsuperscript{81}, renal cell carcinoma\textsuperscript{85}, and pancreatic ductal adenocarcinoma, where it is a potential prognostic factor, associated with stage and recurrence\textsuperscript{106}. Studies show that induced overexpression of miR-218-5p in many of these cancers reduces proliferation, migration, and invasion \textit{in vivo}.

In breast cancer, invasive ductal carcinoma had significantly less miR-218-5p expression compared to ductal carcinoma \textit{in situ}, suggesting an association with invasiveness\textsuperscript{107}. It is also a member of a miRNA signature predictive of ER expression in breast cancer. Increased miR-218-5p expression is associated with increased probability of tumours being ER\textsuperscript{+}\textsuperscript{44}. Interestingly, exogenous overexpression of miR-218-5p in glioblastoma reduces proliferation, as well as expression of Ki-67, suggesting a role in the proliferation processes associated with this factor that might also occur in breast cancer, correlating with reduced prognosis in luminal tumours\textsuperscript{108}.

Many validated targets of miR-218-5p are relevant to cancer. It targets \textit{VOPP1} and \textit{IKBKB}, a member of the IkB kinase complex, both of which regulate IkB\textalpha (\textit{NFKBIA}), itself an important regulator of NF-\kappaB\textsuperscript{75}. Reduction of miR-218-5p expression results in increased downstream NF-\kappaB activity, resulting in reduction in apoptosis, increased proliferation, and increased migration and invasion through MMP expression\textsuperscript{76}.
MiR-218-5p also regulates ROBO1, a component of the SLIT-ROBO pathway normally important in axon guidance but deregulated in cancer to promote cellular migration. Interestingly, each copy of the two mir-218 genes lies intronic in a member of the slit family, mir-218-1 in SLIT2, and mir-218-2 in SLIT3. SLIT2 targets ROBO1 as its receptor, activating PI3K, in turn activating AKT, and contributing to tumourigenicity. Another target of miR-218-5p in the AKT pathway is RICTOR, a component of the mTORC2 complex that activates AKT, contributing to cell growth and proliferation.

Other targets are known to contribute to a cancer phenotype, but the specific function is not as well defined. Regulation by miR-218-5p of a number of targets is associated with increased cell growth and proliferation. MiR-218-5p directly targets HOXB3 and BMI1, regulators of tumour suppressors. BMI1 specifically inhibits p53, and low expression of miR-218-5p leads to a reduction of apoptosis. Another target that reduces apoptosis is BIRC5, an inhibitor of caspase function. Cell cycle gene CDK6 is targeted as well, regulating its function in activating cell growth.

MiR-218-5p also has targets involved in cellular migration and invasion, important components of breast cancer recurrence. It directly targets N-cadherin (CDH2), an important component of metastasis, and increased expression of CDH2 contributes to increased migration and invasion. It is a marker of the mesenchymal phenotype, with high expression after EMT, when E-cadherin has low expression. Other target genes involved with cell movement are LASP1, involved in movement of actin filaments, PXN, involved in the regulation of focal adhesions, and GJA1, a component of gap junctions. Expression of all three of these genes is associated with increased migration and invasion. Targets of miR-218-5p contributing to the migration and invasion phenotype, but with undetermined pathways, are LAMB3, CAV2, and HMGB1, a chromatin protein.

In contrast to the majority of literature on miR-218-5p, one study found increased expression of the miRNA in breast cancer metastases to bone. Acting as an oncogene, it directly targets and down regulates three inhibitors of the Wnt pathway, leading to increased activity of the pathway, resulting in proliferation and migration. This function of miR-218-5p might be specific to metastases. In primary tumours, reduced expression would contribute to metastasis by increasing growth and invasion. However, in secondary tumours, increased miR-218-5p might
promote MET and activate alternative growth pathways, such as Wnt, allowing micrometastases to flourish.

Based on the functions of the miRNAs I validated as being differentially expressed, I selected miR-218-5p for functional analysis in an \textit{in vivo} model of luminal breast cancer. I selected MCF7 cells for my model, as they are ER+, HER2-, have similar expression of miR-218-5p as my luminal tumour recurrence groups, and are commonly used in the literature. To analyze its function, I transfected either mimics or inhibitors specific to miR-218-5p into the cells, then compared the tumourigenicity phenotype to cells transfected with mimics or inhibitors to a scrambled sequence, acting as a negative control in my experiments. I selected miR-21-5p as a positive control, as the functional effects of altering this miRNA in MCF7 on cell growth and migration have been published\textsuperscript{53}.

