MiRNAs as Oncologic Biomarkers for Breast Cancer

A thesis submitted to the National University of Ireland, Galway for the degree of Doctor of Philosophy in the School of Medicine

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This thesis is dedicated to my parents,

Tina and Brendan McDermott

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Abbreviations

AAV	Adeno-associated virus
AC	Doxorubicin/cyclophosphamide
AGO	Argonaute
AJCC	American Joint Committee on Cancer
AMO	Anti-miRNA oligonucleotide
ANN	Artificial Neural Networking
AP	Alkaline Phosphatase
ARE	AU-rich element
AU	Adenylate-uridylate
AUC	Area under the curve
ATCC	American type Culture Collection
BAN	1-bromo-4-methoxybenzene
BCIP	5-brom-4-chloro-3'-indolylphosphate
BCS	Breast conserving surgery
CA	California
CA 15-3	Carcinoma Antigen 15-3
CDK	Cyclin-dependent kinases
cDNA	Complementary DNA
CEA	Carcinoembryonic Antigen
Cl	Chloride
CI	Confidence intervals
CO_2	Carbon dioxide
CRF	Case report form
Cq	Quantification cycle
C_T	Cycle threshold
DCIS	Ductal Carcinoma *in situ*
ddH_2O	Double-distilled water
DFS	Disease free survival
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
dNTP	Deoxyribonucleotide
E	PCR amplification efficiencies
EC	Endogenous control
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
EU	European Union
FAM	6-carboxyfluorescein
FDA	Food and Drugs Administration
FFPE	Formalin fixed paraffin embedded
FGF	Basic Fibroblast Growth Factor
FISH	Fluorescent *in situ* hybridisation
FRET	Fluorescence Resonance Energy Transfer
g	Gram
g	G-force
GC	Guanine-cytosine

GCP	Good clinical practice
GEO	Gene Expression Omnibus
GME	Global mean expression
GR	Glucocorticoid receptor
GRUHG	Galway Roscommon University Hospital Group
GP	General practitioner
GUH	Galway University Hospital
HBOC	Hereditary Breast and Ovarian Cancer
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HER2/*neu*	Human epidermal growth factor receptor
HRT	Hormone replacement therapy
IAC	Inter-assay controls
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICORG	All Ireland Cooperative Research Group
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
IMS	Industrial methylated spirits
Inv.	Invasive
ISH	*In situ* hybridization
K2E	Dipotassium EDTA
LAF	Laminar air flow
LNA	Locked nucleic acid
MBD	Methyl-CpG binding domain
mg	Milligram
Mg	Magnesium
miRISC	miRNA-associated RNA-induced silencing complex
miRNA	microRNA
mRNA	Messenger RNA
miR-scr	miR-scramble
ml	Millilitre
mm	Millimeter
mM	Millimolar
NBT	4-nitroblue tetrazolium
Nt	Nucleotide
NFR	Nuclear fast red
MRI	Magnetic resonance imaging
NA	Not applicable
NAC	Neoadjuvant chemotherapy
nm	nanometre
NPM1	Nucleophosmin 1
NSABP	National Surgical Adjuvant Breast and Bowel Project
NUI	National University of Ireland
OCP	Oral contraceptive pill
OS	Overall survival
PBS	Phosphate buffered saline

pCR	Pathological complete response
PCR	Polymerase chain reaction
PFS	Progression free survival
PI	Propidium iodide
PR	Progesterone receptor
r	Pearson correlation coefficient
RAKE	RNA-primed array-based Klenow enzyme
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA	Ribonucleic acid
ROC curve	Receiver Operator Characteristic Curve
rpm	Revolutions per minute
RQ	Relative quantity
RQ-PCR	Relative Quantification Polymerase Chain Reaction
S	Svedberg units
SAGE	serial analysis of gene expression
SEER	Surveillance, Epidemiology and End Results
SERM	Selective oestrogen receptor modulator
snoRNA	Small nucleolar RNA
SSC	Saline sodium citrate
TAN	Tumour associated normal
TLDA	TaqMan low density array
TNM	Tumour Node Metastases
UICC	Stage of breast tumor according to the International Union Against Cancer staging criteria
UK	United Kingdom
US	United States
USA	United States of America
UTR	Untranslated region
WHI	Women's Health Initiative
Yrs	Years
°C	Degrees Celsius
µg	microgram
µL	Micro litre
µM	Micro metre
ΔG	Free energy
$\Delta\Delta C_T$	Comparative cycle threshold

Communications Originating from this Work

Peer Reviewed Papers

Identification and validation of oncologic miRNA biomarkers for luminal A-like breast cancer

AM McDermott, N Miller, D Wall, LM Martyn, G Ball, KJ Sweeney, MJ Kerin
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AM McDermott, HM Heneghan, N Miller, MJ Kerin
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Published Abstracts

MiR-15 family miRNAs: Aberrant expression in breast cancer

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Luminal A breast cancer: Identification of novel circulating biomarkers

AM McDermott, N Miller, G Ball, KJ Sweeney, MJ Kerin
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Poster Presentations

The *miR-15* family exhibit differential expression in breast cancer
AM McDermott, HM Heneghan, D Wall, N Miller, MJ Kerin
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Vancouver, BC, Canada, 20th-25th January 2013

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Abstract

Understanding of the molecular complexity underlying breast cancer has greatly advanced in recent years. Distinct phenotypic subtypes have been identified each of which carry prognostic and predictive implications. Despite these advances, delays in diagnosis resulting in haematogenous dissemination and development of distant metastases remains the leading cause of death for breast cancer patients. This prevalent heterogeneous disease is in critical need of circulating biomarkers to expedite accurate diagnosis and provide more specific individualised treatment modalities in an effort to alleviate the global social and economic burden of this cancer. The knowledge that miRNA expression is altered in breast cancer, coupled with their presence in circulation and promising biomarker characteristics has placed them at the forefront of translational research with applicability to diagnostics, prognostics and prediction. This study was undertaken to evaluate the potential of miRNAs as circulating diagnostic and predictive biomarkers in breast cancer, and to advance understanding of their downstream targets and cellular location.

Accurate miRNA quantification requires rigorous normalisation of raw data using stably expressed endogenous controls. At the outset of this study there was a dearth of systematically tested control miRNAs for use in the circulation. Using both miRNA microarrays and RQ-PCR, *miR-16* and *miR-425* were thus identified as the most stably expressed endogenous controls for RQ-PCR in blood specimens for breast cancer studies.

To identify circulating miRNA expression patterns associated with Luminal A (ER+PR+HER2/*neu*-) tumours, expression profiling of 667 miRNAs on 20 blood samples (10 Luminal A; 10 controls) was performed. Artificial neural networking analysis of the dataset identified 76 differentially expressed miRNAs. Validation in a larger cohort revealed a profile (*miR-29a*, miR-*181a* and *miR-652*) with sensitivity and specificity of 77% and 74%, respectively, to identify Luminal A tumours, providing insight into the molecular mechanisms underpinning this common subtype.

In the next phase of the study, miRNAs belonging to the *miR-15* family were shown to be aberrantly expressed in blood and tissue of women with breast cancer. Combination of *miR-15a, miR-195* and *miR-497* provided a sensitivity and specificity profile of 82% and 76.5%, respectively, for breast cancer detection. In an effort to elucidate the functional role of the *miR-15* family in carcinogenesis *in silico* analysis was performed to identify miRNA-mRNA duplex interactions, which were further examined by comparison of the expression of putative target genes (CCND1, FGF2 and BCL2) with miRNA expression in breast tumours.

To gain insight to the cellular location of *miR-195* and *miR-497*, miRNA *in situ* hybridisation was performed. In this manner *miR-195*, was identified as being localised to stromal and tumour epithelial cells. Both *miR-195* and *miR-497* expression *in vitro* were affected by treatment with a demethylation agent, indicating a role for epigenetic modification, possibly explaining their altered expression in the breast cancer state.

Finally, the application of miRNAs as predictive biomarkers for guiding and monitoring response to chemotherapy was assessed by tracking their expression in serial blood samples taken from patients undergoing neoadjuvant treatment for breast cancer.

The data presented herein reveals that miRNAs are exciting molecules with the potential to revolutionise breast cancer diagnosis and treatment in pursuit of individualised targeted cancer treatment for patients.

Chapter 1
Introduction

1.1 Cancer

The global burden of cancer is continuing to rise; it is the principle cause of death in developed countries and the second leading cause of death in developing countries (1). In 2008 alone, roughly 169 million years of healthy life were lost worldwide due to cancer and its accompanying morbidity and mortality (2). This epic health problem is increasing in incidence, with a projected 27 million annual diagnoses expected by the year 2050 (3). The cause is multifactorial, and can be attributed to an aging population and poor lifestyle choices such as the lack of physical activity, smoking, obesity and poor diet, amongst others.

1.2 Breast cancer

Breast cancer is the most frequently diagnosed malignancy in women. Despite being highly curable if diagnosed and treated early, breast cancer is the leading cause of female cancer-related death, accounting for 23% of overall cancer cases and 14% of cancer mortality in the developed and developing worlds (1, 4). The worldwide incidence is over 1,300,000 with over 2,800 new cases diagnosed in Ireland alone annually (3, 5). In 2008, Ireland was estimated to have the fourth highest incidence and mortality of breast cancer out of 27 countries. Disease progression culminating in hematogenous spread of malignant cells from the primary tumour to distant organs with subsequent proliferation into metastases remains the leading cause of death for breast cancer patients (6). This can be related to late presentation and subsequently delayed diagnosis, as well as a failure to respond to conventional therapies in some cases. Breast cancer is a disease in critical need of additional means to expedite accurate and early diagnosis and provide more specific treatment modalities in order to reduce the global social and economic burden of this cancer.

1.2.1 Breast cancer aetiology and risk factors

The exact underlying aetiology of breast cancer remains largely unknown. However a number of risk factors associated with an increased risk of developing breast cancer have been identified.

Gender and age: Female gender and increasing age are well established risk factors. Breast cancer is 100 times more frequently diagnosed in females than in males (4). The total and age-specific incidence follows a bimodal distribution with the first peak in the early 50's

and the second peak in the early 70's (7). The probability of a woman developing breast cancer based on age was derived from data in the Surveillance, Epidemiology and End Results (SEER) database in the US between 2006 and 2008 (Table 1.1)(8).

Table 1.1 Age dependent probability of a woman developing breast cancer

Age Bracket (years)	Probability of developing breast cancer
Birth to 39	0.49 (1 in 203 women)
40 to 59	3.76 (1 in 27 women)
60 to 69	1.53 (1 in 28 women)
≥70	6.58 (1 in 15 women)
Birth to death	12.29 (1 in 8 women)

Hormonal and reproductive factors: Exposure to steroid sex hormones has been linked with breast cancer. The main risk factors include early onset of menses, late onset of the menopause, older age at first pregnancy and nulliparity (9, 10). The association between exogenous hormonal use (oral contraceptive pill and hormonal replacement therapy) and breast cancer is controversial. There is conflicting data on the oral contraceptive pill (OCP) and breast cancer risk. Several large observational studies have failed to provide concrete evidence that rates of breast cancer are higher in OCP users compared to non-users (11-13). One pooled analysis reported a small increased risk (RR 1.07) of breast cancer in OCP users compared to women who had never used the OCP (14). Data surrounding the risk of breast cancer and HRT is more convincing. The results of two large observational studies suggested an increased risk of breast cancer with hormone replacement therapy (15, 16).The Women's Health Initiative (WHI) randomised controlled trial confirmed an increased risk of breast cancer with use of combined oestrogen-progestin postmenopausal hormone replacement therapy (HRT) (17). The risk of invasive breast cancer was increased with combined HRT (HR 1.2) when compared to the placebo group after 5.6 years of follow up. Interestingly, however, unopposed oestrogen only HRT conveys a reduced risk of breast cancer (18). Short term combined HRT (<3 years duration) appears to be safe with respect to breast cancer risk.

Family history: A positive family history of breast or ovarian cancer is an important risk factor, particularly if a first degree relative is affected at a young age (age <50 years). Other factors include two or more first degree relatives with breast or ovarian cancer or male breast cancer. In a study of over 50,000 women with breast cancer, there was an increased

risk of almost two-fold if the woman had one affected first degree relative, and increased by threefold if she had two first degree relatives with breast cancer (19). Most breast cancer is sporadic (>90% cases), but a positive family history may suggest the present of a genetic mutation, such as a *BRCA1* or *BRCA2* mutation or another cancer syndrome (*PTEN, TP53*).

Other risk factors: Previous personal history of breast cancer and lifestyle factors such as obesity, diet, smoking and physical activity are also risk factors for breast cancer.

1.2.2 Breast cancer diagnosis

The diagnosis of breast cancer involves the combination of clinical examination, radiological imaging and an invasive tissue biopsy, providing histological confirmation.

Early breast cancer frequently produces no symptoms or clinical signs. Patients may report a mass in the breast or axilla, skin changes, bloody discharge or nipple changes. A clinical examination is performed to inspect the breasts for any visible changes and to palpate the breasts and axillae for discrete masses.

Mammography is the current gold standard modality for breast cancer diagnosis, with screening mammography now offered in many developed countries including the UK and Ireland. The sensitivity and specificity of mammography is acceptable for screening use, with estimates of sensitivity ranging from 67 to 95% and specificity ranging from 82 to 97%. However these sensitivity and specificity parameters are largely dependent on other factors such as patient age, professional experience of the radiographer/radiologist and breast density, with the latter recently reported as less significant than originally perceived (20-22). Indeed the histological and molecular subtype of breast tumours has also been reported to impact on the verity of mammography. Invasive lobular carcinoma (ILC) accounts for 6-10% of all breast cancers diagnosed, with an increasing incidence (23, 24). This histological group, although typically hormone receptor positive and of Luminal A subtype, present a diagnostic challenge , with mammography having a lower sensitivity in comparison to invasive ductal carcinoma (25). The sensitivity for ILC ranges from as low as 57 to 71% with a similarly abhorrent false negative rate, a recent study reporting 28.9% (26, 27). Triple negative breast tumours (ER-PR-HER2/*neu*-) are an additional subgroup that are frequently undetected by routine mammography (28).

Table 1.2 Screening mammography[1,2]

Screening Mammography	
Sensitivity	62-90%
Specificity	82-97%
Recall after initial screening	63 per 1000 screened
Overall Recall Rate	46 per 1000 screened
Cancers Diagnosed	7 per 1000 screened

Magnetic resonance imaging (MRI) and ultrasound of the breast could improve breast cancer detection rates, but the role of these imaging modalities in diagnostics is still in evolution (29). In any case imaging techniques alone are unlikely to suffice for conclusive diagnosis as intrinsic subtype, which largely predicts the course of management, cannot be determined from imaging alone.

The final component of triple assessment is a breast biopsy. This is usually performed as a core biopsy in the outpatient setting. It is at this stage that histological presence of carcinoma is confirmed, along with determination of hormonal and HER2/*neu* receptor status.

1.2.3 Breast cancer pathology

The term 'breast cancer' refers to a wide range of lesions forming in the breast tissue. Adenocarcinoma of the breast (breast carcinoma) is the most common form of breast cancer, arising from the epithelial layers of the milk ducts (ductal) and/or lobules (lobular) of the mammary gland. This accounts for over 95% of all malignant breast tumours. The remaining tumours (<5%) arise from other cell types present in the breast and include Pagets disease of the nipple, phyllodes tumour and angiosarcoma (30).

Despite the common epithelial origin of most (>95%) breast tumours, breast carcinoma is a heterogeneous disease, with marked variation in macroscopic appearance, behaviour, management and response to treatment. A single breast tumour may be comprised of a combination of histological tumour types.

[1] IARC, Screening Techniques, 2002
[2] BreastCheck Programme Report 2009/2010

There are two main distinctions to make:

- *In situ* (non-invasive) carcinomas

By definition this is comprised of malignant cells that have not breached the epithelial basement membrane. Ductal carcinoma *in situ* (DCIS) is the most commonly encountered *in situ* carcinoma which, as the name suggest, arises from the ducts. There are several different types of DCIS which are largely classified based on their architectural pattern (cribiform, papillary, micropapillary or solid), their grade (high intermediate or low) and the presence of comedonecrosis. DCIS is considered highly curable. Lobular carcinoma *in situ* (LCIS) is less common and is a pre-cancerous lesion.

Figure 1.1 Ductal carcinoma *in situ*

Several foci of DCIS with a single area of microinvasion (arrow) are visible[3].

[3] http://tgmouse.ucdavis.edu/JENSEN-MAMM2000/BRCA-1/brca-1.html

- Invasive (infiltrating) carcinoma

Invasive carcinomas are lesions in which the tumour cells have invaded the epithelial basement membrane to the breast stroma and thus have the potential for metastasis. This group includes several distinct histological subtypes (31) :

A. Invasive ductal carcinoma (Figure 1.2): 70-80%
B. Invasive lobular carcinoma (Figure 1.3): 10-15%
C. Mixed (ductal and lobular) carcinoma: <5%
D. Inflammatory: 2-3%
E. Colloid: 2-3%
F. Medullary: <2%
G. Tubular: <2%
H. Papillary: 1%

Figure 1.2 Invasive ductal carcinoma

A moderately well differentiated invasive ductal carcinoma with moderate variation in nuclei size and shape (Grade 2), no tubule formation (Grade 3) and few mitoses (Grade 1)[4].

[4] http://tgmouse.ucdavis.edu/JENSEN-MAMM2000/BRCA-1/brca-1.html

Figure 1.3 Invasive lobular carcinoma

Invasive lobular carcinoma which characteristically forms 'indian file' strands of infiltration into the stroma[5].

1.2.4 Breast cancer metastasis

Invasive breast cancers largely spread by either local invasion (into surrounding structures such as pectoralis major) or metastases. If tumour cells invade and enter the lymphatic system, lymph node involvement will ensue. The sentinel lymph node is the first node draining a breast tumour and can be used to determine nodal involvement (Figure 1.4). Once the nodes are involved the cancer can spread in the lymphatics to include the axillary, internal mammary, supra-clavicular and infra-clavicular lymph nodes. Breast cancer cells can also enter the peripheral circulation and result in distant metastases to the lung, bone, brain and liver.

[5] https://mywebspace.wisc.edu/wwolberg/breast/lob_iv.html

Figure 1.4 The sentinel lymph node

The sentinel lymph node is the first node to drain a breast tumour. Involvement of the sentinel lymph node confirms that further lymphatic metastases may have occurred.

1.2.5 Breast cancer classification

Breast cancers can be classified by a number of different systems; each provides information regarding prognosis and response to treatment. Breast cancer staging is most commonly performed using the TNM system of the Union for International Cancer Control (UICC-TNM staging system). This system has been accepted by both the UICC and the American Joint Committee on Cancer (AJCC). It considers tumour size, nodal involvement and the presence or absence of distant metastases (Table 1.3).

Table 1.3 The UICC-TNM staging system

Primary Tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Tis(DCIS)	Ductal Carcinoma in situ
Tis(LCIS)	Lobular Carcinoma in situ
Tis(Pagets)	Pagets disease of the nipple with no associated invasive carcinoma
T1	Tumour ≤2cm in greatest dimension
T2	Tumour >2cm but ≤5cm in greatest dimension
T3	Tumour >5cm in greatest dimension
T4	Tumour of any size directly extending into the chest wall and/or to the skin
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastasis to movable ipsilateral axillary node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed or matted, or in clinically detected ipsilateral internal mammary nodes in the absence of clinically apparent axillary nodes
N3	Metastases to ipsilateral infraclavicular lymph node(s) with or without axillary node involvement or in clinically detected ipsilateral internal mammary lymph node(s) with clinically apparent axillary node involvement or involvement of ipsilateral supraclavicular lymph node(s) with the presence or absence of axillary or internal mammary node involvement
Distant metastases (M)	
MX	Presence or absence cannot be assessed
M0	No distant metastases
M1	Distant metastases

The various components of the TNM classification system permit breast tumours to be classified into prognostic groups, commonly referred to as cancer staging (Table 1.4).

Table 1.4 Anatomic stage

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage 1A	T1	N0	M0
Stage 1B	T0	N1mi*	M0
	T1	N0	M0
Stage 2A	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage 2B	T2	N1	M0
	T3	N0	M0
Stage 3A	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage 3B	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage 3C	Any T	N3	M0
Stage 4	Any T	Any N	M0

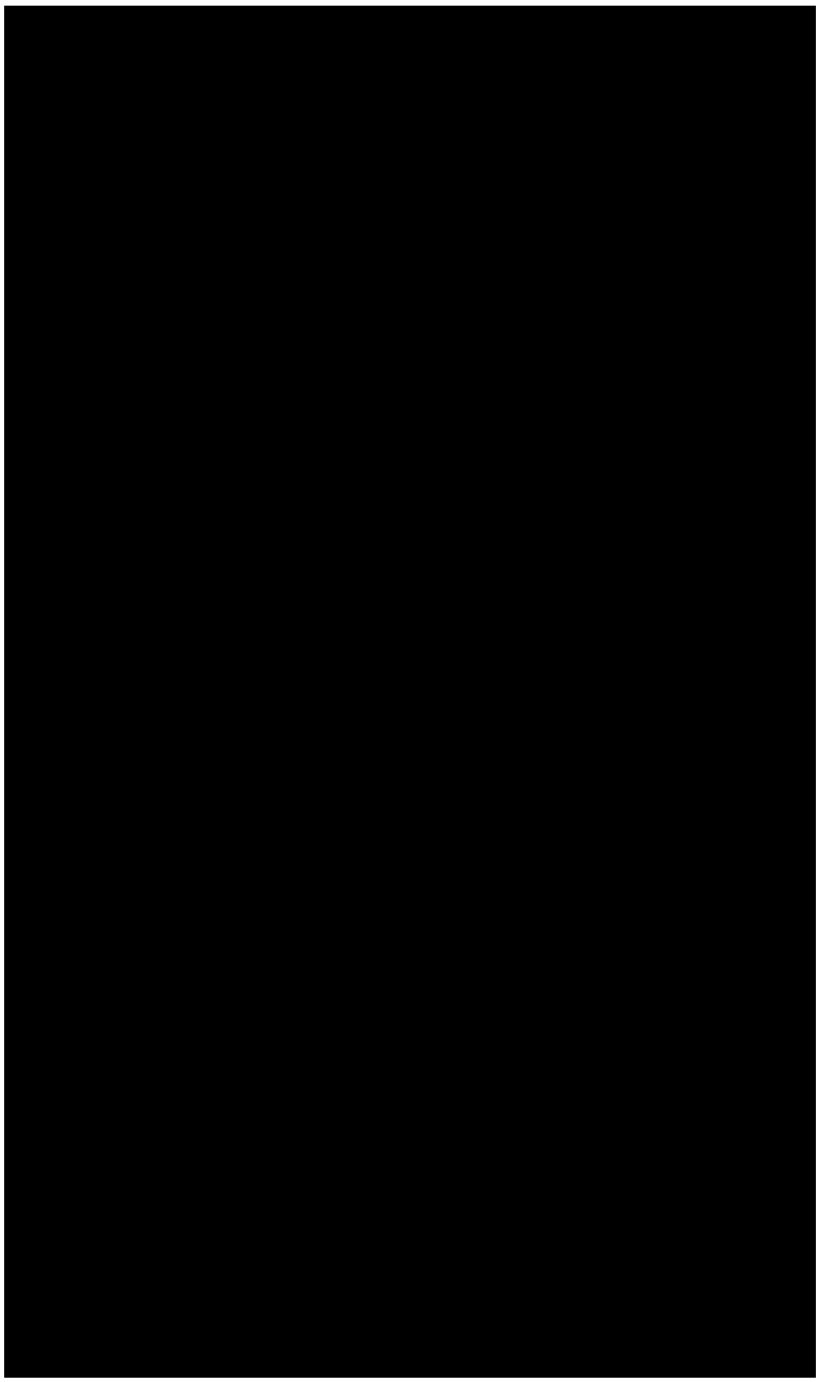
Tis: *In situ;* N1mi*: micrometastases <2mm

These staging systems are important, as they facilitate in determining the severity of a patients cancer, permitting appropriate management with surgical and adjuvant treatment strategies. In addition staging facilitates in prognostication. However, the TNM staging system has limitations. In recent times additional factors have become important in predicting response to treatment and determining prognosis, namely oestrogen, progesterone and HER2/*neu* receptor status.

1.2.6 Markers in breast cancer

Oestrogen receptor, progesterone receptor and HER2/*neu* receptor status are routinely assessed on newly diagnosed breast tumours. The hormone (oestrogen, ER, and progesterone, PR) receptors predict response to hormonal therapies and are present on up to 80% of breast tumours. In general, these tumours (Luminal A and Luminal B subtype) have a more quiescent course. The presence of ER or PR receptors is most commonly confirmed by immunohistochemistry (IHC) which exploits antibodies that bind specifically to the receptor in a tissue specimen (Figure 1.5). However, this technique is subject to both antibody and pathologist accuracy, and is therefore highly subjective.

Figure 1.5 ER scoring by immunohistochemistry



The QUICK score is used to determine hormone receptor status with a maximum potential score of 8, and greater than 2 being considered positive. The top image shows a breast tumour which is strongly ER positive, with a QUICK score of 8/8. The middle image shows a tumour with staining of a reduced intensity which the pathologist has scored a 5. However this is highly subjective, with the potential for an alternative score in the hands of a separate operator. The bottom image shows no staining[6].

[6] http://archive.ispub.com/journal/the-internet-journal-of-pathology

HER2/*neu* (Human epidermal growth factor receptor 2) amplification occurs in up to 25% of breast tumours and denotes a likely response to treatment with trastuzumab (a monoclonal antibody) and other targeted therapies (32). Other anti-HER2/*neu* treatments include pertuzumab (monocolonal antibody), lapatinib (tyrosine kinase inhibitor) and trastuzumab emtansine (a conjugate of trastuzumab and mertansine, a cytotoxic agent) (33-35). HER2/*neu* testing is highly subjective, with two main strategies currently employed in most centres. HER2/*neu* status is initially assessed by IHC, which detects receptor overexpression. Although cheap and relatively straightforward to perform, this technique is extremely subjective, with receptor status determined by the pathologist based on percentage and intensity of staining (Figure 1.6). This is followed by further, more objective, investigation by fluorescent *in situ* hybridisation (FISH) in equivocal cases which detects gene amplification. FISH provides more accurate results but is more costly and labour intensive to perform (36).

Figure 1.6 HER2/*neu* scoring for breast cancer

Immunohistochemistry (IHC) for the HER2/*neu* receptor is scored from 0 to 3 based on the percentage and intensity of staining positivity as demonstrated in the images above[7]. 0 and 1+ are considered HER2/*neu* negative while 2+ and 3+ are considered positive. This scoring system is extremely subjective.

[7] http://latestbreastcancer.blogspot.ie

Breast cancer is a disease without a useful circulating biomarker to facilitate early detection. Carcinoma Antigen 15-3 (CA 15-3) and Carcinoembryonic Antigen (CEA) are circulating biomarkers. However their clinical application in breast cancer management is, if any, confined to detecting and monitoring disease recurrence and progression. These markers are merely elevated in 10% of stage 1 and 20% of stage 2 disease, precluding any usefulness in the diagnostic arena (37-39).

Much current cancer research is focused on the identification of circulating cancer-specific biomarkers for application to disease diagnostics, as well as predicting and monitoring response to disease and tumour recurrence. There are no reliable circulating biomarkers for breast cancer. This prevalent disease is in need of a minimally invasive biomarker which may be used in combination with radiological imaging to facilitate early subtype specific tumour diagnosis. Blood presents an excellent medium for biomarker discovery; it is minimally invasive and simple to obtain during routine clinical examination. Moreover, blood circulates throughout the body delivering nutrients and carrying proteins (including miRNAs), hormones and cells while eliminating waste substances, thereby reflecting the summation of physiological and pathological processes occurring in an individual at any one time.

1.2.7 Breast cancer pathogenesis and molecular biology

As mentioned above the majority of breast cancers arise from epithelium of the mammary gland. We do not yet fully understand the processes leading to breast carcinogenesis but it is likely that most invasive breast cancers arise through a sequence of molecular variations at the cellular level, resulting in the following:

1. Hyperplasia: An increase in cellularity
2. Atypical Hyperplasia: Emergence of atypical breast cells
3. Carcinoma *in situ*
4. Invasive carcinoma

Breast cancer is a heterogenous group of diseases which exhibit great clinical and biological diversity. This was initially recognised over a hundred years ago when it was noted that removing the ovaries of women with breast cancer improved prognosis for a subset of patients. The early unravelling of the molecular events culminating in breast cancer

implicated oncogenes such as the *c-myc* oncogenes, cyclin-*D1, CDK-1* and the tyrosine kinase family, as well as tumour suppressor genes, predominantly p53 (40).

Molecular profiling techniques have advanced over the past two decades facilitating major advances in our understanding of this diverse disease entity. Gene expression profiling is a common technique in molecular biology, permitting the simultaneous assessment of thousands of genes thereby presenting a global snapshot of cellular activity and function. Variations of this technique can be used to determine the presence and activity of predefined or undefined target genes by analysing the presence of their mRNA transcripts. The gene expression profiling era has heralded major advancements in our molecular understanding of cancer. It has also advanced the clinical management of several cancers by providing more elaborate classification systems, cultivating diagnostic and prognostic acumen and providing novel disease modulating and therapeutic targets.

Perou and Sorlie conducted pioneering work in this field of research and employed micro-array generated gene expression signatures which confirmed that breast cancer is a heterogenous disease process, which can be stratified into 4 distinct epithelial subtypes (41-43). These subtypes are luminal A (ER+PR+HER2-), luminal B (ER+PR+HER2+), basal (ER-PR-HER2-), HER2/*neu* over-expressing (ER-PR-HER2+) and normal-breast-like. These molecular subtypes largely correlate with ER (oestrogen receptor), PR (progesterone receptor) and Her2/*neu* status (Figure 1.7). Each subtype is associated with distinct natural history, clinical behaviour and prognosis (44). A recent study by the Cancer Genome Atlas Network substantiated the presence of the four main breast tumour subtypes (45).

Figure 1.7 Molecular subtypes of breast cancer

Gene expression patterns of 85 experimental breast tissue samples were analysed by hierarchical clustering. The tumours were separated into distinct subtypes based on differences in gene expression which broadly correlated with hormone and HER2/*neu* receptor status[8].

The molecular alterations and initiating events responsible for the phenotypically distinct subtypes remain to be fully elucidated. Further insight into the molecular mechanisms underlying tumourigenic transformation is clearly warranted for the identification of additional molecular predictors and disease biomarkers in the clinical management of breast cancer.

The concept of molecular subtypes of breast tumours was readily accepted by the academic and clinical community. Patients are categorised according to their ER, PR and HER2/*neu* receptor status, and as such have different adjuvant therapy options, and ultimately distinct prognoses. Molecular profiling techniques lend themselves tremendously well to translational research. The literature contains several examples of distinct gene signatures with predictive or prognostic capabilities for patients with breast cancer, two of which are validated and available commercially, Oncotype DX®[9] and Mammaprint (46-49). Oncotype DX® (Genomic Health Inc.) conducts a 21-gene profile (16 discriminator genes and 5 reference genes, Table 1.5) on a section of formalin fixed paraffin embedded tumour tissue which is used to generate a recurrence score (49). This score determines the likelihood of deriving a clinical benefit from adjuvant chemotherapy in addition to the 10-year risk of breast cancer recurrence. Mammaprint® is a 70 gene

[8] Adapted from Sorlie *et al*, Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications, PNAS 2001 98(19): 10869-74.
[9] http://www.oncotypedx.com/

prognostic profile which was developed by van't Veer *et al.* Similar to Onco*type* DX this signature requires fresh frozen breast tumour tissue for analysis.

Table 1.5 Gene panel included in Oncotype DX®

Proliferation	Invasion	HER2
Ki-67 STK15 Survivin Cyclin B1 MYBL2	Stromyelysin 3 Cethepsin L2	GRB7 HER2
Oestrogen	**Reference**	**Other**
ER PR Bcl2 SCUBE2	Beta-actin GAPDH RPLPO GUS TRFC	GSTM1 CD68 BAG1

Both of these tests are performed in single sites across Europe and the USA. Their incorporation into clinical use is an excellent example of translational medicine and individualised patient management, with results from these tests informing decisions on the risk of recurrence and need for chemotherapy. However, a section of tissue must be available for analysis. There is a notable lag time between diagnosis (biopsy) and prognostic score result. Additionally, these tests are expensive to perform, a growing concern in the current era of health care economics. Although these gene signatures and resulting commercial tests provide additional prognostic information and a more individualised approach to breast cancer management, they are not without their limitations. There are many inherent weaknesses in the use of these messenger RNA signatures alone but their discovery has unveiled an exciting approach to the use of molecular biology in prognostication and predicting response to treatment.

1.2.8 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy (primary systemic chemotherapy) has become popular for early operable breast cancer. Early experiences have demonstrated that response to therapy is not uniform; some patients respond better than others, with the range of response reported to be as varied as 4 to 34% with response rates as high as 50% in HER2/*neu* overexpressing subsets (50-52). Some patients do not derive any benefit, and in essence suffer toxic side effects in vain. The NSABP-B18 study has shown that a

pathological complete response (pCR) to neoadjuvant chemotherapy is a good prognostic factor for women with breast cancer (51). Onco*type* DX® and Mammaprint®, discussed above, provide additional information on the likelihood of a patient to benefit from adjuvant chemotherapy. However, there is currently no clinical test (tissue- or circulation-based) for neoadjuvant chemotherapy that can accurately predict responders from non-responders, or that can be exploited to monitor treatment response in real time. With the increasing patient awareness of the toxicity of chemotherapy and the evolving era of individualised, patient-centred cancer management there is a need to develop an accurate, acceptable clinical test that would identify patients who are most likely to obtain pCR from neoadjuvant treatment, and thus spare the group of patients who are unlikely to respond from the toxic effects of treatments.

1.3 Introduction to microRNAs

Mi(cro)RNAs are a class of small, non-coding RNA molecules that play important roles in most biological processes by regulating gene expression at the post-transcriptional level. They were first discovered almost 20 years ago, and have since become the focus of much scientific and translational research. MiRNAs are known to play functional roles in both the normal and pathological state. Aberrant miRNA expression has been described in several pathological processes, including carcinogenesis (53). Indeed, investigation into their altered expression in cancer unveiled their dual roles, as oncogenes and tumour suppressor genes.

MiRNA expression profiles can classify tumours by type or clinico-pathological characteristics, which has potential utility in cancer diagnostic, prognostic and predictive settings. This would permit more appropriate treatment selection, allow close surveillance of response to treatment and even spare those patients who have early disease or will not respond to systemic treatment, from the toxicities associated with adjuvant chemotherapy.

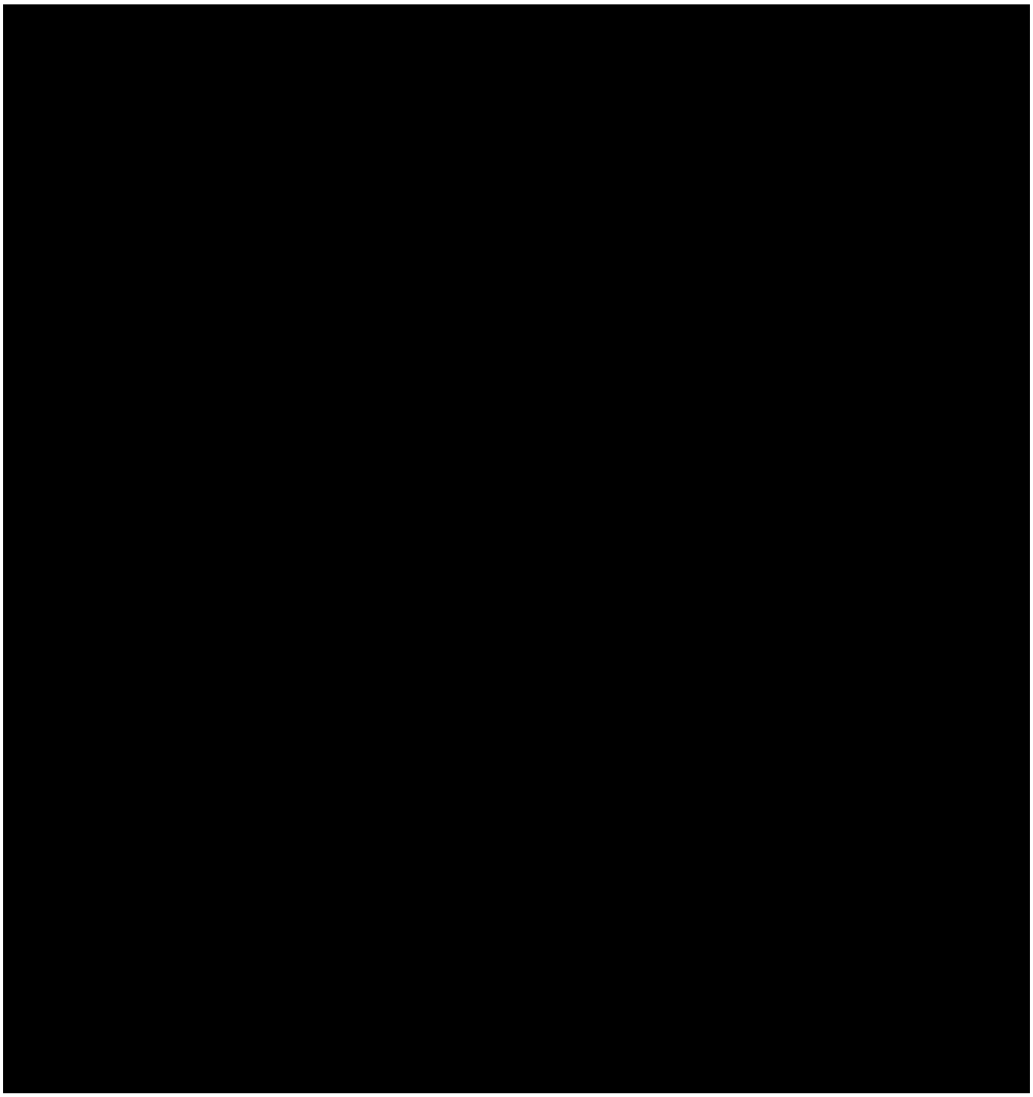
There has been an explosion of interest in miRNAs over the past five years, which is reflected in the rapidly expanding body of miRNA related research. At the time of writing, there were 30,424 mature miRNA sequences described in primates, worms, birds, rodents, flies, plants, fish and viruses[10].

[10] miRBase Release 20 June 2013 ftp://mirbase.org/pub/mirbase/CURRENT/README

1.3.1 MiRNA biogenesis

The biogenesis of human miRNA (Figure 1.8) originates in the nucleus, where there is transcription of a large primary (pri-) miRNA by RNA polymerase II or III. 70% of human miRNAs are transcribed from introns and/or exons, suggesting that regulation of this process is under gene promoter control. The remaining 30% of pri-miRNAs are located in intergenic regions, and so have independent promoters (54, 55). Pri-miRNAs can be up to several hundred or thousand nucleotides in length and contain at least one miRNA stem loop. This single unit may contain up to six precursor (pre-) miRNAs, which are produced by the cleaving action of the RNase III enzyme Drosha, combined with the microprocessor complex subunit DGCR8. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a hairpin structure that is critical for their transport to the cell cytoplasm by the energy-dependent Exportin-5 (56). Once in the cytoplasm this hairpin can then be cropped by the RNAase III enzyme Dicer, to produce a double stranded structure, miRNA:miRNA*, consisting of the miRNA and its complement. This multistep process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC interacts with its target mRNA and exhibits its cellular effects (57).

Figure 1.8 MiRNA biogenesis and processing



This is a simplified representation of the steps involved in miRNA biogenesis and processing in human cells[11]. This multistep process begins in the nucleus of the cell, where there is transcription a large primary (pri-) miRNA by RNA polymerase II. This large pri-miRNA is then cleaved by the RNase III enzyme Drosha, coupled with the microprocessor complex subunit DGCR8, to produce pre-miRNA. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a stem loop structure for their transport to the cell cytoplasm by Exportin-5 (5). Once in the cytoplasm, this hairpin structure is cropped off by the RNase III enzyme, Dicer, producing the double stranded miRNA:miRNA* duplex. This process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC targets complementary mRNA sequences and exerts its cellular effects, via transcriptional cleavage or transcriptional repression.

[11] McDermott *et al*, Pharmaceutical Research 2011

1.3.2 MiRNA function

MiRNAs have been implicated in almost every part of the cell cycle by 'finely tuning' gene expression. They exhibit their function by sequence specific modulation of gene expression at a post-transcriptional level. It is estimated that miRNAs govern over 30% of protein coding genes in this way (58). MiRNAs have been shown to exhibit high evolutionary conservation across species but with variation in expression levels of specific miRNAs in different cell types and at alternative stages in development, implying important regulatory functions (59).

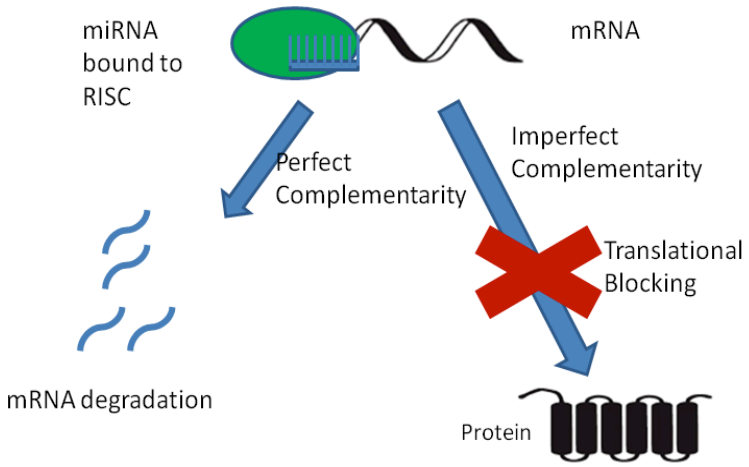
The 'seed-sequence', the short region of importance in miRNA target recognition, extends from bases 2 to 8 on the 5' tail of the mature miRNA strand (55). Each miRNA has two possible mechanisms of action, determined by the degree of complementarity between the miRNA seed sequence and its mRNA target, which is governed by Watson and Crick base pairing (Figure 1.9). Firstly, if the target mRNA and miRISC have perfect base pairing homology, the mRNA is cleaved and degraded through activation of the RNA-mediated interference pathway. Secondly and more commonly, miRNAs modulate their gene targets by repression of protein translation. MiRNAs exhibit this effect by imperfectly binding to partially complementary sequences located often in the 3' untranslated region (UTR) of target mRNAs, although miRNAs can also bind to the coding region and 5'UTR of target genes (60). The proposed mechanism by which imperfect pairing between a miRNA and its target results in translation inhibition or repression is that efficiency of translation is reduced consequent to various mismatched 'bulges' in the central region, or to a lesser extent the 3'end, of the miRNA (Figure 1.10). These bulges appear to affect the strength with which the miRNA binds to its mRNA target and can affect the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA (61). Each human miRNA has the capacity to influence several mRNA targets through imperfect base pair homology.

Several mechanisms by which miRNAs result in translational repression have been suggested, including blocking the initiation of translation, de-adenylation (poly(A) tail shortening) or the recruitment of translational blockers. mRNA degradation or cleavage is typically believed to occur as a result of decapping and subsequent 5' to 3' decay.

Recently, a new hypothesis has been introduced, the 'translational-repression-triggers-mRNA-decay' hypothesis (62). Ameres *et al* postulate, with the support of biochemical and

kinetic analysis models, that miRNAs may initially result in mRNA translational repression which then triggers mRNA decay (63-65). This could be as a consequence of translational blocking rather than as a direct effect of a specific miRNA or AGO protein.

Figure 1.9 MiRNA function



It is generally accepted that the mechanism by which miRNAs exert their effects on gene expression is dependent on the degree of complementarity between the miRNA molecule and the target mRNA. Perfect complementarity results in mRNA degradation. While imperfect complementarity, more commonly encountered, results in translational repression.

Figure 1.10 A typical pattern of base pairing, with imperfect complementarity, between a miRNA and its target mRNA

Typically, the miRNA binds to a specific site or sites within the 3′UTR region of the mRNA sequence. According to thermodynamic analysis, some degree of complex formation occurs along the entire miRNA–mRNA duplexed region. Base pairing is particularly weak in the central region due to mismatched 'bulges' in the miRNA sequence (A), and particularly strong at the 5′ end (seed region) of the miRNA (B). Base pairing between *let-7* miRNA and *hbl-1* mRNA in *C. elegans* is shown as an example[12].

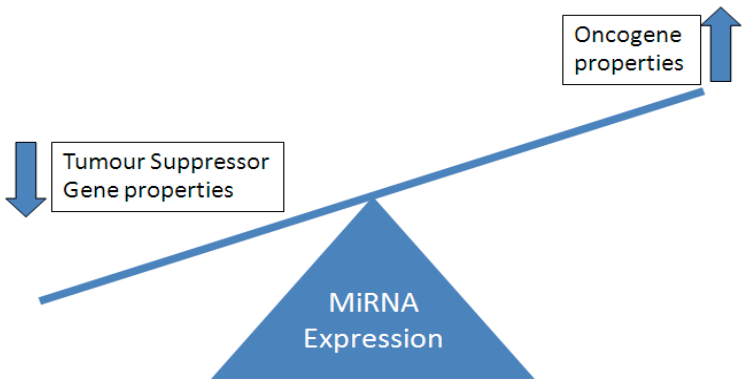
[12] Lin *et al,* 2003

MiRNA research is still in its infancy with debate surrounding the exact mechanism of function. There is general agreement that miRNAs act at the post-transcriptional level, but several reports in high impact journals have suggested that some miRNAs may exert their effects before or after the initiation of translation (66, 67). Similarly, it is widely accepted that miRNAs result in repression of gene expression. However, there are isolated reports of specific miRNAs resulting in gene activation. This phenomenon tends not to be a direct consequence of miRNA binding, but more so a downstream effect of translational cleavage or repression (68, 69). There is, however, an example in the literature of a miRNA directly causing an increase in gene expression. Vasudevan *et al* report that on cell cycle arrest, *miR-369-3* associates with an AU-rich element (ARE) in the 3'UTR of the tumour necrosis factor alpha gene resulting in activation of translation (70, 71).