I confirmed that transfection of mimics or inhibitors to miR-21-5p and miR-218-5p affected the expression of their respective selected published targets. While a target of miR-21-5p had reduced expression in cells transfected with a mimic, and increased expression in cells transfected with an inhibitor as expected, targets of miR-218-5p only showed reduced expression in cells transfected with a mimic. This could be due to the relatively low endogenous expression of miR-218-5p, resulting in little functional change when inhibited.

Based on the potential function of miR-218-5p’s published targets, I chose the XTT cell quantification assay, measuring cell number, the scratch wound assay, measuring migration, and transwell assay, also measuring migration. Altering the function of miR-21-5p or miR-218-5p had no effect on cell growth, based on the XTT cell quantification assay. Results that showed similar numbers of live cells in both experimental cells and control cells were consistent, suggesting little direct functional effect of these miRNAs on growth in MCF7 cells.

For the migration assays, results were highly variable. Overall, migration in MCF7 cells is low, as expected of a cell line with an epithelial phenotype, regardless of experimental condition. For the scratch wound assay, 24 hours of movement resulted in only a small migration distance. The experiments had a range of migration distances; cells transfected with a mimic or inhibitor for miR-21-5p and an inhibitor for miR-218-5p averaged the same migration distance as the control cells. However, cells transfected with a mimic for miR-218-5p had no migration
over the 24 period on average, representing a highly significant reduction in migration ability in those cells.

Unlike the scratch wound assay, the transwell migration assay had no reduction in migration in cells transfected with a mimic for miR-218-5p. All experimental conditions had the same average number of migrated cells as the control cells. Similar results were seen in the transwell Matrigel assay, where no differences in the number of migrated cells were seen for any experimental condition or control. In both types of transwell experiments, overall low numbers of cells migrating or invading increased variability, resulting in inconclusive results.

Based on my array results showing that miR-218-5p is expressed significantly less in breast tumours from patients who experienced a recurrence of their cancer, compared to tumours in patients who did not experience a recurrence, as well as the literature, I believe that this miRNA has a tumour suppressive effect in luminal breast cancer. The results of the scratch wound assay, demonstrating significantly reduced migration in cells transfected with a mimic miR-218-5p appear to confirm these results, although not conclusively.

It is possible that the lack of changes in cellular proliferation or survival could be due to miR-218-5p not having a driving role in affecting cell growth in MCF7 cells. The absence of changes in any cells transfected with an inhibitor to miR-218-5p could be due to its low endogenous expression, as demonstrated by the absence of increased expression in the miRNA’s targets. My results showing an absence of changes in phenotype in MCF7 cells transfected with inhibitors or mimics to miR-21-5p do not agree with a published study demonstrating increases in cell growth and migration when miR-21-5p’s activity was exogenously increased53. There are multiple possibilities for the dichotomy of results. My functional analysis used a relatively new type of mimic and inhibitor for transfection different from previous studies, including different oligonucleotide chemistry. Additionally, differences in transfection, cell culture or assay protocol could have played a role in the difference of results. Due to many miRNAs having functions responding to environmental factors, even relatively minor variables such as type of culture plate or flask has been shown to affect miRNA expression24.

Overall, I found nine miRNAs with significantly reduced expression in luminal breast tumours from patients who experienced a recurrence of their cancer, compared to tumours from patients without recurrence. Four of these miRNAs, miR-135a-5p, miR-140-5p, miR-200a-3p,
and miR-218-5p, were selected for validation and confirmed as being significantly differentially expressed. In an *in vitro* cell line model of luminal breast cancer, ectopic overexpression of miR-218-5p resulted in a significant reduction in cellular migration in a scratch wound assay, compared to unaltered control cells. My results suggest that miRNA expression plays a role in luminal breast cancer recurrence, and at least one of those miRNAs may have a functional role in that recurrence.


6 Future Studies

To determine if there were miRNAs associated with recurrence in luminal breast cancer, I analyzed the expression of 377 miRNAs on 39 luminal tumour samples. In addition to the significantly differentially expressed miRNAs, these data can be used for further analysis. The statistical methods used for the current work required a miRNA to be expressed in at least five samples in each recurrence group. Of the 179 miRNAs that did not meet these criteria, 119 had expression in at least one sample. There may be miRNAs that were expressed in a number of samples from one recurrence group, but less than five in the other group, which are differentially expressed but not detected by this analysis.

The expression data could also be analyzed using different characteristics. It is possible that there are miRNAs that are differentially expressed between tumours based on age of onset or menopausal status. Alternatively, as the current analysis was supervised; an unsupervised clustering analysis could be done on the tumour dataset, to see if the overall expression patterns naturally cluster into groups that correlate with the recurrence groups.

Additionally, a number of the samples in this analysis also have mRNA expression array data available. By using an algorithm such as GenMIR++ on both datasets, relationships between miRNAs and potential target mRNAs can be identified. This will determine which targets follow the expression trends and suggest which pathways are functionally involved in the expression changes.