1.3.3 The role of miRNAs in cancer

MiRNAs function at the initiation, promotion and metastatic stages of many malignancies. They are intricately involved in tumourigenesis and so, the ability to identify specific miRNAs as diagnostic, prognostic, predictive and therapeutic targets has rapidly become a focus of research worldwide. In the cancer state, there is a large body of evidence demonstrating how miRNAs function as oncogenes and tumour suppressor genes. Interestingly, a single miRNA can act dually, as both an oncogene and a tumour suppressor gene, depending on the specific cancer type (Figure 1.11). For example, *miR-125b* is known to have varying expression levels in different tumours; down-regulation is observed in anaplastic thyroid and serous ovarian carcinoma, indicating a potential tumour suppressor role (72, 73) while oncogenic properties (over-expression) are noted in prostate cancer (74). This is an important concept, as it highlights how each cancer must be considered a distinct disease entity.

Figure 1.11 Dual functionality of miRNAs



A single miRNA can act a tumour suppressor gene, or an oncogene depending on the direction of dysregulation, depending on the cancer and cell type. A miRNA with tumour suppressor qualities is underexpressed in the cancer state compared to the 'normal' state. While a miRNA with oncogenic (oncomiR) properties is typically overexpressed in the cancer state.

1.3.4 MiRNA identification and profiling in cancer

Cancer comprises a heterogeneous group of complex diseases, several of which share fundamental pathological processes. Our improved understanding of miRNA function has allowed us to better understand the complexity of human malignancies, particularly the pathways involved in tumour initiation and progression. Indeed miRNA expression profiling of a range of human tissues, both normal and pathological, enhances our understanding of the developmental stages of several cancers. Specific profiles of dysregulated miRNAs can be created for almost every type of tumour. The use of miRNA expression profiles has several advantages over their predecessors, mRNA profiles, predominantly in permitting the classification of poorly differentiated tumours (75, 76). Unique miRNA signatures highlight the potential of miRNAs as cancer-specific diagnostic biomarkers, and also unveil the potential therapeutic strategy of restoring the miRNA expression profile to normal as an anti-cancer treatment approach. There are a range of different techniques available for high-throughput miRNA expression profiling, some of the more common techniques are outlined below.

1.3.4.1 Oligonucleotide miRNA-microarray

Oligonucleotide based miRNA-microarray analysis was first described in 2004 (77). It is the most commonly used method for genome-wide assessment of miRNA expression in human

cancers, particularly in large studies. The microarray chip is printed with the sense strand, the miRNA gene specific oligonucleotide probe on the microarray hybridizes with labelled cDNA of the miRNA targets. The cDNA is synthesized by reverse transcription. The results are obtained after staining with streptavidin conjugates and detected by laser scanning. This method is easily standardized and can be applied reliably to a large sample size. It is also cheaper to perform than some of the more novel approaches, permitting simultaneous analysis of over 300 miRNAs. However, drawbacks include a reduced specificity in detecting structurally similar miRNAs.

1.3.4.2 Bead-based flow cytometric technology

Bead-based flow cytometric technology is a highly specific method of miRNA expression profiling. The technique was first described by Lu *et al* in 2005 (75). Polystyrene beads are coated with antisense oligonucleotide probes which hybridize with biotin-labelled PCR amplicon dsDNA (target). Staining for streptavidin–phycoerythrin is performed before results are obtained from signal detection using flow cytometry. Bead-based technologies are technically more challenging to perform, allowing only small numbers of miRNAs to be studied at a time. In addition it has been suggested that bias may be introduced to this technique; the small RNA component of the sample must be enriched prior to commencing by fractionation. Bias could also be introduced during PCR. However, this method permits higher miRNA specificity than oligonucleotide-based microarrays.

1.3.4.3 Tag-based sequencing methods

Other technologies in this field include the tag-based sequencing methods; miRNA serial analysis of gene expression (miRAGE or SAGE) (78) and RNA-primed array-based Klenow enzyme (RAKE) assay (79). SAGE involves the serial analysis of miRNA expression by small RNA purification, tagging and cloning. However its widespread use is restricted due to these laborious and expensive steps. The RAKE assay includes on-slide application of the Klenow fragment of DNA polymerase I. This assay is sensitive and specific; additionally it permits the discovery of novel miRNAs. However it is also labour intensive and requires a large amount of RNA.

1.3.4.4 Deep sequencing technology

Recent advances in scientific technology permit genome wide sequence analysis; deep sequencing technology, also called next generation sequencing, is one such large-scale

platform. The application of this technology permits the simultaneous sequencing of millions of sequences on a single sample. Deep sequencing is free from many of the limitations confronted by other methods; there is no cross-hybridization and no prior sequence knowledge is required. Deep sequencing can be considered 'unbiased' in this regard and is an excellent choice for diseases in which molecular profiling has previously been unfruitful. The major difficulty associated with this method of miRNA expression profiling is the large amount of data that confronts the researcher. Advances in bioinformatic tools have permitted informative results to be deduced from the data produced during such experiments (80-83). Indeed, some researchers consider this complex data analysis and large numbers of generated sequences to be a disadvantage associated with deep-sequencing. In addition, this method is expensive to perform.

1.3.4.5 The future of miRNA expression profiling techniques

The techniques discussed above can only be applied to samples or specimens that have been archived, as cell lysis or fixation is required. However, it is well known that miRNA expression patterns are likely to be transient, and reflect the host or disease micro-environment at that particular time. It could be that a miRNA profile of a specific cancer state represents a stage in the natural history of tumour development, constantly evolving with tumour progression. The next step in miRNA expression profiling would be to devise profiling techniques which could be applied *in vivo*. Molecular imaging is one such technique which permits the evaluation of the dynamic functioning of miRNAs within living cells. This remarkable advance supports repeated quantitative imaging of tumour and stem cells. Molecular imaging is superior in this regard as it permits remarkable insight into miRNAs that are dysregulated in carcinogenesis for consideration as novel treatment strategies.

1.3.4.6 Validation techniques: Northern blotting and real time quantitative PCR

The high-throughput technologies outlined above aid in the identification of novel disease-specific miRNA targets and miRNA signatures for diagnostic and therapeutic approaches. It is, however, necessary to validate these findings using additional techniques. The most commonly employed validation methods are northern blotting and quantitative real time polymerase chain reaction (RQ-PCR). Northern blotting requires large amounts of total RNA, is time consuming and requires handling of radioactive material (if conducted according to standard protocol). RQ-PCR requires less RNA, is a simple technique to

perform, and is more commonly employed in validation experiments. It is considered the gold standard technique for RNA quantification (84). Real-time quantitative (RQ-PCR) is widely used to quantify miRNA expression due to its sensitivity, specificity, speed, simplicity and the small amounts of template RNA required.

1.3.5 Endogenous controls for RQ-PCR

Target miRNA expression levels are normalised to those of a control(s) in order to differentiate true biological variation from experimentally induced artefacts. To accurately quantify miRNA expression by RQ-PCR, samples are assayed during the exponential phase of the PCR reaction. It is during this time that the amount of target miRNA is presumed to double with each cycle, free from the influence of limiting reagents. Relative quantification of miRNA expression implies the comparison of cycle threshold (C_T), the cycle number at which fluorescence signals are detected above background, to C_T values of an endogenously expressed control RNA. Accuracy of this method depends on the choice of endogenous control. Alternative methods of normalisation, such as normalisation to the global mean and use of spike-in controls, among others have also been described (85). Irrespective of the approach, the normalisation technique and specific control RNA(s) selected influence the results produced from RQ-PCR and thus validity. The selection of a suitable EC(s), with which to normalise RQ-PCR data, is an important first step in the accurate and reliable determination of miRNA expression levels.

Ideally a reliable EC(s) remains stably expressed regardless of disease status or other clinical parameters. A set of robust ECs that are stably expressed across all body tissues, fluids and disease pathways has yet to be described, and is unlikely to exist. Several studies based on tissue have reported the use of small RNAs (such as *U6, RNU44* or *RNU48*) or *miR-16* to normalise miRNA expression data (86-90). However, use of these reference genes cannot simply be applied to miRNA analysis in blood or other body fluids as miRNA expression patterns are disease-specific and perhaps specimen-type specific (91). Despite the plethora of studies on circulating miRNA profiles to discriminate between normal and pathological disease states, there have yet to be conclusive reports of appropriate ECs. This remains a significant issue that must be addressed to substantiate biomarker discovery and validate single miRNA expression analysis using RQ-PCR.

Few validated endogenous controls for miRNA research in breast cancer have been described. Initial miRNA studies on breast tissues by Mattie *et al* normalized miRNA expression to *miR-16* and *let-7*, which were later shown to be stably expressed across malignant, benign and normal breast tissue by Davoren *et al* (86, 92). Early studies on systemic miRNAs in breast cancer normalized to *miR-16* (93, 94). Additional studies, on breast and other cancers, have suggested alternative EC candidates such as *U6, RNU44, RNU48*, *miR-142-3p*, *miR-484*, *miR-191* and *miR-425* (87, 95-102). However, there is a lack of validated reports of suitable ECs for circulating miRNAs.

1.3.6 In situ hybridisation for miRNAs

The molecular profiling and validation miRNA techniques described above provide quantitative data about miRNA expression in tissue or biological fluids, but are unable to provide information regarding a specific cellular source of miRNAs. The origin of dysregulated miRNAs in the cancer state therefore cannot be identified, particularly in cases where heterogeneous specimen sources are used. *In situ* hybridisation provides information on expression of individual miRNAs as well as the cellular location within different tissues and cell populations (103). Advancements in locked nucleic acid (LNA) technology have progressed this field permitting semi-quantitative miRNA expression data and miRNA localisation within the cellular microenvironment (Figure 1.12).

Figure 1.12 *In situ* hybridization of *miR-126*

MiR-126 can be readily appreciated (stained purple) in the tumour associated vasculature[13].

[13] http://www.bioneer.dk

1.3.7 Epigenetic regulation of miRNAs

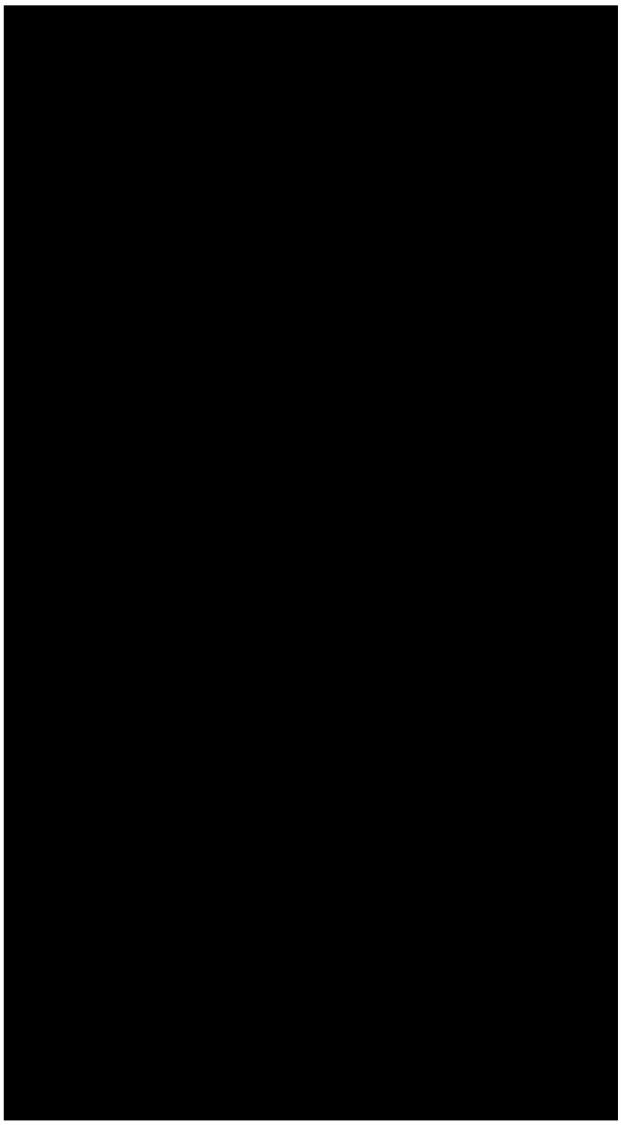
Epigenetics pertains to the inheritance of gene expression alterations with no change in the DNA sequence (104). There is an expanding body of evidence to suggest that epigenetic and genetic variation may be responsible for altered expression of a number of miRNAs (105, 106). DNA methylation is a type of epigenetic modification which causes phenotypic alterations as a consequence of gene expression repression. Hypermethylation of promoter DNA has been shown to silence the expression of some specific miRNAs and has been implicated in tumourigenesis (106). DNA methylation in mammals predominantly occurs at the C-5 position of complementary CpG by DNA methyltransferases (107). DNA methylation of CpG islands was found to be at promoter regions of tumour suppressor miRNAs, with under-expression in the cancer state.

1.3.8 MiRNA profiling in breast cancer

Breast cancer is at the forefront of miRNA research. Iorio *et al* conducted one of the first global miRNA profiling studies on breast tumour and normal breast tissue specimens using miRNA microarrays. Twenty-nine miRNAs with altered expression (both under and over-expressed) in breast cancer were identified (108). Mattie *et al* provided one of the first reports of distinct miRNA profiles for differing breast tumour subtypes, namely miRNA subsets of ER and HER2/*neu* receptor status (86). Interestingly, there was some degree of overlap in the altered miRNAs between these two early studies including *miR-125b, miR-145* and *miR-21*.

Since then, there have been numerous reports of altered tissue miRNA expression patterns correlating with molecular subtype and hormonal receptor status (109-111). Blenkiron *et al* were among the first to establish miRNA expression profiles for intrinsic breast tumour subtypes (109). This group analysed the expression of 309 miRNAs in 93 human breast tumour specimens. A total of 133 miRNAs were expressed in normal breast and tumour tissue, with 38 miRNAs displaying differential expression across subtypes (Figure 1.13). Furthermore, Luminal A and basal tumours could be correctly classified using their miRNA expression profile alone.

Figure 1.13 Individual miRNAs and clinicopathological variables



B basal; H HER2/*neu* overexpressing; LA Luminal A; LB Luminal B; Normal-like; - negative; + positive
This heat map presents the relationship between miRNA expression and subtype, as well as the clinicopathological variables ER status and grade. There are 31 miRNA, each with at least one significant (adjusted $p<0.01$) association[14].

As mentioned above, several tools have been developed to predict response to treatment, such as Oncotype DX and Mammaprint. However these tools are largely based on messenger RNA profiles and as such are associated with inherent weaknesses. Recent evidence suggests that miRNA signatures provide complementary and additional

[14] Blenkiron *et al*, 2007

information to traditional molecular marker studies (75, 112). The role of miRNA expression profiling to correctly classify breast tumours according to molecular subtype is an excellent example of translational research. These expression profiles unveil the potential application of miRNA panels as prognostic and predictive tools. Lowery *et al* have identified subsets of miRNAs which correlate with ER, PR and HER2/*neu* receptor status using a miRNA microarray (111). MiRNA expression was compared against 29 early stage breast tumour and tumour associated normal (TAN) tissues. Artificial neural networking was employed as the bioinformatics method to identify those miRNAs with differential expression between tumour and normal breast tissue. These candidate miRNAs were then validated in a larger cohort using RQ-PCR. The miRNA signatures are presented in Table 1.6 below.

Table 1.6 MiRNA signatures of ER, PR and HER2/*neu* receptor status[15]

ER status	PR status	HER2/*neu* status
miR-342	miR-520g	miR-520d
miR-299-3p	miR-377	miR-181c
miR-217	miR-527-518a	miR-302c
miR-190	miR-520f-520c	miR-376b
miR-125b		miR-30e-3p
miR-218		

Indeed miRNA profiles have also been generated to discriminate between a number of other histological variables, including DCIS compared to invasive carcinoma, unilateral compared to bilateral breast cancer and lobular breast cancer (113-115).

1.3.9 Circulating miRNAs in breast cancer

MiRNAs were originally studied in tissue, but tumour specific miRNAs were first described in serum of patients with diffuse large B-cell lymphoma (116). Lawrie *et al* reported that *miR-155*, *miR-210* and *miR-21* were over-expressed in the sera of patients with diffuse large B-cell lymphoma compared to the serum of healthy controls. These seminal findings unveiled an exciting role for miRNAs as circulating biomarkers of health and disease. Since 2008 there has been a surge of interest in this promising and lucrative field, evident by the rapidly expanding body of literature focusing on circulating miRNAs as cancer-specific biomarkers. Indeed recent studies have even explored the possibility of miRNA

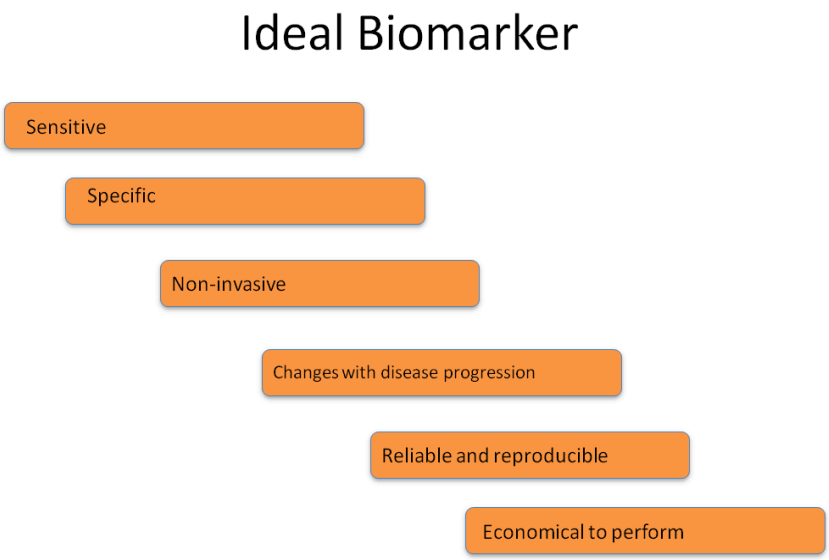
[15] Lowery et al, Breast Cancer Res 2009;11(3):R27

quantification in other body fluids, perhaps tailored to the specific disease entity; salivary miRNAs for oral squamous cell carcinoma (96) and urinary miRNAs for bladder carcinoma (117), for example.

1.3.10 MiRNAs as biomarkers

The characteristics of an ideal biomarker are outlined in Figure 1.14. A single molecule is unlikely to fulfil all the criteria but circulating miRNAs appear to be promising biomarker candidates. They are relatively stable compared to other RNAs and can be obtained using a simple blood test. They can endure most processing and storage handling, including extremes of temperature, low pH, high pH, prolonged storage and repeated freeze-thaw cycles (118). Specific circulating miRNAs have been shown to have acceptable sensitivity and specificity profiles in discriminating against those with cancer and healthy controls. Heneghan *et al* published one of the first reports of the use of circulating miRNAs for breast cancer detection. *MiR-195* was reported to be significantly elevated (p=0.001) in the blood of women with breast cancer (n=83) compared to healthy controls (n=44) with expression levels in the cancer group returning to those of the controls two weeks post-operatively. This study reported a circulating *miR-195* sensitivity of 85.5% and a specificity of 100%. In a subsequent study, Heneghan *et al* reported that elevated circulating *miR-195* was a breast cancer specific phenomenon, following comparison of *miR-195* expression with controls, prostate cancer, renal cell carcinoma, colon cancer and melanoma. Several studies have also since demonstrated that breast cancer-specific miRNAs are detectable in the circulation revealing their potential application as circulating disease specific biomarkers (94, 119, 120).

Figure 1.14 Characteristics of an ideal biomarker

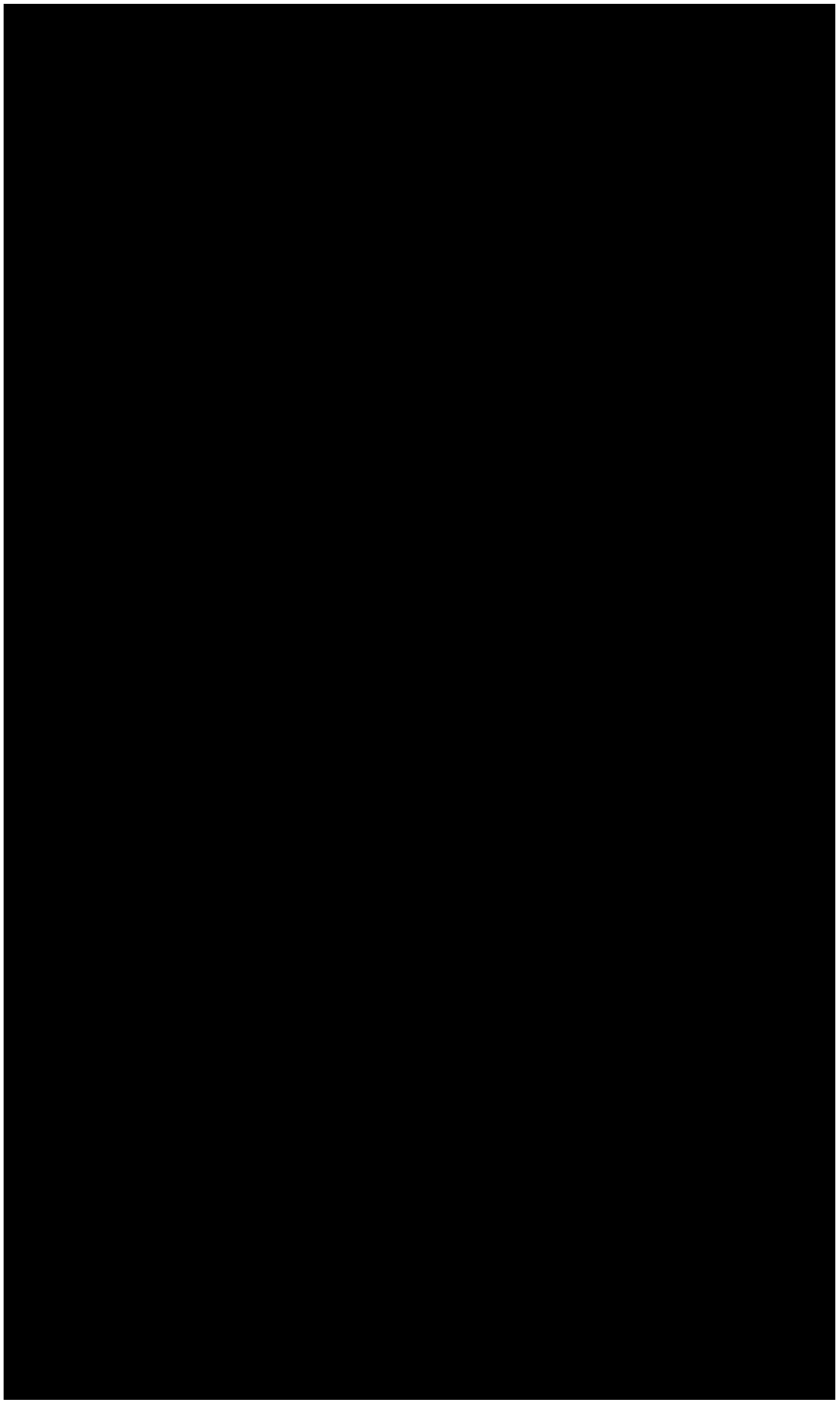


In addition to the apparent ability to reflect tumour type, miRNAs have other attributes that make them appealing biomarker material, namely the ability for miRNA profiles to translate beyond a simple 'yes/no' cancer diagnosis, and instead provide more detailed information about the tumour. Distinct miRNA signatures have been shown to correlate with clinicopathological parameters in tissue, and this may also be true of circulating miRNAs. Speculation that circulating miRNA profiles could reflect not only the tumour tissue-type, but also the intrinsic molecular subtype thus acting as a 'fluid biopsy' would be particularly valuable in breast cancer where management, even immediately following diagnosis, is governed by hormonal and HER2/*neu* receptor status, largely conveying molecular subtype. The specific miRNAs that are actively secreted or passively released by the tumour microenvironment may reflect the subtype specific genetic and epigenetic alterations at a cellular level of that intrinsic subtype (Figure 1. 15).

There are several mechanisms by which miRNAs are potentially shed and subsequently transported in the systemic circulation. MiRNAs may be selectively packaged into exosomes, small lipoprotein vesicles originating from the endosome, which are released from the cell by fusion with the cell membrane (121). Microvesicles also package miRNAs for exportation and are formed by simple blebbing of the plasma membrane. MiRNAs may also be passively released or secreted from the cell free from encapsulation by lipoprotein bodies. It has also been demonstrated that a large proportion of circulating miRNAs are not

encompassed in lipoprotein particles, but rather bound to protein complexes such as nucleophosmin 1(NPM1) and Argonaute2 (Ago2) (122, 123).

Figure 1.15 MiRNAs as circulating biomarkers for breast cancer

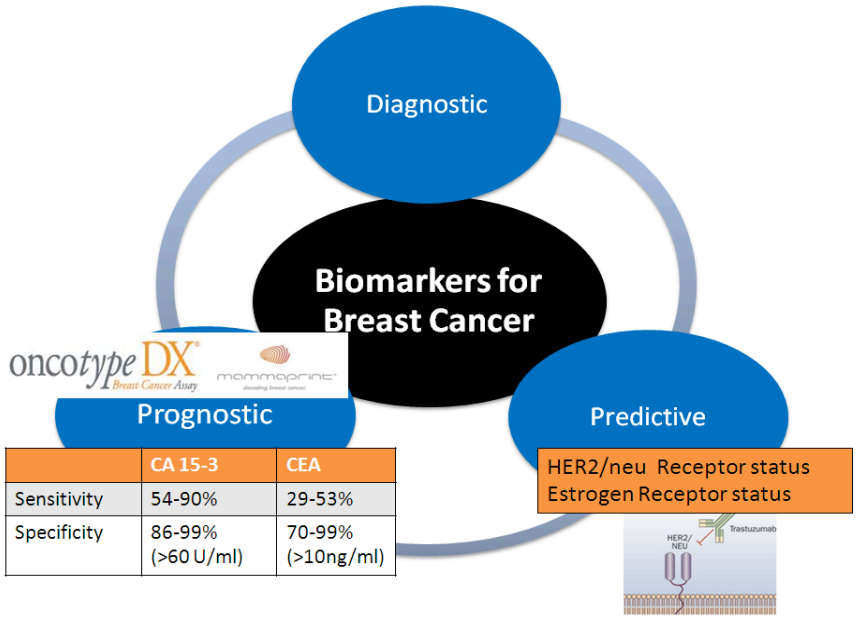


MiRNAs are released into the circulation by either active secretion or passive leakage[16].

[16]Adapted from Cortez MA *et al* (2011) MicroRNAs in body fluids-the mix of hormones and biomarkers. Nature reviews Clinical oncology 8 (8):467-477

The application of miRNAs as disease biomarkers is not confined to the diagnostic arena. MiRNA profiles reflecting molecular subtypes could inherently predict response to hormonal and HER2/*neu* target therapies. It is also plausible to suggest that miRNA profiles could be described to predict chemo sensitivity and radio-sensitivity, as well as to monitor response to such adjuvant therapies. *MiR-125b* has been associated with chemotherapeutic resistance in breast cancer, exhibiting a higher pre-treatment expression level in non-responders (124). High pre-treatment circulating *miR-210* has been shown to be predictive of trastuzumab resistance in women undergoing neoadjuvant chemotherapy (125). However, additional large scale studies are required to appraise the application of circulating miRNAs in predicting and monitoring response to treatment. A miRNA profile with the capacity to perform this task would be invaluable, as it would spare patients who are unlikely to derive any benefit from adjuvant treatments, which would have abundant positive collateral effects.

Figure 1.16 Current biomarkers for breast cancer



	CA 15-3	CEA
Sensitivity	54-90%	29-53%
Specificity	86-99% (>60 U/ml)	70-99% (>10ng/ml)

Biomarkers for breast cancer can be classified into diagnostic, predictive and prognostic. There are currently no diagnostic biomarkers for this disease. HER2/*neu* is predictive of response to treatment with traztuzumab. ER receptor positive tumours respond to hormonal therapy. Prognostic biomarkers include circulating CA-15-3 and CEA for late stage disease. Oncotype DX® and Mammaprint® are gene based predictive tools performed on a specimen of breast tumour tissue.

1.4 Study rationale

A key facet of miRNAs is their functional involvement in almost all developmental, physiological and pathological processes. They exert their effects by finely tuning gene expression at the post transcriptional level. However, their ease of detection and quantification in the circulation coupled with altered expression levels has revealed their remarkable potential as disease biomarkers, for application to diagnostics, prognostics and prediction as well as a role as disease modulators and therapeutic targets. The intimate involvement of miRNAs in almost all physiological and pathological processes and subsequent presence in the blood means that the minutiae of cellular functioning could potentially be detected at a systemic level. In essence, a miRNA 'fluid biopsy' from the peripheral circulation could prove as informative as a traditional tissue biopsy.

Further investigation into miRNAs with previously documented associations with breast cancer, as well as the identification of novel miRNAs, and their downstream mRNA targets, will broaden our understanding of these tiny RNA molecules, their role in carcinogenesis and the molecular alterations underpinning the heterogeneous disease process of breast cancer. Moreover, the identification of miRNAs with a potential application as biomarkers for breast cancer would fulfil a long awaited and coveted role in breast cancer management.

The fundamental aim of this study was to investigate the application of miRNAs as circulating oncologic biomarkers for breast cancer using the following approaches:

1. **Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies**
    a. A prerequisite to accurate interpretation of RQ-PCR data is robust data normalisation. A commonly used method is to compare the cycle threshold (C_T) of target miRNAs with those of a stably expressed endogenous (EC) miRNA(s) from the same sample. Despite the large number of studies reporting miRNA expression patterns, comparatively few appropriate ECs have been reported thus far. The purpose of this study was to identify stably expressed miRNAs with which to normalise RQ-PCR data derived from human blood specimens.

b. The effect of normalisation using different endogenous controls on relative expression of target miRNAs was then assessed.

2. Identification and Validation of Oncologic miRNA Biomarkers for Luminal A Breast Cancer

a. The aim of this study was to further our understanding of the molecular subtypes of breast cancer by identifying circulating miRNAs with altered expression in Luminal A breast cancer. Luminal A breast cancer was selected as the model for this study as it is the most common breast cancer subtype. Microarray profiling followed by analysis with artificial neural networking techniques were employed to identify circulating miRNAs that are differentially expressed in women with Luminal A breast cancer (ER positive, PR positive, HER2/*neu* negative) in comparison to healthy controls, in an effort to further understand the molecular alterations underpinning this common type of breast cancer.

b. Expression of candidate miRNA targets was then validated in a larger cohort of blood and tissue specimens using RQ-PCR.

c. The association of circulating miRNA expression with clinicopathological variables such as grade and stage of disease was then investigated to determine if such differences could be detected in the circulation.

d. The effectiveness of the newly identified candidate miRNAs for Luminal A breast cancer as circulating diagnostic biomarkers in the clinical setting was then evaluated.

3. Differential Expression of *miR-15* Family Members in Breast Cancer

a. MiRNA related research to date has mainly focused on individual miRNAs. A miRNA family is a group of miRNAs which share a common 'seed sequence' which is critical in determining binding and therefore function. *MiR-195*, a member of the *miR-15* family, has been reported as having altered expression in the circulation of women with breast cancer. The aims of this study were to profile the *miR-15* family members (*miR-15a, miR-15b, miR-195, miR-424* and *miR-497*) in the circulation and tissue of women with breast cancer.

b. Despite the increasing body of literature surrounding tumour and circulating miRNAs as biomarkers for breast cancer, few studies have sought to identify the relationship between the two. Matched tumour tissue and blood samples were exploited to determine whether circulating *miR-195* and *miR-497* expression profiles reflected that of the tissue.

c. To investigate the clinical utility of circulating *miR-15* family members as oncologic biomarkers for breast cancer by evaluating their sensitivity and specificity profiles in discriminating between women with breast cancer and healthy controls.

d. Each miRNA has the potential to act on multiple mRNA targets. Similarly each mRNA can be targeted by multiple miRNAs. Predicting putative mRNA targets for each miRNA can be facilitated by the use of *in silico* analysis. To further the understanding of the role of *miR-15* miRNAs in cancer putative mRNA targets were identified.

4. Analysis of *miR-195* and *miR-497* in the tumour microenvironment

a. MiRNA quantification by RQ-PCR provides precise information about the relative quantity of miRNAs, but lacks the ability to provide critical spatial information on the location of miRNAs within the tumour microenvironment. The overall aim of this study was to advance our understanding of *miR-195* and *miR-497* in the tumour microenvironment. The initial aim was to determine where in the tumour microenvironment *miR-195* and *miR-497* were located using *in situ* hybridisation (ISH).

b. We then wished to assess *miR-195* and *miR-497* expression in different breast cancer cell lines, to determine if all cell lines displayed a similar expression pattern or if *miR-195* expression is dependent on molecular subtype.

c. There is a growing body of evidence that suggests that epigenetic and genetic variation may be responsible for altered expression of a number of miRNAs. DNA methylation is a type of epigenetic modification which causes phenotypic

alterations as a consequence of gene expression repression. Finally we wished to evaluate epigenetic modulation by methylation altered *miR-195* and *miR-497* expression *in vitro*.

5. Circulating miRNAs: Novel breast cancer biomarkers and their use for guiding and monitoring response to chemotherapy

a. With the increasing patient awareness of the toxicity of chemotherapy and era of individualised, patient-centred cancer management plans there is a need to develop an accurate, acceptable clinical test that would identify patients who are most likely to obtain pCR from neoadjuvant treatment, and thus spare a proportion of patients from the toxic effects of treatments from which they will derive no benefit. The aim of this study was to establish a multicentre prospective clinical trial to recruit patients undergoing neoadjuvant chemotherapy for newly diagnosed breast cancer so that fluctuations in circulating miRNA expression levels over the course of neoadjuvant chemotherapy could be assessed.

b. The relationship between circulating miRNA profiles and patients' intrinsic subtype of breast cancer was investigated.

c. In an effort to evaluate if circulating miRNA profiles could be used as predictive tools of response to treatment, the relationship between miRNA expression profiles and response to treatment was examined.

d. The relationship between miRNA expression levels and other existing clinicopathological parameters was also evaluated, specifically nodal status, ER status and HER2/*neu* receptor status.

Chapter 2
Materials and Methodology

2.1 Discipline of Surgery BioBank

2.1.1 Introduction

Biomedical research is dependent on the availability of human biological material (specimens) for clinical trials and scientific investigations. The Surgery Research BioBank at University Hospital Galway has been in existence since 1992. Approval was initially granted by the Galway Roscommon University Hospital Group (GRUHG) Clinical Research Ethics Committee to store breast cancer-related tissue specimens and clinically relevant data, subject to obtaining written informed consent from each patient. In recent years, the BioBank has expanded, following approval from the GRUHG Clinical Research Ethics Committee, to include research specimens from patients with other cancer types, namely prostate, colorectal, renal cell carcinoma and melanoma. The sample type composition of the Biobank is outlined in Table 2.1, correct as of May 2013. The BioBank is also permitted to store omental and subcutaneous fat samples to conduct studies on metabolism and obesity.

Table 2.1 BioBank composition

Group	Whole Blood	Serum/Plasma	Tissue
Breast	2036	1423	1359
Colorectal	131	156	488
Renal/Bladder	34	68	56
Control	1373	164	64

Tissue number includes tumour, TAN and benign (i.e. not healthy volunteers)
There is a large no. of whole blood controls due to specific population genetics breast study.

Blood, tumour and tumour-associated normal (TAN, macroscopically normal appearing tissue adjacent to the tumour site) tissue specimens have been routinely collected from patients undergoing treatment for these pathologies. To date there are over 4,000 patients entered in the system. The number of usable biological samples is in excess of 10,000. Shire is a Microsoft Access-based laboratory information management system (LIMS) that generates unique anonymized patient identifiers and records clinical details pertaining to the samples.

2.1.2 Sample Collection (for Breast Cancer Patients)

Patients attending Galway University Hospital (GUH) are invited to participate in the breast cancer BioBank at the time of diagnosis. The research programme is described in detail by the clinician and specialist breast care nurse, patients are then provided with an information leaflet (Appendix 1.1) further describing the biobanking process and its implications. Time is allowed for questions, and written informed consent obtained if the patient is willing to participate in the programme. Consenting patients agree to donate blood and/or tissue specimens before or during the course of their treatment. Blood samples (whole blood, serum and plasma) are typically collected pre-operatively. Tissue specimens are retrieved from patients at the time of diagnosis (core biopsy) or during surgical resection of the tumour.

2.1.3 Blood collection

Whole blood samples are routinely collected pre-operatively by peripheral venous sampling. Three bottles are collected in total; one 10ml in vacuette EDTA K3E blood bottle (Grenier Bio-one), one 5ml Vacuette EDTA K3E blood bottle (Grenier Bio-one)and one 4ml vacutainer Serum Separator Tubes II (Becton Dickinson). One EDTA bottle is processed for plasma (5ml) and the others remain unprocessed (10ml, whole blood). Samples for serum collection are left to clot at room temperature for 30 minutes and all samples destined for serum and plasma preparation are centrifuged at 2000rpm at 4°C for 10 minutes. The plasma and serum are aspirated by pipetting (typically 1.5 to 2 mL volumes), aliquoted into pre-labelled tubes and stored at -80°C until required. Unprocessed EDTA blood samples are stored at 4°C until required.

2.1.4 Tissue collection

As per standard operating procedures, tissue samples are obtained either pre-operatively, at the time of diagnosis by core biopsy, or at the time of surgical resection of their tumour. The type of tissue specimens routinely collected include primary breast tumour tissues from patients undergoing surgical management of their breast tumour, macroscopically normal breast tissue from areas adjacent to the breast tumour (TAN), normal breast tissue retrieved during reduction mammoplasty and benign breast tissue. Resected tissue specimens are dissected to reach 0.5cm in at least one dimension, and are then submerged in a sterile storage tube containing 5 volumes RNA Later® solution (Qiagen)). RNA-later is a stabilisation reagent that permeates tissues to rapidly stabilise cellular RNA to protect it from degradation. Tubes containing tissue and RNA-later are then transferred to the lab

and stored at -4°C for approximately 24 hours. Once the sample has been logged into the Shire Database, the RNA-later solution is decanted, and specimens are archived at -80°C.

2.1.5 Anonymization of patient data

Demographic and specific clinicopathological data pertaining to all samples used is recorded in the Shire database (Appendix 1.2). This generates unique alphanumeric codes and assigns a specific storage location for each sample. All samples are relabelled with these anonymized codes prior to storage. The range of data entered into Shire includes:

Patient demographics

Breast cancer risk factors

Menopause status at diagnosis

Tumour size

Tumour grade (Table 2.2)

Hormone receptor status

HER2/*neu* receptor status

Nodal status

Presence or absence of distal metastases

TNM staging

This data is derived from a separate database designated for the collection of clinicopathological information (Breast Cancer Database) information which is fed-back to the department from clinical notes, Consultant correspondence (Surgical, Oncology, Radiotherapy), radiology reports, pathology reports and multidisciplinary meetings. Breast carcinomas are routinely histologically graded by the Nottingham modification of the Scarff-Bloom-Richardson grading system which considers the specimens tubularity, nuclear size and shape and hyperchromatic figures (Table 2.2).

Table 2.2 Nottingham modification of the Scarff-Bloom-Richardson (SBR) grading system

Tubule Formation		Mitotic Count		Nuclear Pleomorphism	
Majority of tumour (>75%)	1	0-9 mitoses/10 hpf	1	Small regular uniform cells	1
Moderate degree of tumour (10-75%)	2	10-19 mitoses/10 hpf	2	Moderate nuclear size and variation	2
Little or none (<10%)	3	≥20 mitoses/10 hpf	3	Marked nuclear variation	3

Combined Histological Grade	
Low grade (I)	3-5
Intermediate grade (II)	6-7
High grade (III)	8-9

Hpf, high powered field

2.1.6 Patient consent

Prior written informed consent was obtained from each participant in each cohort as described above (Appendix 1.1) and all studies were approved by the Galway University Hospital Ethics Committee review board.

2.2 Study Groups

At the start of each experiment, specific study groups were chosen using data from the Breast Cancer Database according to the clinical and pathological criteria under investigation. In this way suitable candidates were identified to address specific questions as outlined in the following section.

2.2.1 Circulating miRNA analysis

2.2.1.1 Breast cancer patients

A cohort of 67 patients was identified for whom pre-operative blood samples were available or could be obtained. This group was representative of a typical group of symptomatic breast cancer patients. This group or subsets of this group were used for all studies performed on circulating miRNAs in breast cancer. Blood samples were collected prospectively from these patients prior to surgical resection of their tumours. All patients had histologically confirmed breast cancer, and no patient in this cohort had received neoadjuvant chemotherapy or radiotherapy prior to sample acquisition. Relevant demographic and clinicopathological details were retrieved from the prospectively maintained Breast Cancer Database (Table 2.3). The histological tumour profile of patients in this study reflected those of a typical breast cancer clinic.

Table 2.3 Clinicopathological data for circulating miRNA analysis

Breast Cancer		**Blood**
Number		**67**
Age (years)		58.37 (±14.05)
Intrinsic Subtype		
	Luminal A	52
	Luminal B	6
	HER 2/*neu*	3
	Basal	4
	Missing	2
Histological Subtype		
	DCIS	2
	Invasive Ductal	53
	Invasive Lobular	3
	Other	9
Grade		
	1	8
	2	43
	3	12
	Missing	4
Total Tumour Size (mm)		35.92 (±25.80)
	Missing	14
Invasive Tumour Size (mm)		28.03 (±19.68)
	Missing	34
Lymph Node Status		
	Positive	34
	Negative	28
UICC Stage		
	1	19
	2	23
	3	18
	4	2
	Missing	5
Menopausal Status		
	Pre	20
	Peri	8
	Post	39
Control		
Number		**56**
Age (years)		50.04 (±23.37)

mm,millimeters; Inv. T size, Invasive Tumour size; UICC, Stage of breast tumour according to the International Union Against Cancer staging criteria; ER, Oestrogen receptor; PR, Progesterone receptor: HER2/*neu*, Human epidermal growth factor receptor; -, negative; +, positive; NA not applicable; All control subjects had no personal or family history of breast or ovarian cancer and were clinically well at the time of sampling.

2.2.1.2 Control samples

Control blood samples were obtained from healthy individuals with no known previous malignancy, no family history of breast or ovarian cancer and no active inflammatory condition. Participants were recruited from a number of sources within the same catchment area as the cancer group including a GP practice, nursing homes, healthy women accompanying others to outpatient appointments and young females working in other disciplines in the university. A total of 56 controls were collected for this study (Table 2.3). They were of similar age range and ethnic background to the cancer patients.

2.2.2 Breast tissue cohort

To investigate the expression of individual miRNAs in breast cancer tissue, a cohort of breast tumours (n=20) and matched TAN tissues (n=10) representing Luminal A, Luminal B, HER2/*neu* and basal breast cancer was identified. The cohort of breast tumour and TAN specimens was representative of a typical breast cancer cohort with a mean age of 52.7 years, and a predominance of Luminal A subtype. Clinical and pathological data relating to the clinical samples are presented in Table 2.4.

Table 2.4 Clinicopathological data for breast tumours

Breast Tumours		**Number**
Number		**20**
Mean age, years (range)		52.70 (±7.09)
Median Whole. T size (mm)		34.1 (±42.5)
Histological Subtype		
	Invasive ductal carcinoma	20
Grade		
	1	2
	2	3
	3	13
	Missing	2
UICC stage		
	Stage 1	6
	Stage 2	13
	Stage 3	1
Intrinsic Subtype		
	Luminal A	9
	Luminal B	3
	HER2/*neu* overexpressing	6
	Basal	2
Menopausal Status		
	Pre	7
	Post	13
Nodal Status		
	Positive	1
	Negative	19
Tumour Associated Normal (TAN)		
Number		10

A subset of the tissue cohort (n=15) included patients for whom blood samples and tissue samples were available. This group served as a group for whom the relationship between tumour and circulating miRNA expression could be examined. This cohort is outlined in Table 2.5.

Table 2.5 Clinicopathological details for tissue and blood matched cohort

Sample Number	Age (years)	Tumour size (mm)	Stage (UICC)	Grade	Intrinsic Subtype	Nodal Status
R10-1062	60	20	1	2	Luminal A	Negative
R10-1094	50	22	2	2	Luminal A	Negative
R10-1273	33	27	2	3	Basal	Negative
R10-1277	56	53	2	2	Luminal A	Positive
R10-1387	59	120	3	2	Luminal A	Positive
R10-1526	46	38	3	1	Luminal A	Positive
R11-0033	55	61	3	2	Luminal A	Positive
R11-0070	76	45	2	3	Luminal A	Positive
R11-0344	68	10	1	1	Luminal A	Negative
R11-0355	57	30	3	3	Basal	Positive
R11-0365	53	44	3	2	Luminal A	Positive
R11-0533	73	17	1	2	Luminal A	Negative
R11-0541	41	34	3	2	Luminal A	Positive
R11-0634	50	25	2	2	Luminal A	Positive
R11-0707	58	*	2	2	Luminal A	Positive

No., number; T size, tumour size (mm)

2.2.3 Neoadjuvant study cohort

To determine whether circulating miRNAs could be used as biomarkers for monitoring and predicting response to chemotherapy in the neoadjuvant setting, a multi-centre clinical trial was established in collaboration with ICORG, the All Ireland Cooperative Clinical Research Group. The purpose of this study was to recruit patients with a newly diagnosed breast tumour planning to undergo neoadjuvant chemotherapy and to monitor their miRNA levels at several clinically relevant time-points.There were 30 patients enrolled in this study (Table 2.6).