While at least two of the differentially expressed miRNAs have been preliminarily validated in an external data set, the overall miRNA expression profiles in my luminal tumour cohort may be representative of luminal tumours as a whole, and the differentially expressed miRNAs might indicate the risk of recurrence for other luminal patients. These miRNAs, or a subset of them, could represent a class prediction signature. To create an actual function based on the expression of these miRNAs predicting the recurrence risk in an unknown luminal tumour, the strength of each gene’s expression to predict one group or the other must be weighted, and then a rule developed with the weighted expression values.

Once an algorithm is developed, it must then be tested on an independent set of new luminal tumours, to determine its accuracy, and specifically its false positive and false negative
rate, and the algorithm adjusted accordingly. An ideal prognostic signature has the lowest false negatives as possible, as it is better for patients who do not need it to receive extra treatment, rather than patients who would benefit going without treatment. It must then be validated on larger datasets with more patients using alternative platforms, and success compared to current standards, before a miRNA gene expression signature to predict luminal breast cancer prognosis can be ready for clinical use\textsuperscript{48}.

The advantage of using miRNA analysis in the clinical setting compared to mRNA gene signatures is the stability of miRNA in FFPE samples, which is the standard for biopsy and surgical sample preservation, and used for histology analysis\textsuperscript{40}. Unlike mRNAs, which degrade during the fixation process, miRNA are stable due to their short length, and can be extracted from FFPE for analysis\textsuperscript{60}.

While ISH on FFPE samples was unsuccessful in this study due to the low expression of the miRNAs of interest, other methods are available that include PCR amplification \textit{in situ}, which may prove more successful for these conditions\textsuperscript{63}. When the technology has matured, it may be possible to profile the expression of differentially expressed miRNAs directly on a histology panel along with ER or HER2. They could also potentially be analyzed in a high throughput manner on tissue microarrays, improving their usefulness in the clinical setting\textsuperscript{12}. It is possible that a luminal prognosis miRNA expression signature, once validated, could be measured \textit{in situ}. This would greatly decrease the cost and improve the clinical usefulness of a signature.

While TaqMan RT-qPCR profiling has been considered the “gold standard” of miRNA analysis, new next generation RNA sequencing (RNA-Seq) technology could eventually replace it as the miRNA analysis method of choice. This method sequences all RNA in a sample, then maps all the RNA “reads” to the genome. Expression levels can be measured as number of reads relative to a spiked in control sequence of known concentration, or relative to an endogenous control\textsuperscript{111}. The primary advantage of RNA-Seq as a miRNA tool is its ability to provide comprehensive analysis of miRNA expression. The technology allows for measurement of novel miRNAs that do not have primers available, it can determine single nucleotide polymorphisms and mutations within miRNAs, and it can detect miRNA posttranscriptional modifications and those with multiple isoforms\textsuperscript{112}. Combining expression analysis with mutation and
posttranscriptional modification analysis would allow an expression signature to be augmented with mutations or isoforms of pathological relevance.

Functional analysis of miR-218-5p in a luminal breast cancer model demonstrated an association with cellular migration. This was a limited investigation, and a more comprehensive analysis of miR-218-5p’s effect on migration is warranted before definitive conclusions can be made. There are many different types of experiments available for assaying cellular viability and migration and invasion. Other growth conditions, such as in 3D culture, and methods of determining cell viability could be used, including cell cycle analysis and cell sorting. Alternatively, caspase assays could be used to determine if apoptosis was having an effect on cell proliferation and viability. Cellular migration and invasion can be measured by different kinds of membranes and pore sizes in transwell assays, and chemoattractants could be used to increase overall movement. Migration can also be assayed on various surfaces using the microliter-scale assay, and invasion assayed through thin slices of tissue to determine biologically relevant invasion ability. Alternative luminal breast cancer cell lines, such as T47D or ZR751, or a transfection vector with alternative chemistry or non-transient expression would provide additional support to these results and assist in functional characterisation. Assuming miR-218-5p, or other differentially expressed miRNAs, is confirmed to be involved in migration or other phenotypes, it should then be investigated in other models of breast cancer, such as in mice. Assessing tumour growth and metastases when miR-218-5p has been overexpressed or inhibited will provide information more directly relevant to luminal tumours.

MiRNA-based therapies are currently under investigation, either replacing under-expressed miRNAs or introducing miRNA inhibitors. One miRNA-based therapy has entered clinical trials in humans. It is not impossible that a therapy for luminal breast cancer based on restoring tumour suppressive miRNA function might be developed. Ultimately, miRNAs in luminal breast cancer might not just predict prognosis, but improve it as well.
References