Table 2.6 Clinicopathological details of neoadjuvant chemotherapy cohort

Number		**30**	**%**
Age (years)		50.3 ± 10.4	NA
Intrinsic Subtype			
	Luminal A	17	56
	Luminal B	4	14
	HER 2/*neu*	3	10
	Basal	6	20
Histological Subtype			
	Invasive Ductal	29	97
	Invasive Lobular	1	3
Grade			
	1	1	3
	2	15	50
	3	14	47
Total Tumour Size (Radiological, mm)		39.1 ± 20.6	NA
	Missing	5	17
Total Tumour Size (Histological, mm)		34.1 ± 17.7	NA
	Missing	4	14
Lymph Node Status			
	Positive	24	80
	Negative	5	17
	Missing	1	3
Response to NAC			
	Poor	2	7
	Partial	19	63
	Good	6	20
	Complete	3	10

2.2.5 In situ hybridisation cohort

A cohort of 10 Luminal A breast cancer patients for whom paraffin tissue blocks were available were selected for inclusion in the *in situ* hybridisation study. Their clinicopathological details are outlined in Table 2.7 below.

Table 2.7 Clinicopathological details of patients included in ISH study

Cases	Age (yrs)	Inv. T size (mm)	Whole T size (mm)	Histological Subtype	Nodal Status	Grade	UICC Stage	ER	PR	HER2/ neu	Intrinsic Subtype	Controls	Age (yrs)
1	70	22	22	Inv. Muc.	-	2	1	+	+	-	Luminal A	1	81
2	52	15	15	Inv. Ductal	-	2	2	+	+	-	Luminal A	2	61
3	60	13	20	Inv. Ductal	-	2	2	+	+	-	Luminal A	3	82
4	50	22	22	Inv. Ductal	-	2	1	+	+	-	Luminal A	4	76
5	46	38	38	Inv. Ductal	+	1	2	+	+	-	Luminal A	5	74
6	59	120	120	Inv. Ductal	+	2	3	+	+	-	Luminal A	6	87
7	56	53	53	Inv. Lobular	+	2	2	+	+	-	Luminal A	7	94
8	55	61	61	Inv. Ductal	+	2	3	+	+	-	Luminal A	8	94
9	44	45	45	Inv. Ductal	-	2	2	+	+	-	Luminal A	9	96
10	75	50	60	Inv. Ductal	+	2	3	+	+	-	Luminal A	10	72

2.3 RNA extraction

Depending on the sample type, differing RNA extraction protocols were used. In this study RNA was extracted from blood, tissue (tumour and normal) and cell lines. RNA isolation from whole blood was performed using Trizol, a phenol-chloroform based extraction technique. Total RNA was extracted from breast tissues (tumour and normal) and cell lines using Qiagen kits.

2.3.1 RNA extraction from whole blood

Total RNA was extracted from whole blood samples by firstly lysing blood samples in Trizol (3 mL Trizol/I mL whole blood) supplemented with 200µL 1-bromo-4-methoxybenzene (BAN, Molecular Research Centre). RNA was separated from DNA, protein phases and cellular debris by centrifugation at 4°C for 15 minutes at 12,000g. The upper clear aqueous phase (approximately 600-1000 µL) were removed from each tube and transferred to fresh 2mL collection tubes. RNA was precipitated from solution by the addition of 1mL isopropanol and centrifugation at 12,000g for 8 minutes at 18°C. Supernatants were carefully removed and the RNA pellets were washed with 1mL 75% ethanol. The ethanol wash was repeated to improve the 260/280 nm spectrophotometry ratio. Following ethanol removal, RNA pellets were air-dried, and then re-solubilised in a total of 60 µL nuclease free water. Three µL aliquots were retained for RNA concentration and integrity analysis, prior to storage of the remainder at -80°C.

2.3.2 RNA extraction from tissue samples

Tissue samples (10-100 ng) were removed from -80^{o}C storage and homogenised in 1 mL Trizol using a bench-top homogeniser (Kinematica). In between samples, the homogeniser probe was cleaned rigorously using RNase free water (Qiagen), RNase Zap (Ambion) and 100% ethanol. Sample homogenisation was performed in batches of tissue types *i.e*. tumour and TAN samples were homogenised on separate occasions to minimise any potential cross-contamination between tissue types. Once homogenised, samples were stored at -80°C for at least 24 hours to facilitate lysis before continuing with RNA. In most instances, due to their small sizes, core biopsies could not be homogenised in this manner and were disrupted using a mortar and pestle, or manually minced using scalpel blades.

To extract RNA from homogenised tissue, the RNeasy Mini Kit® (Qiagen) and manufacturer's protocol were used. Homogenates were removed from -80^{o}C storage and

allowed to gently defrost on ice. Once the samples had thawed 200 µL of chloroform was added per mL Trizol (Life Technologies). Samples were vortexed for 15 seconds left to stand at room temperature for 5 to 10 minutes. Homogenates were centrifuged at 12,000g for 15 minutes at 4°C to precipitate insoluble material and selectively isolate RNA from DNA and protein phases. RNA migrated to the upper aqueous phase, which was aspirated by pipetting (approximately 600 µL) to a fresh 1.5mL eppendorf tube. Three and a half volumes of 100% ethanol (2.1mL for each 600 µL) were added to each sample, and mixed thoroughly by vortexing, before 700 µL aliquots of the RNA-ethanol mixture was pipetted onto RNeasy columns (Qiagen) and centrifuged at maximum speed for 21 seconds at 4°C. This step was repeated until the whole sample passed through the RNeasy column. The extracted RNA was DNase-treated by firstly washing the columns with 350µL of buffer RW1 and the addition of DNase I in Buffer RDD (Qiagen) for 15 minutes at room temperature. Columns were washed a second time with buffer RW1 to remove traces of DNase. RNA bound to the membrane was twice washed with 500 µL Buffer RPE (Qiagen), before samples were eluted in chilled nuclease free water (60 µL) in total. Three µL aliquots of RNA were retained for RNA concentration and integrity analysis. The remaining RNA (approximately 57 µL) was transferred to RNA storage tubes and stored at -80°C until required.

2.3.3 Cell lines

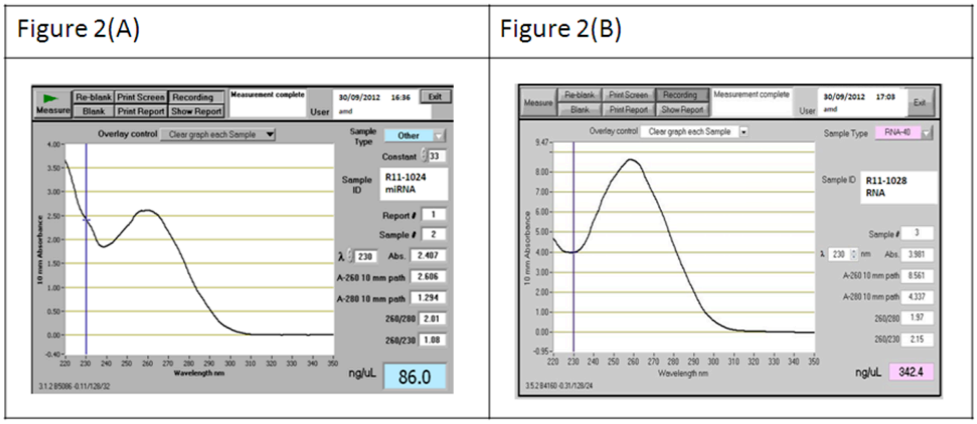
Cell pellets were lysed in Trizol and either processed immediately or stored at -80°C prior to extraction of RNA, which was conducted according to the co-purification technique above.

2.4 Analysis of RNA concentration

NanoDrop spectrophotometry (NanoDrop 1000 Spectrophotometer, NanoDrop Technologies) was used to assess the concentration and purity of extracted RNA. This instrument measures the absorbance at different wavelengths, the ratios of which were used to assess the purity of extracted RNA. The instrument displayed absorbance spectra for each sample. High quality RNA had absorbance peaks centred at a wavelength of 260 nm(A_{260}). The ratio of A_{260}/A_{280} was used as a measure of nucleic acid purity: ratios of ~2 were generally accepted as 'pure' for RNA. If the ratio was appreciably lower, the presence of protein, phenol or other contaminants was inferred. A second ratio to consider was the A_{260}/A_{230}. Values between 1.8 and 2.2 indicated high purity, with lower ratios indicating the presence of contaminants such as the carry-over guanidinium salts. The extinction

coefficient was used to convert peak absorbance to concentration. For RNA the extinction coefficient is 40 (ng/µL RNA / A_{260}). An extinction coefficient of 33 (ng/µL RNA / A_{260}) was used to determine miRNA concentration.

Figure 2.1 Nanodrop spectrophotometry readings



The concentration and purity of (A) miRNA assessed using the NanoDrop1000® spectrophotometer with a constant value of 33 and (B) large RNA assessed using the NanoDrop1000® spectrophotometer with a constant value of 40

NanoDrop measurements were made as follows: The apparatus pedestal was cleaned and 1 µL nuclease free water was used to 'blank' the spectrophotometer. Then 1 µL of the RNA aliquot was carefully pipetted onto the pedestal. The sample arm was used to compress the sample, resulting in formation of a sample column which was held together by surface tension. Spectral measurements were made with a path-length of 0.1cm. The RNA concentrations were then automatically calculated using the following formula:

$$\text{RNA concentration (ng/µL)} = (A_{260} \times e)/b$$

A_{260}: Absorbance at 260nm
e: extinction coefficient (ng-cm/mL)
b: pathlength (cm)

2.5 Analysis of RNA integrity

The quality of the extracted RNA was assessed using an Agilent 2100 Bioanalyzer instrument (Agilent Technologies) with Agilent Small RNA kits to determine the miRNA component of extracted RNA and Agilent 6000 Nano kits to determine its integrity. This

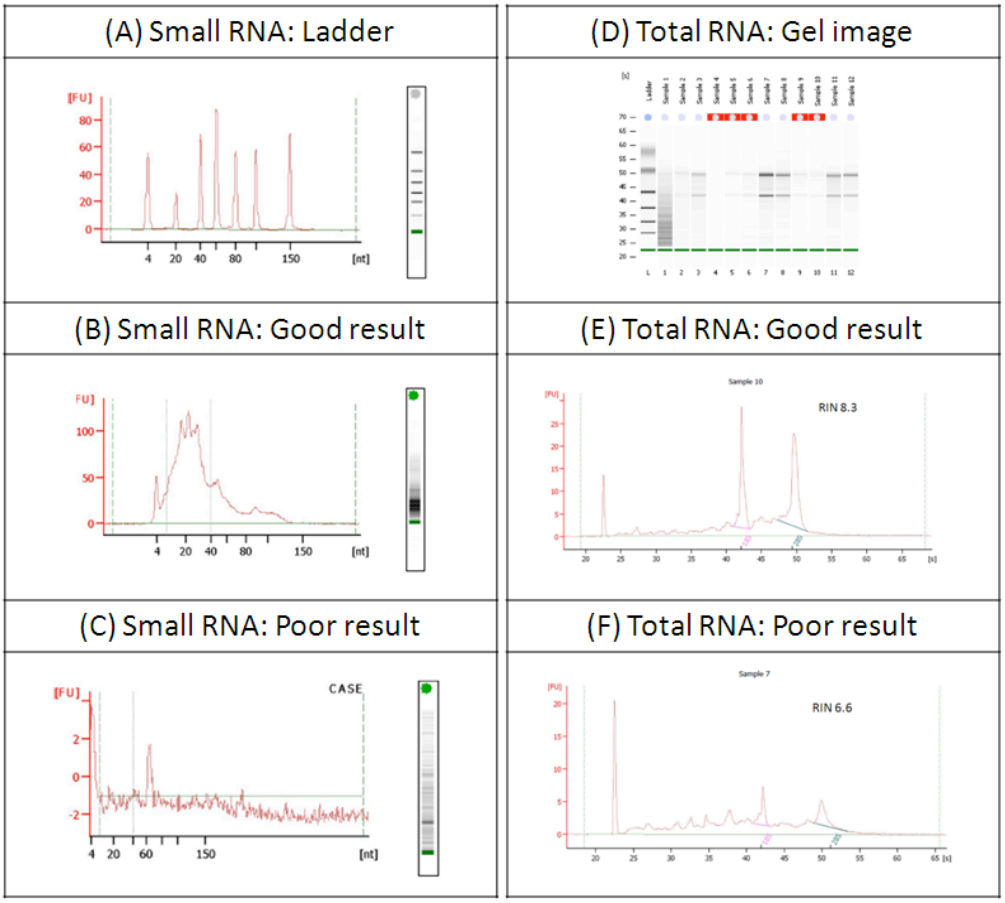
microfluidics-based platform was used for sizing, quantification and quality control of RNA molecules via visual representation of RNA integrity and ribosomal ratios. The assays enabled rapid and highly sensitive visualization and quantification of miRNA in total RNA extracts and were used as a quality control step in the development of miRNA purification protocols.

Agilent RNA chips contained a series of interconnected micro-channels that separated nucleic acid fragments based on their size (weight) as they were forced through it by electrophoresis. The separated fragments were subsequently detected using laser-induced fluorescence detection and the software generates an electropherogram and gel-like image, as well as displaying sample data such as RNA concentration. The chip contains sample wells, gel wells and a well for the ladder, the external standard. The micro-channels were constructed in glass to create interconnected networks among these wells. These channels were filled with a polymer and fluorescence dye during chip preparation, followed by the ladder and 11 samples in the wells. When all of the wells of the chip have been filled, an integrated electric circuit was created. This chip was then placed in the Agilent 2100 Bioanalyser. The 16 pin electrode provided one electrode for each well of the RNA Chip. The charged RNA molecules were separated based on their size, as small molecules travel faster than larger ones. The fluorescent dye was incorporated into the RNA strands, data was generated as these RNA-dye complexes are detected by laser-induced fluorescence. An RNA ladder was run on every chip as an external standard which acts as a reference for data analysis. The ladder contained 6 RNA fragments ranging in size, and the software compared the unknown RNA (sample) to the ladder fragments to deduce the ribosomal RNA peaks and the concentration of the unknown sample.

2.5.1 Agilent total RNA 6000 Nano kit

RNA degradation is a gradual process resulting in a decrease in the 18S to 28S ribosomal band ratio and an increase in the baseline signal between the two ribosomal peaks. The RIN software tool takes the entire electrophoretic trace into account and classifies eukaryotic total RNA, from 1 to 10, with 1 being the most degraded and 10 the most intact. The RIN removes user-dependant RNA quality interpretation and is independent of the amount of RNA used (once total RNA >50ng/µl) and the sample type. For miRNA profiling, using samples with a RIN number equal to or greater than 7 has been recommended (126).

Figure 2.2 Agilent small RNA assay



The integrity of extracted RNA was assessed using the Small (A, B, C) and Total (D, E, F) Agilent kits respectively. The Small RNA ladder is displayed in (A). With good and poor quality sample results in (B) and (C) respectively. The gel image from the large RNA kit is displayed in (D) where the ladder is visible on the left hand side. Good (RIN 8.3) and poor (RIN 6.6) quality samples are visible in (E) and (F), respectively.

Table 2.8 Agilent analysis of samples used in microarray

Sample Number	MiRNA Content (%)	Average Size (nt)	Spectrophotometry Conc. ng/µl
Cancer Group			
1	14	15	237
2	84	32.8	270.55
3	69	19	201.7
4	94	21	194.35
5	85	37	243.6
6	91	24	236.44
7	97	21	355.5
8	81	24	172.96
9	87	23	277.9
10	91	23	196.93
Control Group			
1	98	34.9	222.15
2	91	32.3	244.4
3	79	27	180.1
4	100	33.4	143.7
5	40	15	273.55
6	81	25	402.6
7	87	18	217.2
8	89	22	290.1
9	92	26	349.8
10	68	26	252.0

%; per cent, nt; nucleotide
This table shows the integrity and quantity of miRNA isolated from the blood samples included in the microarray experiment, assessed using Nanodrop spectrophotometry and the Agilent Bioanalyser NanoChip, respectively.

RNA 6000 chips were primed in accordance with the manufacturer's protocols, as outlined in the following: The gel was prepared by pipetting 550 µL of gel matrix into a spin filter and centrifuging at 1500g for 10 minutes at room temperature. The filtered gel was then aliquoted into 65 µL aliquots and stored in 0.5ml RNAase-free microfuge tubes. The gel-dye mix was then prepared. The dye concentrate was vortexed for 10 seconds and then spun down before 1 µL was pipetted into 65µL of filtered gel. This mixture was mixed by pipetting, followed by vortexing and then centrifugation at 13,000g for 10 minutes at room temperature. Next, a new Nano RNA Chip was placed on the chip priming station and 9 µL of the gel-dye mix is then loaded into the first gel well. The gel was encouraged to pass through the micro-channels as the chip priming station was closed for 30 seconds. The plunger was then released, and observed to have moved back beyond the 0.3mL mark.

After a 5 second interval the plunger was retrieved to the 1mL mark. Additional gel-dye mix was pipetted into the next gel wells to be filled. Then 5µL of RNA 6000 marker was pipetted into all 12 sample wells and the ladder well. The ladder is loaded, with 1 µL pipetted into the ladder well. 1 µL of each of the 12 samples was pipetted into each of the sample wells. The chip is placed in the adapter and vortexed for 1 minute at 2400rpm. The chip was placed on the Bioanalyzer instrument and run within 5 minutes. The electrode pins were cleaned with RNAase ZAP for 30 seconds, followed by RNAase-free water for one minute and left to air dry immediately prior to use. RNA samples (50-500 ng/µL) were prepared and heat-denatured at 70°C for 2 minutes, prior to loading in 1 µL volumes on Agilent chips.

2.5.2 Agilent small RNA kit

The small RNA assay was used for its ability to resolve high resolution in the 6-150 nucleotide range. This enabled confirmation of miRNA retrieval and comparison of the small RNA component between samples.

The Small RNA Assay was conducted in accordance with the manufacturer's guidelines, as summarised below. RNA samples (1-100 ng/µL) were prepared and heat-denatured together with 1 µL ladder, at 70°C for 2 minutes and stored on ice until ready for loading. The electrode pins were cleaned with RNAase ZAP for 30 seconds, followed by RNAase-free water for one minute and left to air dry immediately prior to use. The chip priming station was prepared by moving the chip to the lowest position. The electrode pins were cleaned with RNAase free water for 5 minutes prior to use. The reagents were allowed to reach room temperature over 30 minutes during which time they were protected from light.

The gel was prepared by pipetting the complete volume (approximately 650 µL) of the gel matrix into a spin filter and centrifuging at 10,000g for 15 minutes. The gel-dye mix was prepared carefully pipetting 2 µL of dye into 40 µL of filtered gel followed by centrifugation at 13,000g for 10 minutes at room temperature. Next, a new Small RNA chip was placed on the chip priming station and primed with the addition of 9 µL of the gel-dye mix into the first gel well. The gel was encouraged to pass through the micro-channels as the chip priming station was closed for 60 seconds. The plunger was released, and observed to have moved back beyond the 0.3 mL mark. After a 5 second interval the plunger was retrieved to the 1mL mark.

The following components were loaded into their respective wells: conditioning solution (9 µL); marker (5 µL) and ladder (1 µL). RNA samples were added to the chip in 1 µL volumes (11 in total). The chip was placed in its adapter and vortexed for 1 minute prior to loading onto the Bioanalyzer instrument and initiating the run.

2.6 Reverse transcription of miRNA to complementary cDNA

RNA cannot be amplified by PCR in its native state due to the presence of uracil (U) bases. To circumvent this, samples are reverse transcribed whereby retroviral reverse transcriptase is used to generate a complimentary cDNA template, which can be amplified by PCR. Due to their short lengths (18-22 nucleotides) miRNAs have presented a significant technical challenge with regard to sensitive and specific quantification. The introduction of stem-loop primers for reverse-transcription has greatly improved RT efficiency and specificity due to the base stacking of the stem which enhances the thermal stability of the RNA–DNA heteroduplex. Furthermore, spatial constraint of the stem–loop is believed to improve the assay specificity in comparison to conventional linear RT primers.

Figure 2.3 Specificity of TaqMan stem-loop RT primers (adapted from Chen *et al* (127)).

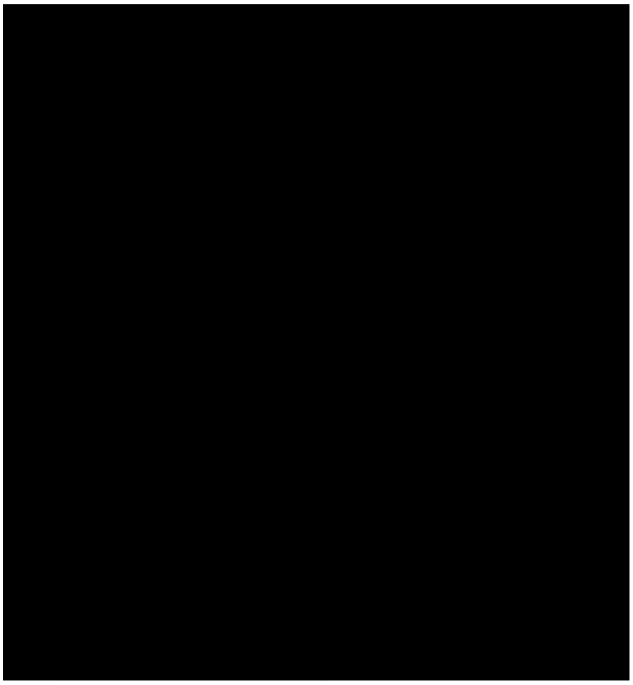


Table 2.9 Sequence-specific stem-loop RT primers

miRNA	Stem-loop primer sequence	Length
miR-21	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA	50 nt
miR-16	GTCTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA	49 nt
miR-26b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCTAT	50 nt

Where sequences were available as above, primers were synthesised by Eurofins MWG Operon (Germany), otherwise assays containing miRNA-specific stem-loop primers were purchased from Life Technologies.

To generate first strand cDNA, RNA samples (typically 100 ng from blood, 5 ng from tissue) were reverse-transcribed using High Capacity cDNA Reverse Transcription kits (Life Technologies). Starting quantities of miRNA were diluted as necessary based on concentration to achieve final volumes of 5µL.

Table 2.10 MiRNA dilutions for reverse transcription

Shire Number	Origin	miRNA Conc. @ 33	Dilution	Dilution Factor	ng/µl	Volume 1ng	Volume 100ng	Volume Water
R11-0767	Blood	88.76	None	1	88.76	0.0113	1.13	3.87
R11-0133	Blood	180.62	1+1	2	90.31	0.0110	1.11	3.89
R11-0505	Blood	205.5	1+2	3	68.52	0.0146	1.46	3.54
R11-1331	Tissue	443.16	1+99	100	4.43	0.2257	1.13	3.87
R11-1590	Tissue	754.13	1+99	100	7.54	0.1326	0.66	4.34

RNA was diluted to achieve a workable concentration such that final volumes would not exceed 5 µl. Typically a total of 100 ng miRNA are used for cDNA synthesis originating from blood, while 5 ng of miRNA from tissue are added.

Individual miRNAs were reverse transcribed using the following reagents (Appendix 1.3).

Reagent	Volume Per Sample
miRNA (100ng blood/5ng tissue)	5.0 µL
dNTP mix (100mM)	0.17 µL
10X RT Buffer	1.65 µL
Nuclease Free Water	4.57 µL
RNase inhibitor (20U/ µL)	0.21 µL
Stem loop primer (50nM)	3.1 µL
MultiScribe RT (50U/ µL)	1.1 µL

Samples were then incubated on an AB7900 GeneAmp thermal cycler (Life Technologies). Each batch of reactions included a negative control which contained all reaction components except miRNA. The incubation proceeded as follows:

Temperature	Duration (minutes)
16 °C	30
42°C	30
85°C	5
4°C	Indefinitely

2.7 Real-time quantitative polymerase chain reaction (RQ-PCR)

Real-time polymerase chain reaction is used to simultaneously amplify and quantify targeted cDNA molecules in real time, as the reaction is progressing. Repeated cycles of heating and cooling (thermal cycling) using specific primers and Taq polymerases results in the exponential amplification of target sequences. In this study TaqMan PCR was used, an approach that harnesses the 5'-3' exonuclease activity of Taq polymerase to cleave dual-labelled 'TaqMan' probes. This cleavage releases fluorescence the accumulation of which is measured during the exponential phase of PCR reaction, typically cycles 15-25. As the reaction progresses, the cDNA generated as a result of the PCR reaction is used as a template for replication in subsequent cycles, hence the chain reaction.

Typically, PCR reactions consist of 40 thermal cycles. In this study the following PCR cycling conditions were used throughout:

1. Denaturation: Reactions were heated to 95°C for 15 seconds to induce melting of the cDNA/RNA template, yielding single stranded molecules.
2. Annealing: Reactions were cooled to 60°C for 60 seconds, enabling the sequence-specific primers to anneal to the now-single stranded cDNA template. DNA synthesis was then initiated by Taq polymerase bound to the primer-template structure.
3. Elongation: Taq polymerase synthesised a new DNA strand complementary to the DNA template by adding dNTPs in the 5' to the 3' direction.

There are four reaction phases of PCR:

1. Baseline: the initial cycles during which there is little change in fluorescence signal despite the theoretical doubling of PCR product

2. Exponential phase: during which the amount of amplified product doubles (assuming 100% efficiency) during each cycle.
3. Linear phase: the reaction rate reduces as the reagents are consumed and the enzymes lose their activity.
4. Plateau phase: the reagents and enzymes have been completely exhausted. The reaction has stopped.

Figure 2.4 Phases of PCR

The phases of PCR for a positive sample (blue) and a negative sample (green)[17].

When the amplified product has accumulated enough to reach a detectable fluorescent signal this is known as the threshold cycle (Ct). The greater the amount of starting template the lower the threshold cycle. Analysis of cycle threshold can be used to estimate gene expression levels by comparison of Ct values to either a standard curve (absolute quantification) or to an endogenous control (relative quantification). The latter technique is the most widely used approach, and was used in these studies.

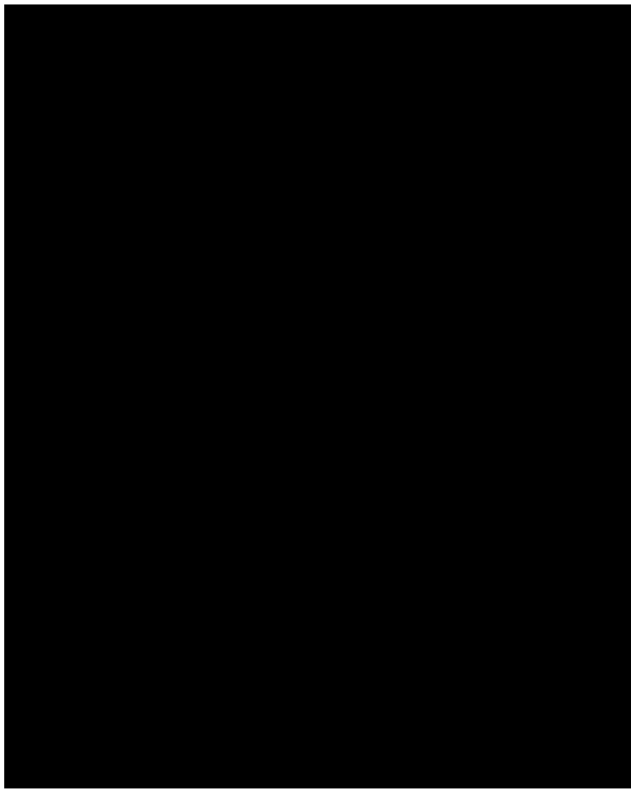
[17] https://www.abbottmolecular.com/us/technologies/real-time-pcr/maxratio-data-analysis.html

Table 2.11 RQ-PCR terminology

Term	Definition
Amplicon	The short segment of DNA generated by the PCR process.
Calibrator	A sample used as the basis for comparative expression analysis.
Cycle Threshold (C_T)	The fractional cycle number at which the fluorescence generated within a reaction crosses the threshold for detection.
Endogenous Control	A miRNA or snoRNA contained in a sample/expressed in a sample that is used to normalise target miRNA quantities for relative quantification.
No Template Control (NTC)	PCR wells containing all the PCR components except the RNA (substitute water). If the NTC generates a signal, it might indicate that the RT or PCR reagents were contaminated with RNA.
Target miRNA	A miRNA of interest
TaqMan Probes	TaqMan probes are a hydrolysis probe that depend on the 5'- 3' endonuclease activity of Taq polymerase to cleave a dual fluorescently labelled probe (FAM™- labelled MGB) during hybridization to the complementary target sequence. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher component coupled to the 3' end. TaqMan probes hybridize to a specific internal region of a PCR product. In the unhybridized state, the proximity of the covalently attached dye and quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the Taq polymerase cleaves the 5' end of probe (Figure 2.5 below). This decouples the fluorescent and quenching dyes and Fluorescence Resonance Energy Transfer (FRET) no longer occurs. Fluorescence subsequently increases in each cycle, proportional to the amount of probe cleavage that occurs.[18]

[18] http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/ProjTaqMan.shtml

Figure 2.5 TaqMan hydrolysis probes



MiRNA-specific cDNA was quantified by RQ-PCR using TaqMan primers (forward and reverse) and probes (Life Technologies) as illustrated above. During the reaction, TaqMan probes anneal to their complementary sequence between the forward and reverse primers. Taq polymerase moves along the DNA strand, cleaving the TaqMan probe and releasing its fluorescence. In this way, only probes which are annealed to the target sequence are cleaved and fluoresce and strictly only specific amplification is detected[19].

RQ-PCR reactions were conducted in final volumes of 10µL, using an AB7900HT instrument (Life Technologies). MiRNA specific cDNA was synthesised as described above. The reactions were prepared on Fast 96 well plates (Life Technologies). Where sequences were available, primers and probes were obtained from Eurofins MWG Operon (Germany). For these miRNAs, reaction wells consisted of the following:

Reagent	**Volume per sample**
First strand miRNA-specific cDNA	0.7µL
TaqMan Fast Master Mix (2x)	5.0 µL
TaqMan probe	0.5 µL
Forward Primer (1.5µM)	1.5 µL
Reverse Primer (0.7µM)	0.7 µL
Nuclease free water	1.68 µL

[19] http://upload.wikimedia.org/wikipedia/en/0/07/Taqman.png

Otherwise assays containing miRNA-specific stem-loop primers and probes were purchased from Life Technologies, requiring the following reaction:

Reagent	**Volume per sample**
TaqMan Fast Master Mix (2x)	5.0 µL
miRNA PDAR	0.5 µL
Nuclease free water	3.8 µL

Standardised 'Fast' thermal cycling conditions were applied, consisting of 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. All reactions were performed in triplicate. RT-PCR plate templates were used for experimental set-up, as illustrated in Appendix 1.4.

2.7.1 Accuracy of Results

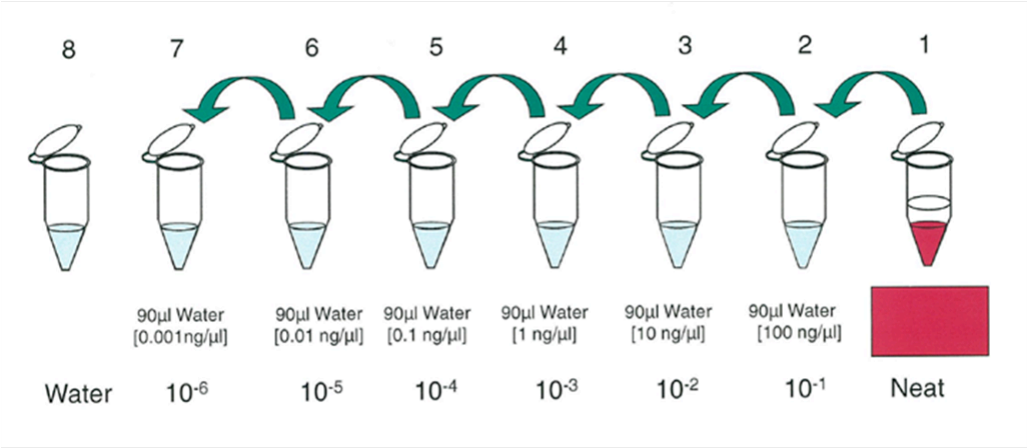
Accuracy of RQ-PCR results on individual runs was ensured by the following measures:

- *MiR-26b* cDNA synthesised from colorectal tumour tissue served as an inter-assay control and calibrator and was included on each 96 well PCR plate. A threshold of 0.28 standard deviations was set for intra- and inter-assay replicates.
- The standard deviation of C_T values of triplicate samples was <0.28 for each miRNA target in each sample. If the standard deviation was equal to or greater than 0.28, the accuracy of the data was questioned and the run was repeated until such accuracy was achieved.
- PCR plates frequently included samples of mixed groupings, for example cancers mixed with controls, or various time-points such as diagnosis on the same plate as pre- and post-neoadjuvant chemotherapy samples.
- Pipetting was performed accurately with regularly calibrated pipettes using barrier pipette tips. Very low volume pipetting (<0.6µl) was avoided where possible with dilutions made where necessary. Sealed plates were centrifuged at 2000 g prior to the PCR run.
- PCR plates were prepared in specifically designed Class II safety cabinets.

2.8 PCR amplification efficiencies

As outlined above, reactions with optimised conditions the exponential phase should signify doubling of the amplification product during each cycle, i.e. the amplification efficiency should approach 100%. Amplification efficiencies of the RQ-PCR miRNA assays were determined using 10-fold serial dilutions (neat to 10^{-6}) of cDNA (Figure 2.6), which were amplified using the same conditions as outlined in Section 2.7 above.

Figure 2.6 Dilution series for PCR efficiency cDNA



Six 10-fold serial dilutions are generated from the neat cDNA sample (on right). Ten micro litres of neat cDNA are added to 90 µL NFW. This mixture is thoroughly mixed by pipetting before 10 µL is added to the next 90 µL NFW. This process continues as illustrated above until a concentration of 10^{-6} is achieved.

Dilution curves were then produced by plotting Ct (on the y-axis) against the dilution factor (on the x-axis). The slope of the curve was then used to calculate the amplification efficiency (E) for each RQ-PCR miRNA assay as follows:

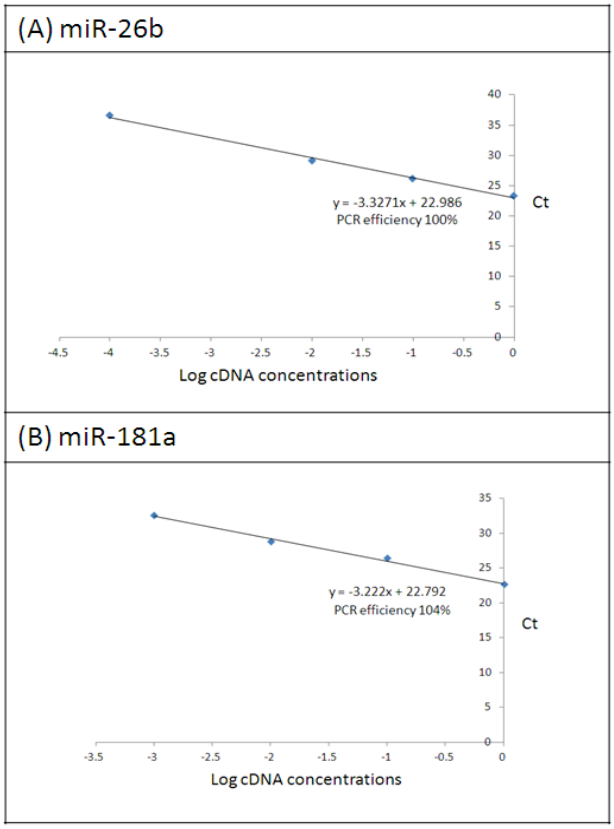
$$E = (10^{-1/slope} - 1) \times 100$$

Slope = that of the dilution curve

Amplification efficiencies of between 90-110% are required for robust, reproducible and reliable RQ-PCR assays. All assays used met this cut-off. Calculating the amplification efficiency has important implications on calculating miRNA expression levels. At 100% efficiency there is exact doubling of the amount of DNA at each cycle and Ct values of the dilution series are 3.3 cycles apart, while at 90% efficiency the amount of cDNA increases from 1 to 1.9 at each cycle, similarly for 80% and 70% it will be 1.8 and 1.7 respectively. A minor difference in efficiency results in a large difference in the amount of final RQ-PCR

product as this efficiency is applied in converting the raw Ct value to the relative expression value, as outlined in the equation below. Calculating the accurate amplification efficiency is important.

Figure 2.7 Dilution curves used to calculate PCR efficiencies



PCR amplification efficiencies were calculated for each candidate miRNA and *U6* RQ-PCR assay using the formula $E = (10^{-1/slope} - 1) \times 100$, where the slope is of the plot of Ct versus the log input of cDNA concentration. This figure displays dilutions curves for *miR-26b* (A) and *miR-181a* (B) with PCR efficiencies of 100% and 104%, respectively.

Table 2.12 PCR efficiencies for target miRNAs and *U6*

Target	PCR efficiency (%)
U6	90
miR-15a	100
miR-15b	105
miR-16	98
miR-19b	96
miR-26b	100
miR-29a	98
miR-93	107
miR-181a	104
miR-182	100
miR-195	103
miR-223	102
miR-301a	108
miR-423-5p	97
miR-424	104
miR-497	100
miR-652	110

2.9 Endogenous controls

Reliable RQ-PCR data is dependent on the ability to correct for variation between reactions. Variations may be introduced during any step from sample preparation through to target amplification. Normalising RQ-PCR data to an endogenous control (EC) is the most accurate method of correcting for technical or biological sources of variation. Such variation can be introduced as a consequence of differences in sample collection, RNA extraction, RNA quality, enzymatic efficiencies or even sample to sample inconsistencies in cellular subpopulations. The accuracy of RQ-PCR gene expression analysis is critically dependent on accurate normalisation of the data. Incorrect and inappropriate conclusions can be drawn from erroneous normalisation of the data (92, 128, 129). Reliable ECs exhibit the following characteristics; they are ideally stably expressed across the entire sample cohort, are expressed along with the targets of interest in the sample cohort, and demonstrate equivalent storage stability, extraction and quantification efficiency as the targets of interest (130). Few ECs that fulfil these criteria are known. To date, a reliable stably expressed EC has yet to be identified across all tissue types, or in the circulation of those across all disease states. In breast cancer studies, reliable ECs should be unaffected by factors such as grade and hormone receptor status, as well as being stable in benign breast disease or control cases.

Several ECs have been evaluated for quantification of mRNA expression in several disease states, but there is lack of similar studies reporting validated EC genes for miRNA expression analysis. *MRPL19* and *PPIA* are validated EC genes for mRNA expression analysis in breast tissue (131). *U6* is frequently used as a normaliser for RQ-PCR expression analysis. However, the application of small nucleolar RNAs for miRNA expression determination is controversial, as RNA extraction efficiency, reverse transcription and amplification by PCR may be affected by longer RNA strands (132).

The Discipline of Surgery, NUI Galway, published the first report of reliable ECs for miRNA expression analysis for breast tissue, advocating a combination of *miR-16* and *let-7a* (92). At the time of writing, there was no consensus in the scientific community on the most suitable EC(s) for circulating miRNA expression analysis in breast cancer. However, *miR-16* has been shown to be stably and substantially expressed in numerous tissue types, both in healthy individuals and across those with a variety of diseases (132). The expression of *miR-16* in the circulation has also been demonstrated to be relatively stable, both in benign disease processes and malignancy (116, 133, 134). These studies suggest that circulating *miR-16* is the most reliable EC in this context to date. It was used to normalise the initial studies.

2.10 Relative quantification

To precisely and reliably determine gene expression values, raw fluorescence data generated at the end of the fast PCR run by the PCR instrument (AB7900HT, Applied Biosystems) were converted to Ct values by SDS RQ Manager Software (Applied Biosystems). This data was then imported to qBase PLUS Software (Biogazelle) which was used to scale raw data to an internally defined calibrator (Cal) and an EC, such that the following formula could be applied to generate relative quantities (135):

$$\Delta Ct = \text{Average Ct of test sample} - \text{Average Ct of calibrator}$$

The ΔCt values were then converted to a linear format using the equation:

$$E^{-\Delta Ct}$$

E=Amplification Efficiency

MiR-16 was used as an endogenous control gene to normalise the RQ expression data to correct from non-biological variation in gene expression potentially introduced during the RQ-PCR process. Then, to reliably determine the expression of a target miRNA relative to the expression gene, the comparative Ct (ΔΔCt) method (136) was used as follows:

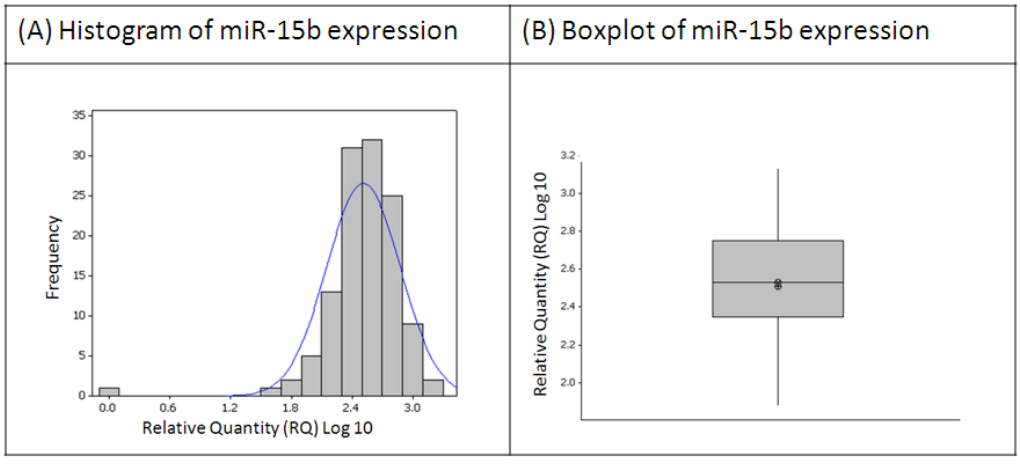
$$\Delta\Delta Ct = \underbrace{(Ct\ \text{Target gene})-(Ct\ EC)}_{\text{Test Sample}} - \underbrace{(Ct\ \text{Target gene})-(Ct\ EC)}_{\text{Calibrator}}$$

The ΔΔCt values were converted to a linear format, using $E^{-\Delta\Delta Ct}$.

2.11 Statistical analysis of PCR data

Statistical analysis was performed using Minitab V16.0 (Minitab Ltd, Coventry UK). The Kolmogorov-Smirnov test for normality was conducted and normal distribution of data was also assessed using histograms and boxplots. A log transformation (log_{10}) of the data was performed for statistical analysis. Normal distribution of data following log transformation is displayed in Figure 2.8.

Figure 2.8 Distribution of PCR data



The data is assessed for normality of distribution following log transformation in advance of statistical analysis. Expression data of *miR-15b* is displayed above, with a histogram displaying the normal distribution (A) and a boxplot (B) showing the mean, median (bullets) and interquartile range (grey box).

Parametric tests were used for normally distributed data, where appropriate. One way ANOVA and independent t-tests were used to compare mean RQ miRNA expression of independent samples while paired t-test was utilized to assess miRNA expression across related samples. Mann-Whitney U tests were employed to assess significance of miRNA data across groups in non-parametric datasets, as appropriate. Correlation analysis using Pearson's correlation coefficient was performed. Results with p values less than 0.05 were considered significant. Binary logistic regression was utilized and receiver operator characteristic (ROC) curves were generated to determine sensitivity and specificity of miRNA expression levels in distinguishing between cancer and control groups. The AUC, a surrogate marker for sensitivity and specificity, was then calculated. This was performed uni-variately for single miRNAs and multi-variately for target miRNAs in combination.

2.11.1 Visual presentation of miRNA expression data

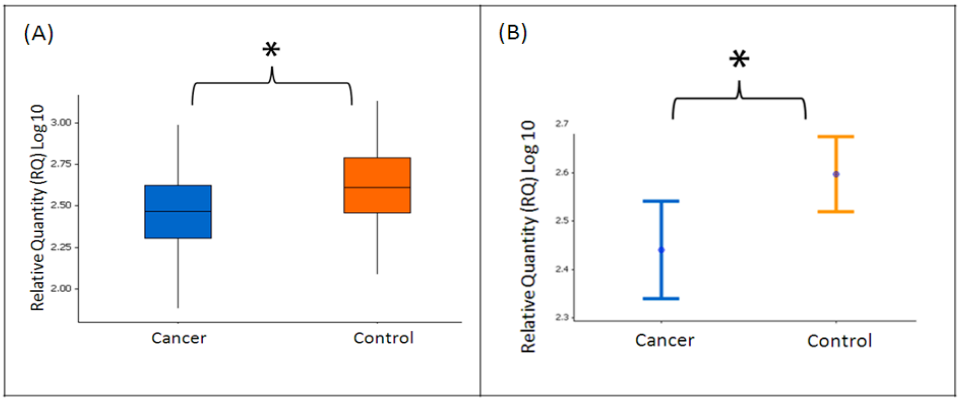
There are a variety of graphs that can be used to visually represent miRNA expression data. MiRNA studies to date have predominantly used boxplots, which depict groups of numerical data (ie. expression data) through their median and quartiles (upper and lower). Boxplots frequently have single lines extending from the box indicating variability outside the quartiles. These graphs display differences in the range and distribution between populations (cancer and control, for example). The degree of dispersion can be appreciated by the shape or spread of the plot. Data is more dispersed in long, thin rectangular shaped boxes.

Interval plots are another method of visually depicting miRNA expression data. Interval plots present the sample mean, and 95% confidence interval. Data from the sample cohort is used to estimate for the entire population, for example all women with breast cancer. The confidence interval represents precision; a 95% confidence interval means that 95% of the time the 'true' population mean would lie within this interval, and the remaining 5% of time the population mean would lie outside the interval.

Interval plots were selected as the graph of choice as they give information about the differences in means, which is what we most commonly report on in miRNA expression studies (t-tests). As such, it is the graph that most accurately reflects the statistical test used. These plots provide more detailed information about the differences between groups and the significance of any such differences. If the confidence intervals between two

groups do not overlap, then there is definitely a statistically significant difference between the groups.

Figure 2.9 Data presentation in boxplots and interval plots



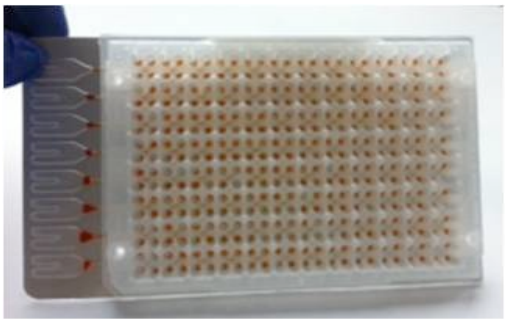
Circulating *miR-15b* expression data in breast cancer (blue, n=67) and control (orange, n=57) groups is presented here by boxplot (A) and interval plot (B). There is a statistically significant difference (t-test, p=0.013) in *miR-15b* expression between the cancer and the control group, which can be more readily appreciated by interval plot.

2.12 TaqMan Low Density Array (TLDA)

For miRNA profiling experiments TaqMan Low Density Arrays (Life Technologies) were used. This high through-put platform consisting of 384 TaqMan miRNA assays spotted onto microfluidic cards. Two of these cards were available for human miRNA profiling: Card A (Figure 2.10) and Card B, representing a total in excess of 667 miRNAs. Each card contained mammalian *U6* assay repeated 4 times for potential data normalization, and an assay unrelated to mammalian species *ath-miR-159a* as a process control.

Figure 2.10 Human TLDA

(A) Image of Human TLDA array Card A



(B) Human TLDA array map

2.12.1 Megaplex reverse transcription

Total RNA (700ng) was reverse transcribed to cDNA using sets of pooled stem-looped RT primers corresponding to the TLDA array cards. In this study pools A and B (Life Technologies) were used, each of which contained 380 specific miRNA RT primers. Reaction mixes (7.5 µL in total) were prepared by the addition of the following reagents:

RT reaction components	**Volume per sample (µL)**
Megaplex RT primers (10x)	0.8
dNTPs with dTTP (100nM)	0.2
MultiScribe reverse transcriptase (50U/µL)	1.5
10x RT Buffer	0.8
$MgCl_2$ (25nM)	0.9
RNase Inhibitor (20U/µL)	0.1
Nuclease Free Water	0.2
Total RNA (700ng)	3.0
Total Volume	7.5

Tubes were sealed and inverted six times, before brief centrifugation. The 7.5 µl reaction mixture was incubated on ice for 5 minutes before thermal-cycling conditions as outlined in Table 2.13, below, on the 7900HT Gene Amp (Life Technologies). The cDNA was stored at -20°C and used in subsequent experiments within one week.

Table 2.13 Thermal cycling conditions

Stage	**Temperature**	**Time**
Cycle (40 cycles)	16°C	2 min
	42°C	1 min
	50°C	1 sec
Hold	85°C	5 min
Hold	4°C	Indefinitely

2.12.2 TLDA RQ-PCR

MiRNA profiling and quantification was performed with TaqMan TLDA array cards A arrays by the addition of the following reagents to 2µL tubes:

Component	**Volume per array (µL)**
TaqMan Universal PCR Master Mix, No AmpErase® UNG, 2x	450
Megaplex™ RT product	6
Nuclease free water	444
Total	900

The mixture was carefully mixed by pipetting, gentle vortexing, and brief centrifugation to eliminate bubbles. The TLDA cards were equilibrated to room temperature before loading 100 µL aliquots of PCR mix to each of the 8 fill ports on the left arm of each reservoir. Cards were centrifuged at 1200 rpm, with an "up-ramp" rate of 3 for 2 minutes for two separate one minute intervals facilitate equal dispersion of the sample throughout the wells on the microfluidic card. The TaqMan array cards were examined to ensure filling was complete before sealing and placing in the 7900 HT instrument (Life Technologies) for thermal cycling.

2.12.3 Array data processing

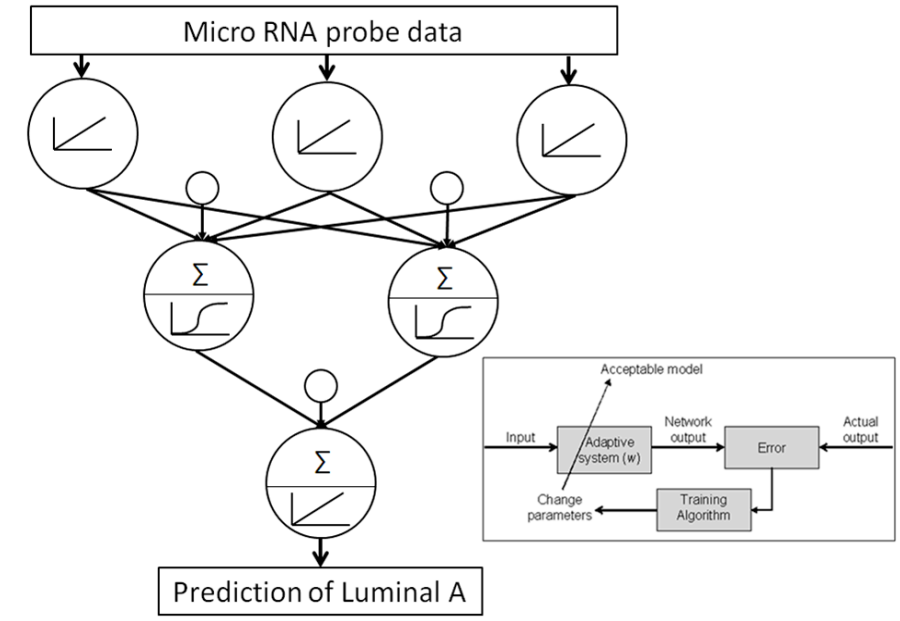
The raw fluorescence data generated at the end of the PCR run by the PCR instrument were converted to Ct values using SDS RQ Manager Software. The data was exported as a flat tab-delimited file for further analysis with qBase PLUS software, to determine the relative expression of the target miRNAs compared to *U6*, the pre-selected endogenous control on the TLDA array cards.

2.12.4 Bioinformatics analysis of miRNA microarray: Artificial Neural Network-based data mining to identify predictive miRNAs

Within this study normalized miRNA array data were analyzed within a nonlinear ANN based data mining algorithm to identify those with altered expression in Luminal A breast cancer. This method comprised a feed-forward back-propagation algorithm utilizing a three layer architecture, a sigmoid transfer function, 2 hidden nodes and early stopping on unseen data full details are described by Lancashire *et al* (137) . Monte Carlo Cross validation was applied to the modeling approach to determine the performance of the miRNA probes on a randomly selected blind subset. This approach addressed issues with false discovery by preventing over fitting, driving the solution to one that has good predictability for a blind population.

The performance of single miRNA probes was determined by developing ANN models using the algorithm described above (each using a single probe intensity from the data), to classify between Luminal A breast cancer and healthy controls (Figure 2.11). This process was repeated for all of the probes on the array and their classification performance on blind data determined. In this way a rank order of miRNAs was determined. From this rank order the key miRNAs were taken forward for validation.

Figure 2.11 Artificial neural networking (ANN)



ANN architecture and algorithm as applied to data mining for miRNA markers of breast cancer.

2.13 In situ hybridisation of miRNA

In order to localise miRNA expression *in vivo* and confirm miRNA expression levels in breast tumours, *in situ* hybridisation (ISH) analysis was performed for *miR-195* and *miR-497* on formalin fixed, paraffin embedded (FFPE) sections of breast tumours using locked nucleic acid (LNA) probes (Exiqon). The breast tumour sections were reviewed by a histopathologist to confirm presence of tumour tissue. The clinicopathological parameters of the patients included in this study are presented in Table 2.7.

2.13.1 Cutting FFPE sections

Before handling the tissue sections, the entire workstation was cleansed with RNase Zap. The FFPE blocks were placed on ice. Slides were prepared and labelled. Two water baths were prepared; one standard water bath with ddH_20 (37°C) and another RNase free Ziehl Neelson jar with RNase free water (room temperature).

When cutting the FFPE sections, new disposable blades were used for each block. The cutting angle was set to 15°. After trimming the outer sections from the block 6 µm sections were cut and placed into the sterile Ziehl-Neehlson jar with room temperature

sterilized ddH_20, to allow any folding to be reversed. The slides were transferred to the heated water bath, where folds were stretched out before being mounted immediately on electrostatic treated slides (Superfrost Plus®, VWR International). The slides were allowed to dry at room temperature for 2 hours prior to storage at 4°C.

2.13.2 LNA probes

DNA oligonucleotides with 30 to 42% locked nucleic acid (LNA) substitutions (138) for *miR-195* and *miR-497* (Exiqon) were utilised. A probe specific for *miR-126-3p* was used as a positive control and a 21-mer scrambled probe with a randomly generated sequence for which there is no known complementary human sequence target was included as a negative control. All LNA-oligos for this study were double-FAM labelled, meaning FAM was attached at both the 5' and 3' end.

2.13.3 ISH analysis

A chromogenic ISH assay was utilised for miRNA detection. Chromogenic ISH takes advantage of a chromogenic precipitate generated during the enzymatic step based on Alkaline Phosphatase (AP) that converts soluble 4-nitroblue tetrazolium (NBT) and 5-brom-4-chloro-3'-indolylphosphate (BCIP) into a water and alcohol insoluble dark blue NBT-BCIP formazan precipitate. Slides are often counter stained with nuclear fast red (NFR) that gives a good contrast to the blue ISH signal when using bright field microscopy. Chromogenic ISH is a well-described and sensitive procedure, suited to miRNA studies as it allows assessment of the ISH signal and tissue morphology simultaneously.

FFPE sections (6 µm) were deparaffinised by placing the slides in xylene for 15 minutes, and then hydrated through five minute incubations in ethanol solutions (99% 3 coplin jars, 96% 2 coplin jars, 70% 2 coplin jars) ending up in PBS (2 coplin jars) before being mounted onto flow through slide chambers and placed in an automated hybridization instrument (Tecan Freedom Evo, Tecan).

In the Tecan instrument, the slides were pre-digested by treatment with proteinase K reagent (15µg/ml) and incubated at 37°C for 8 minutes. Prehybridization was performed with the addition of 50 µl Exiqon hybridization buffer (Exiqon) and incubated at 62°C for 15 minutes. This was followed by addition of the double-FAM LNA probe diluted in Exiqon hybridization buffer. The slides were stringently washed with saline sodium citrate (SSC)

buffers: 5x SCC, 1x SCC and 0.2x SCC at 62°C over 33 minutes. A blocking step was performed with the addition of blocking solution to each slide and incubated for 15 minutes at room temperature. Alkaline phosphatase-conjugated anti-FAM (prepared by diluting in blocking solution) was added and incubated at 30°C for 30 minutes. Enzymatic development was performed with the addition of NBT and BCIP substrate (Roche) at 30°C for 60 minutes. An optional step was the application of nuclear fast red counterstain (Vector Laboratories). Between 200 and 300 µl of nuclear red counterstain was added to slides undergoing this step and incubated at 25°C for 1 minute.

Finally, slides were placed in staining racks and rinsed in tap water for 10 minutes before undergoing a series of one minute dehydration washes in ethanol: 70% ethanol (2 coplin jars), 96% ethanol (2 coplin jars) and 99% ethanol (2 coplin jars). Slides were mounted (Eukitt mounting medium, VWR), and allowed to settle overnight before analysis with light microscopy the following day.

2.14 Cell Culture

2.14.1 Overview

Cell culture refers to the maintenance and culture of immortalised cells *in vitro*. Immortalised cell lines are populations of cells grown under controlled conditions that are capable of unlimited population doublings. Such 'immortalised' cell lines retain their ability to proliferate and divide indefinitely through a transformation event. This event may occur spontaneously *in vivo* by natural somatic mutation or *in vitro* by chemical or viral induction.

2.14.2 Cell lines

The cell lines used in this study were obtained from the American Type Culture Collection (ATCC). Their characteristics are outlined in Table 2.14.

Table 2.14 Breast cancer cell lines

Characteristic	MCF-10-2A	MCF-7	ZR-75-1	T47D	MDA-MB-231	SKBR3
Morphology	Epithelial	Epithelial	Epithelial	Epithelial	Epithelial	Epithelial
Disease	Fibrocystic disease	Adenocarcinoma	Ductal Carcinoma	Ductal Carcinoma	Adenocarcinoma	Ductal Carcinoma
Receptors Expressed	EGFR, IR, GR	ER	ER, PR	ER, PR, AR, GR, PrR	EGFR	HER2/*neu*, EGFR
Growth	Adherent	Adherent	Adherent	Adherent	Adherent	Adherent
Media Used	DMEM	DMEM	RPMI 1640	RPMI 1640	Leibowitz L15	McCoys 5A

AR: Androgen receptor, DMEM: Dulbecco's modified Eagle's medium, EGFR: Epidermal growth factor receptor, ER: Oestrogen receptor, GR: Glucocorticoid receptor, HER2/*neu*: Human epidermal growth factor receptor, IR: Insulin receptor, PR: Progesterone receptor, PrR: Prolactin receptor, RPMI: Roswell park memorial institute

2.14.3 Asepsis in cell culture

All experimentation for cell culture was carried out in Class II laminar air flow (LAF) hoods (Haraeus) in a designated tissue culture facility. Strict aseptic techniques were observed for all cell culture procedures with surfaces and equipment sprayed with 70% industrial methylated spirit (IMS) both before and after use. Workspaces within LAF hoods were arranged such that clean equipment was situated on the right, and soiled equipment including waste on the left. In addition, these hoods were cleaned rigorously after use.

2.14.4 Cell culture

Cells were cultured in tissue culture flasks (Sarstedt) in a high efficiency particulate air (HEPA Class 100) incubator (Thermo Electron Corporation) at 37°C and 5% CO_2. Sterile water baths provided humidity to the environment. These water baths were changed on a weekly basis. All biological waste was disposed of in an appropriate manner following treatment with 1-3% Virkon (Antec). The characteristics of the cell lines for this study are summarised in Table 2.14. The media used was specific to each cell type; DMEM was used for the MCF-10-2A and MCF-7 cell lines, RPMI 1640 was used for ZR-75-1 and T47D cell lines, MDA-MB-231 cells were cultured in Leibovitz media while McCoys 5A was used for the SK-BR-3 cell line. Media for each cell line was supplemented with 10% FBS, 100 IU/mL penicillin G and 100 mg/mL streptomycin sulphate (Pen/Strep) and 1% L-Glutamine. Cells were maintained at 37°C, 5% CO_2 and a media change three times per week. Cells were typically harvested after 5 days.

2.14.5 Feeding

A daily inspection was performed both macroscopically and microscopically, to assess pH shifts and cell viability respectively. Media was typically replenished every second day. Media was pre-heated to a temperature of 37°C over 10 to 15 minutes in advance of being delivered to the cells. Cell feeding was performed in the Class II LAF hood. Pre-existing media is removed and new media is added to the side of the flask that is opposite the cell monolayer. Flasks are promptly returned to the incubator at 37°C. 8mL of media was used for T-25cm^2 flasks.

2.14.6 Sub-culturing and/or harvesting

When cells reached approximately 80-90% confluency or when required for experimental purposes they were sub-cultured. It was important to perform this step before cells reach 100% confluency as this maintains cells in the active log phase of growth, precluding senescence. Media was preheated, with phosphate buffered saline (PBS, calcium and magnesium free, Lonza) and Trypsin/EDTA (0.25%, Lonza). The cell surface was rinsed with PBS once the media was removed. Trypsin (5 mL) was added to the cell monolayer after washing with 5 mL PBS. Incubation over 1 minute at room temperature was permitted after which time the excess trypsin was removed by pouring. The flasks were incubated at 37°C for between three and five minutes to ensure cell dissociation. To ensure cells were no longer adherent they were inspected under the microscope. Resuspension of cells and deactivation of trypsin was achieved by adding fresh media (5.5 mL) followed by gentle pipetting to encourage separation of cell clumps. The single cell suspension was counted harvested for experimental purposes or reseeded in accordance with experimental requirements.

2.14.7 Cell counting

Cell counting was performed using the automated Nucleocounter® (Chemometec). Cell viability is determined on the principle that viable and non-viable cells have a difference in plasma membrane permeability. Propidium iodide (PI), a fluorescent dye, is exploited to this end. Viable cells exclude this compound; non-viable cells have permeable membranes thus permitting PI to cross into the cell and form a complex with cell DNA. If the aim was to measure total cell concentration, and not just to assess cell viability, cell plasma membranes were disrupted by Lysis Buffer Reagent (Reagent A100, Chemometec) followed by a stabilising buffer (Reagent B, Chemometec). The cells were loaded into the

NucleoCassette, containing immobilised propidium iodide. The number of cells per mL was counted. Cell viability is determined from measuring both the total and the non-viable cell count. The following formula is then applied:

$$V = \frac{C_t \times M_t - C_{nv} \times M_{nv}}{C_t \times M_t} \times 100$$

V; Viability, C; Cell concentration, M: Multiplication factor, t; Total cell count, nv; Non-viable cell count

Figure 2.12 Non-viable and total cell counts using the Nucleocounter

This figure depicts the principle of non-viable and total cell counting using the Nucleocounter[20].
PI: Propidium iodide

2.14.8 Cryopreservation

Cells were cryopreserved to maintain stocks. To do this, cells were firstly trypsinised into a single cell suspension and counted as described above. Cells were frozen using 5% filter-

[20] www.chemometic.com

sterilised DMSO, dimethylated sulphoxide to reduce the formation of crystals which could otherwise have caused cell lysis. Due to its toxicity at 37^{o}C, DMSO was gently cooled on ice in 2 mL cryovials (Nunc) before cells were added. Cryovials were immediately transferred to an isopropanol bath and incubated at -80°C for 3 hours. The isopropanol bath was used to control the decrease in temperature, limiting it to -1°C per minute. Once the 3 hour incubation was complete, the cryovials were transferred to liquid nitrogen (-196°C) for long term storage.

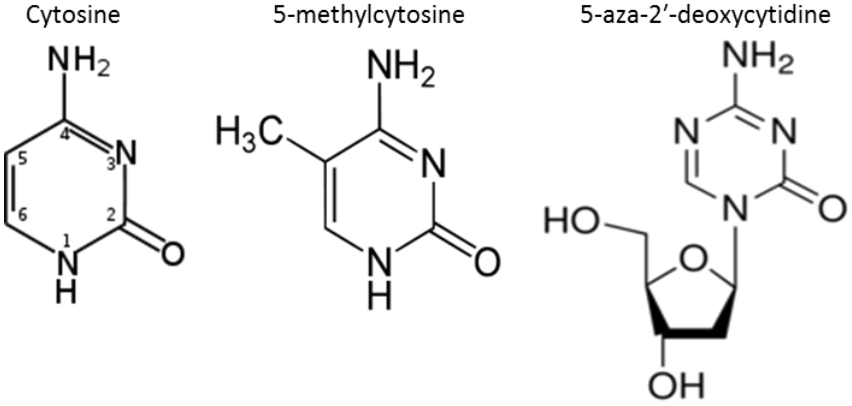
2.14.9 Recovery of cryopreserved cells

Cells were recovered by gentle defrosting of cryovials in a water bath at 37°C followed by immediate re-suspension in cell type-specific culture medium.

2.14.10 Treatment of cells with 5-azacytidine

DNA methylation is an epigenetic phenomenon whereby methyl (CH_3) groups are added to the 5- position of the cytosine pyrimidine ring or the 6- nitrogen of the adenine purine ring of DNA nucleotides resulting in transcription repression, as illustrated in Figure 2.13, 5-azacytidine (5-aza-2'-deoxycytidine) is one of many chemical analogs for the nucleoside cytidine. When these analogs are integrated into growing DNA strands, some, including 5-azacytidine, severely inhibit the action of the DNA methyltransferase enzymes that normally methylate DNA.

Figure 2.13 DNA methylation of cytosine residues



The addition of methyl groups to cytosine DNA nucleotides is catalysed by DNA methyltransferase. 5-aza-2'-deoxycytidine is a de-methylating agent that acts by inhibition of DNA methyltransferase.

Cells (MCF-7) were cultured 24 hours in advance of treatment as described in Section 2.14.4 above. Cells were treated with 5-aza-2'-deoxycytidine (5-Aza-dC) to determine if the expression of specific miRNAs of interest were affected by hypo-methylation. This was achieved by the addition of 5-Aza-dC in 5mM and 10mM concentrations to cell media. The control received normal condition media free from 5-Aza-dC. After 5 days of exposure to the de-methylating agent, the cells were harvested as described in Section 2.14.6.

2.14.11 RNA extraction from cell pellets

A cell pellet was prepared in advance of RNA extraction. The procedure of subculturing and/or harvesting outlined in Section 2.14.6 was adhered to up to the step where 5.5 mL of media was added in order to deactivate the trypsin. The resulting cell suspension was moved from its flask to a 15 mL Sarstedt tube. This was centrifuged at 1,000 rpm for 5 minutes, separating the homogenous cell suspension into an upper aqueous phase of condition media and a lower cell pellet. The cell pellet is frozen at -80°C until extraction. The RNA extraction protocol as described in Section 2.3.2 was then applied.

2.15 Messenger RNA target prediction for miRNAs

2.15.1 Overview

Identification and validation of miRNA binding sites within target genes is a critical step towards furthering current understanding of precisely how miRNAs exert their control over post-transcriptional gene expression. To date comparatively few miRNA: mRNA interactions have been functionally investigated *in vitro* (139-142). In contrast many *in silico* prediction tools have been developed to predict miRNA target sequences on the basis of sequence high throughput homology alignments and incorporation of thermodynamic analysis of duplexed sequences. The complexity of target prediction is accentuated by the fact that each miRNA has potentially hundreds of mRNA target binding sites (58). In addition, a single mRNA could harbour multiple miRNA binding sites, or conversely a single miRNA could theoretically bind to sequences within multiple mRNAs (143). An additional layer of complexity is added to miRNA target prediction due to our limited knowledge of the rules governing interactions between miRNA-mRNA duplexes. MiRNA prediction algorithms provide a practical means of narrowing the funnel of potential mRNA targets to validate experimentally.

2.15.2 The miRNA-mRNA interaction

MiRNA prediction tools rely on the interaction between the seed sequence of the miRNA and its target site on the mRNA. With the exception of a limited number of miRNAs reported to increase target gene expression (69, 71), miRNAs typically silence gene expression by either mRNA cleavage or repression of protein translation. The mechanism of action is determined by the degree of complementarity between the miRNA and its target mRNA. The seed sequence (extending from bases two to eight from the five prime tail of the miRNA) plays a critical role in this process of target recognition, requiring Watson-Crick base pairing (55). Typically the miRNA binds to the 3' UTR of the target mRNA. However, there is potential for miRNAs to bind to 5' untranslated regions (UTRs) and coding sequences (60). Most commonly in mammals, there is imperfect base pairing between the remainder of the miRNA and its target mRNA which results in translational repression as a result of 'bulges' which are created in the central region due to mis-matching of the base-pair sequences.

2.15.3 Target prediction algorithms

There are numerous computational target prediction algorithms, a selection of which is outlined in Table 2.15. Predicting miRNA-mRNA interactions is complex and explains why algorithms based on this feature alone would be inadequate. The differing results produced by alternative algorithms can be explained by the additional features taken into account, including the degree of stringency governing complementarity between the mRNA and the miRNA, free energy, where the target site is positioned (5'UTR, 3'UTR, coding sequence), mRNA target site conservation and other additional attributes (144). Multiple target sites on an mRNA for the same miRNA may enhance the degree of down-regulation, with some algorithms also taking this into consideration (145).

Table 2.15 Computational miRNA target prediction algorithms

Algorithm	URL	Availability	Type of method	Organism	Ref.
Targetscan Human 6.2	http://www.targetscan.org/vert_61/	Online	Complementarity	Human (mammals), Rat, mouse, dog, chicken, chimpanzee, rhesus, cow, opossum or frog	(146)
PicTar March 2007	http://pictar.mdc-berlin.de/	Online	Thermodynamics	Flies, vertebrates, nematodes	(147-149)
DIANA Lab TarBase 6.0	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index	Online	Experimentally validated targets	Homo sapiens	(150)
microRNA.org August 2010 Release	http://www.microrna.org/microrna/home.do	Online	Complementarity	Homo sapiens, mus musculus, rattus norvegicus, drosphilia melanogaster, caenorhabditis elegans	(151-153)
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Download	Thermodynamics and statistical model	Flies	(154)
GUUGle	http://bibiserv.techfak.uni-bielefeld.de/guugle/	Download	Complementarity	Flies	(155)
miRGen++	http://www.psi.toronto.edu/genmir/	Mathlab code	Baynesian interference	Human	(156)
DIANA microT	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index	Online	Thermodynamics	Vertebrates	(157)

Three commonly used target prediction algorithms were used in this study (Table 2.16), namely TargetScan, PicTar and Diana Lab, were selected as each exploited a different method of target prediction (seed complementarity, thermodynamics and experimentally validated targets).

Table 2.16 MiRNA target prediction algorithms used in this study

Algorithm	Parameters	Experimentally supported targets	Sensitivity	Precision
TargetScan	Seed match 3'complementarity Local AU content Position contribution*	21%	12%	51%
PicTar	Binding energy Complementarity Conservation	48%	10%	49%
DianaLab	Validated Targets	100%	NA	NA

* Sites close to 3'UTR ends are promoted, NA; not applicable as all targets have been validated experimentally
Experimentally supported miRNA-mRNA duplex interactions (158); Sensitivity: Proportion of correctly predicted targets to overall correct miRNA-mRNA interactions; Precision: The proportion of correctly predicted target miRNAs to overall total predicted miRNA-mRNA duplex interactions(144)

2.15.4 TargetScan

TargetScan is a freely available online resource which was developed by Lewis *et al* in 2003, for miRNA target prediction in vertebrates (159). It is hosted by the Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA. TargetScan presents a context based score, based on properties such as target site type and position, local AU content, and 3' supplementary pairing, in addition to the seed sequence. A strict 6 nucleotide match comprising bases 2 to 7 is required. Each target site in the 3' UTR is considered to act independently. A mRNA is deemed to be a potential target of a miRNA if there is a conserved seed match and either a conserved anchoring adenosine nucleotide on the 3'UTR downstream of the seed sequence or a conserved m8-t8 match (an AU or GC match between the eighth nucleotide from the 5' end of the miRNA, and the corresponding mRNA 3'UTR). Seed sequence conservation among orthologous 3'UTRs within miRNA binding regions is of the upmost importance in generation of the final context score (160). However, newer versions of TargetScan also consider less conservative interactions.

2.15.5 PicTar

PicTar is a project of the Rajewsky lab at NYU's Center for Comparative Functional Genomics and the MDC, Berlin. It is a freely available online algorithm which searches for near but not complete complementarity between a miRNA and conserved 3'UTRs. The miRNA seed sequence match must commence at nucleotide one or two. It then calculates the free energy (ΔG), or thermodynamic properties, of the resulting duplexes. PicTar considers sequence alignment (and thus conservation) for eight species and miRNAs with multiple alignments are preferred.

2.15.6 DIANALab TarBase

The DIANA-microT algorithm uses a frame of 38 nucleotides in length that is moved along the 3'UTR (157). The DIANA-microT-CDS is the fifth version of this algorithm that identifies targets in both the 3'UTR and CDS regions of mRNAs by considering conservation of both the 3'UTR and CDS, binding, pair stability, AU content and accessibility of target sites through the incorporation of the Sfold web server (161). As well as target prediction algorithms, online resources for experimentally validated targets are available, all of which are hosted by the Alexander Fleming Biomedical Sciences Research Centre, Athens, Greece. DIANA Lab's TarBase was first released in 2005 and contains experimental data on validated miRNA targets for eight species from manually inputted data on recent literature (162). This database not only lists the information regarding the target gene and the miRNA, it also provides key features of the study reporting its interaction. TarBase 6.0 contains a 50 fold target increase and is undated three times annually (150).

It is routine to exploit more than one target prediction algorithm when searching for potential miRNA target sequences, and identify the common targets for further validation. Putative mRNA targets for the *miR-15* family with a potential role in carcinogenesis were evaluated using the aforementioned target prediction programmes (TargetScan, PicTar and DIANA Lab). The generated results were cross-referenced to identify commonly suggested targets. The process yielded putative mRNAs with roles in carcinogenesis.

Chapter 3

Identification and Validation of miRNAs as Endogenous Controls for RQ-PCR in Blood Specimens for Breast Cancer Studies

3.1 Introduction

Accumulating evidence has shown that miRNAs play pivotal roles in regulatory functions pertaining to cell growth, development and differentiation and are associated with a wide variety of human diseases. Despite their discovery over a decade ago, it is only recently that the extent of the complexity of these regulatory molecules is beginning to be understood. Expression analysis studies have revealed differential miRNA expression in tumours compared to normal tissues. MiRNAs have been found to be dysregulated in a wide variety of human cancers. Accordingly, miRNAs have elicited much interest as biomarkers for cancer diagnosis and disease monitoring and are rapidly emerging as novel targets for disease intervention.

Real-time quantitative PCR (RQ-PCR) is widely used to quantify miRNA expression due to its sensitivity, specificity, speed, simplicity and the small amounts of template RNA required. To differentiate true biological variation from experimentally induced artefacts, target miRNA expression levels are normalised to those of a control(s). To accurately quantify miRNA expression by RQ-PCR, samples are assayed during the exponential phase of the PCR reaction during which time the amount of target miRNA is presumed to double with each cycle, without influence from limiting reagents. Comparison of cycle threshold (C_T), the cycle number at which fluorescence signals are detected above background, to an endogenously expressed control RNA is used to determine miRNA expression levels by relative quantification (EC). The accuracy of this method is heavily reliant on the choice of endogenous control. Other methods of normalisation such as normalisation to the global mean, use of spike-in controls, among others have also been described (85). Regardless of the approach, the normalisation technique and specific control RNA(s) used directly influence the results produced from RQ-PCR and thus validity. The selection of a suitable EC(s), with which to normalise RQ-PCR data, is an important first step in the accurate and reliable determination of miRNA expression levels.

Ideally a reliable EC(s) should remain stably expressed regardless of disease status or other clinical variables. A set of robust ECs that are steadily uniformly expressed across all body tissues, fluids and disease pathways has yet to be described, and is unlikely to exist. Several miRNA expression analysis studies based on tissue have reported the use of small RNAs (such as *U6, RNU44* or *RNU48*) or *miR-16* to normalise expression data (86-90). However, use of these reference genes cannot simply be applied to miRNA analysis in blood or other

body fluids as miRNA expression patterns are known to be disease-specific and perhaps specimen-type specific (91). Despite the abundance of studies on circulating miRNA profiles to discriminate between normal and disease states, there have yet to be conclusive reports of appropriate ECs. This remains a significant hurdle that must be addressed to substantiate biomarker discovery and validate single miRNA expression analysis using RQ-PCR.

Breast cancer is a prevalent disease with increasing incidence worldwide. This growing social and economic burden has stimulated the search for novel biomarkers to aid in diagnostics, prognostication and disease monitoring of adjuvant treatment. Few validated endogenous controls for miRNA research in breast cancer have been described. Initial miRNA studies on breast tissues by Mattie *et al* normalized miRNA expression to *miR-16* and *let-7*, which were later shown to be stably expressed across malignant, benign and normal breast tissue by Davoren *et al* (86, 92). Early studies on systemic miRNAs in breast cancer normalized to *miR-16* (93, 94). Additional studies, on breast and other cancers, have suggested alternative EC candidates such as *U6, RNU44, RNU48*, *miR-142-3p*, *miR-484*, *miR-191* and *miR-425* (87, 95-102). However, there is a lack of validated reports of suitable ECs for circulating miRNAs.

3.2 Aims

The aims of this study were:

- To evaluate a panel of candidate ECs (using microarray profiling) to normalize RQ-PCR data derived from blood specimens in breast cancer
- To validate the most stably expressed EC(s)
- In addition we wished to determine the effect of different normalization strategies on target miRNA expression.

3.3 Materials and Methods

3.3.1 Study cohort and sample collection

Blood samples were prospectively collected from 80 women including 50 consecutive patients with a new diagnosis of breast cancer and 30 healthy control participants. All patients had histologically confirmed invasive breast cancer. Samples of venous non-fasting blood were collected in BD vacutainers containing 18mg dipotassium EDTA (K2E) anticoagulant (BD-Plymouth) following written informed consent as described in Section

2.1. Microarray profiling was performed on RNA derived from blood on 10 of the above patients and 10 of the controls. The remaining 40 cases and 20 controls were used to validate candidate ECs and target miRNA expression (Table 3.1). Ethical approval was granted by the Clinical Research Ethics Committee, Galway University Hospital, Ireland.

Table 3.1 Clinicopathological data for blood samples derived from breast cancer cases and controls for microarray and RQ-PCR analysis

Tumours	**Array Number (%) 10**	**RQ-PCR Number (%) 40**
Mean age, years (range)	56.7	56.17
Median whole. T size (mm)	45.6 (±31.27)	30.55 (±19.47)
Missing data	-	2 (5)
Nodal status		
Positive	5 (50)	20 (50)
Negative	5 (50)	20 (50)
Grade		
1	1 (10)	7 (17.5)
2	9 (90)	25 (62.5)
3	-	8 (20)
UICC stage		
Stage 1	2 (20)	15 (37.5)
Stage 2	5 (50)	12 (30)
Stage 3	3 (30)	12 (30)
Missing	-	1 (2.5)
Intrinsic Subtype		
Luminal A	10 (100)	30 (75)
Luminal B	-	2 (5)
HER2/*neu*	-	5 (12.5)
Basal	-	3 (7.5)
Controls	**10**	**20**
Mean Age, years (range)	81.7	49.65

UICC: breast tumour staging according to the International Union Against Cancer Criteria; ER: oestrogen receptor; PR: progesterone receptor: HER2/*neu*, human epidermal growth factor receptor. Luminal A subtype is defined as ER+ve/ PR+ve/ HER2/*neu*-ve. Control subjects had no personal or family history of breast or ovarian cancer and were clinically well at the time of sampling.

3.3.2 RNA extraction and analysis

Total RNA was extracted from 1 mL of blood using Trizol (Life Technologies). RNA concentration and integrity were examined by NanoDrop spectrophotometry (NanoDrop ND-1000 Technologies Inc.) and Agilent Bioanalyzer RNA 6000 Nano Chip Kit Series II (Agilent Technologies) analysis, respectively.

3.3.3 Microarray profiling

Expression profiling of circulating miRNAs was performed on RNA extracted from 20 blood specimens using TaqMan human miRNA microarrays as instructed by the manufacturer (TaqMan Low Density Array Human microRNA Card A, Life Technologies). Megaplex primer pools were used to reverse transcribe RNA samples (700ng) which were then PCR amplified in 2 µL volumes on 384 well pre-configured microfluidic cards. Each card contained TaqMan probes for 377 miRNAs plus 3 pre-defined ECs (*U6* in quadruplicate*, RNU44* and *RNU48*) and a negative control (*ath-miR-159a*).

3.3.4 Candidate EC selection

In addition to the candidate ECs identified by microarray profiling, the expression of 7 additional candidates, as chosen from a review of published studies was investigated in the array dataset (*miR-16, miR-425, miR-484, miR-142-3p U6, RNU44* and *RNU48* (Table 3.2). Three of these (*miR-16, U6,* and *miR-425*) were further validated in a larger cohort of blood from breast cancer patients and controls.

Table 3.2 Candidate endogenous controls

MiRNA Name	Molecule type	Accession Number*	Chr. Location	Blood Component	Ref.
miR-16	miRNA	MI0000070*	**13q14.2**	Whole blood, Serum, Plasma	(93, 98, 116, 119, 163-167)
U6 (RNU6B)	snoRNA	26826**	10p13	Plasma	(95, 168)
RNU48	snoRNA	26801**	6p21.33	Serum	(169)
miR-425	miRNA	MI0001448*	**3p21.31**	Whole blood	(101)
RNU44	snoRNA	26806**	1q25.1	Whole blood	(170)
miR-484	miRNA	MI0002468*	**16p13.11**	Serum	(99)
miR-142-3p	miRNA	MI0000458*	**17q22**	Plasma	(98)

*mirBase database accession number; **NCBI Gene ID ; Chr. Chromosomal

3.3.5 Data analysis

Microarray data was analysed in two ways. Firstly, to identify the most stably expressed miRNA(s) from the panel of 377 miRNAs across the 20 blood samples, global mean expression normalisation was applied (171). MiRNAs were then ranked commencing with those with expression profiles closest to that of the mean. The second approach used the GeNorm algorithm to assess the stability of the 7 candidate ECs chosen from the literature. The most stably expressed ECs were assessed and ranked both individually (M variable) and in combination (V variable) (130).

3.3.6 RQ-PCR validation

Total RNA (100ng) was reverse transcribed using miR-specific stem-loop RT primers (Life Technologies) and components of the High Capacity cDNA Reverse Transcription kit (Invitrogen Life Technologies) according to the manufacturer's protocols. Expression levels of individual miRNAs were detected by subsequent RQ-PCR using TaqMan MicroRNA assays (Invitrogen Life Technologies) and a 7900HT instrument (Life Technologies) using standard thermal cycling conditions in accordance with manufacturer recommendations. PCR reactions were performed in triplicate in final volumes of 10µl on 96 well plates, including inter-assay controls (IAC) to account for variations between runs.

3.3.7 Amplification efficiency

PCR amplification efficiencies (E) were generated using the formula E = (10-1/slope - 1) x 100, using the slope of the semi-log regression plot of Ct versus log input of cDNA (10-fold dilution series of five points). A threshold of 10% above or below 100% was adopted.

3.3.8 PCR data analysis

Cycle threshold (C_T), or quantification cycle (Cq) is the cycle number during a PCR reaction at which the fluorescence generated is sufficient to pass the threshold, ten times the standard deviation of the baseline fluorescence emission. C_T values inversely correlate with the logarithm of the initial expression such that candidates with high expression had low C_T, and vice versa. The threshold standard deviation for intra- and inter-assay replicates was 0.28.

3.3.9 Candidate EC stability analysis

Stability of candidate ECs was assessed using geNorm and NormFinder algorithms. GeNorm is based on the assumption that none of the candidate ECs is co-regulated. It was used to rank ECs according to stability values (M) which represented the variation in expression of candidate ECs in comparison to each other. This was done by selecting optimal pairs of ECs by calculating and comparing M values for all candidates and stepwise exclusion of the least stable EC (130). NormFinder is a Microsoft Excel add-in that can accommodate both inter- and intra-group variation (172), in this case cancer and control, by accounting for variability and bias between groups. It was used to estimate the most stable EC in isolation, and the most stable 2-EC combination. The lower the stability value, the more stable the expression of the EC candidate.

3.3.10 Comparative quantification of target miRNAs relative to EC

Target miRNA (*miR-15b, miR-93, miR-181a* and *miR-652*) expression levels were estimated using qBase software (Biogazelle) to calculate amplification efficiency-corrected relative quantities following normalization to each candidate EC. The comparative cycle threshold ($\Delta\Delta C_T$) method, using the formula $\Delta\Delta C_T = (C_T$ target, test sample - C_T EC, test sample) – (C_T target, calibrator sample - C_T EC, calibrator sample) was applied. To test the effect of alternative EC on target miRNA detection, the expression of miRNA targets (*miR-15b, miR-181a, miR-652* and *miR-93*) with previously documented expression in the circulation of breast cancer patients were measured using the ECs.

3.3.11 Statistical analysis

Statistical analysis was performed using Minitab version 16.0 (Minitab Ltd.). The Kolmogorov-Smirnov test for normality was conducted and parametric tests were used where appropriate. A log transformation ($\log_{10}$) of the data was performed when necessary. Significance of circulating miRNA levels was determined using t-tests or Mann-Whitney U test, as appropriate. Results with p values < 0.05 were considered significant.

3.4 Results

3.4.1 *Identification of candidate ECs (using microarray)*

To identify the most stably expressed miRNAs from the microarray dataset global mean expression (GME) normalization was applied. This involved the use of the mean expression value of all expressed miRNAs in a given sample (in this case, 20 samples) as a

normalization factor for miRNA RQ-PCR. MiRNAs with expression profiles closest to the mean were *miR-103, miR-185, miR-532-3p, miR-194, miR-126, miR-155, let-7e, miR-345, miR-425 and miR-15b* as illustrated in Table 3.3. The first 9 miRNAs on this list were excluded from further EC analysis on the basis of their documented roles in breast cancer (Table 3.3). *MiR-425* was the only miRNA in this group not previously implicated in breast cancer and hence was chosen for further validation by RQ-PCR.

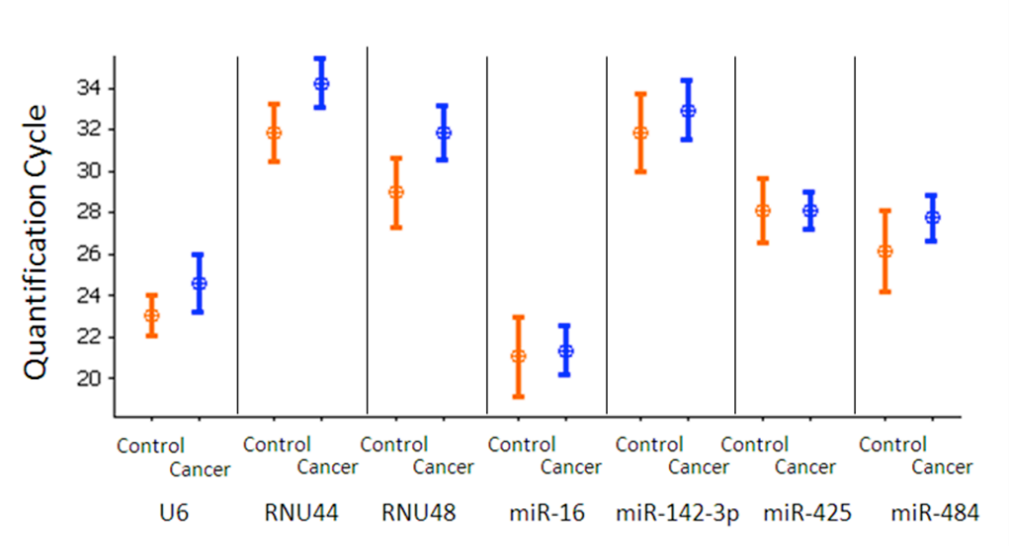
3.3 Global mean expression analysis to determine 10 most stably expressed miRNAs from the microarray dataset

Rank	miRNA	Previous Reports	St. Dev. from GME	Ref
1	miR-103	Upregulated in serum of breast cancer patients	0.121	(173, 174)
2	miR-185	Suppress tumour growth and progression in human ovarian cancers, paediatric renal tumours and breast cancer cell lines	0.128	(175)
3	miR-532	Associated with triple negative(ER-ve/PR-ve/HER2/*neu*-ve) breast cancer in tumour tissue	0.132	(176)
4	miR-194	Upregulated following Traztuzumab therapy in breast cancer cells; overexpression of miR-194 results in cell migration and invasion inhibition in breast cancer cell lines	0.139	(177)
5	miR-126	Under-expressed in breast cancer, with restoration associated with metastases suppression in breast cancer cell lines and breast tumours	0.150	(178-180)
6	miR-155	Over-expressed in breast tissue and circulation of women with breast cancer	0.155	(181, 182)
7	let-7c	Under-expressed in breast cancer with a tumour suppressor role	0.158	(93, 183, 184)
8	miR-345	Targets MRP in multidrug resistant breast cancer cells compared to normal cells	0.158	(185)
9	miR-425	No reports of altered expression or functional role in breast cancer	0.160	-
10	miR-15b	Altered expression in circulation and tumour of those with breast cancer	0.162	Chap. 5

The top 10 most stably expressed miRNAs following normalisation of the microarray data using global mean expression (GME). Nine of these miRNAs have been implicated in breast cancer. *MiR-425* is the only candidate miRNA with no reported association with breast or any other cancer type.

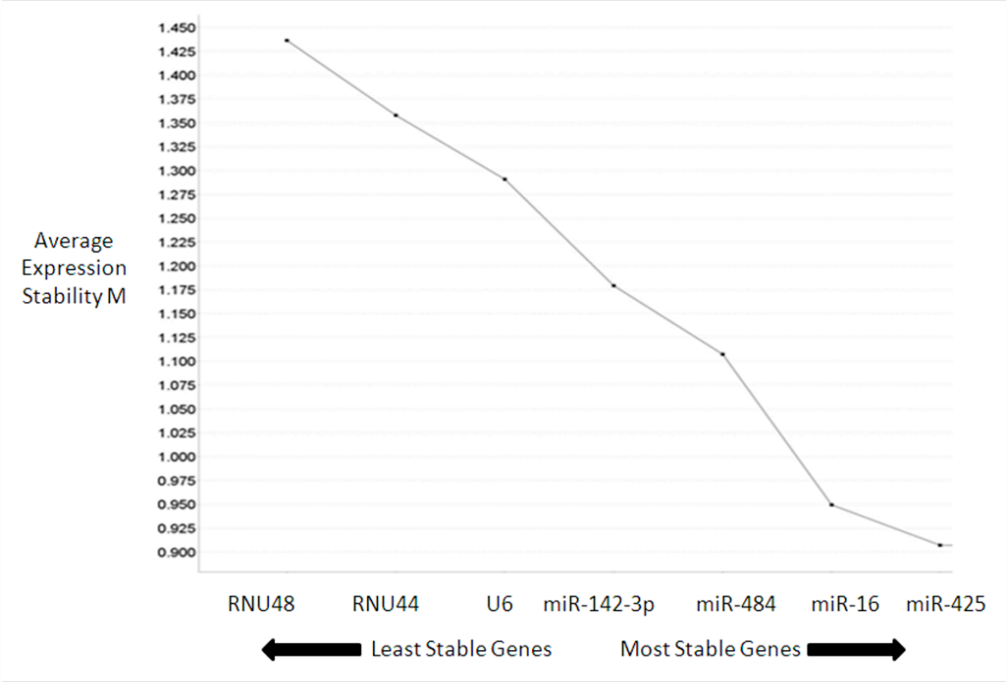
There was no significant difference in expression of the candidate miRNAs and snoRNAs between the cancer and control group across the microarray dataset (Figure 3.1). Expression stability of the means of snoRNAS (*U6, RNU44* and *RNU48*) and miRNAs (*miR-16*, *miR-425*, *miR-142-3p*, and *miR-484*) were assessed using GeNorm (Figure 3.2). *MiR-425* was found to be the most stably expressed, with a geNorm M-value 0.907. RNU48 was the least stably expressed candidate. Combination of *miR-425* and *miR-16* resulted in the lowest V-value of 0.185 (Figure 3.3).

Figure 3.1 Quantity of each candidate miRNA on microarray analysis



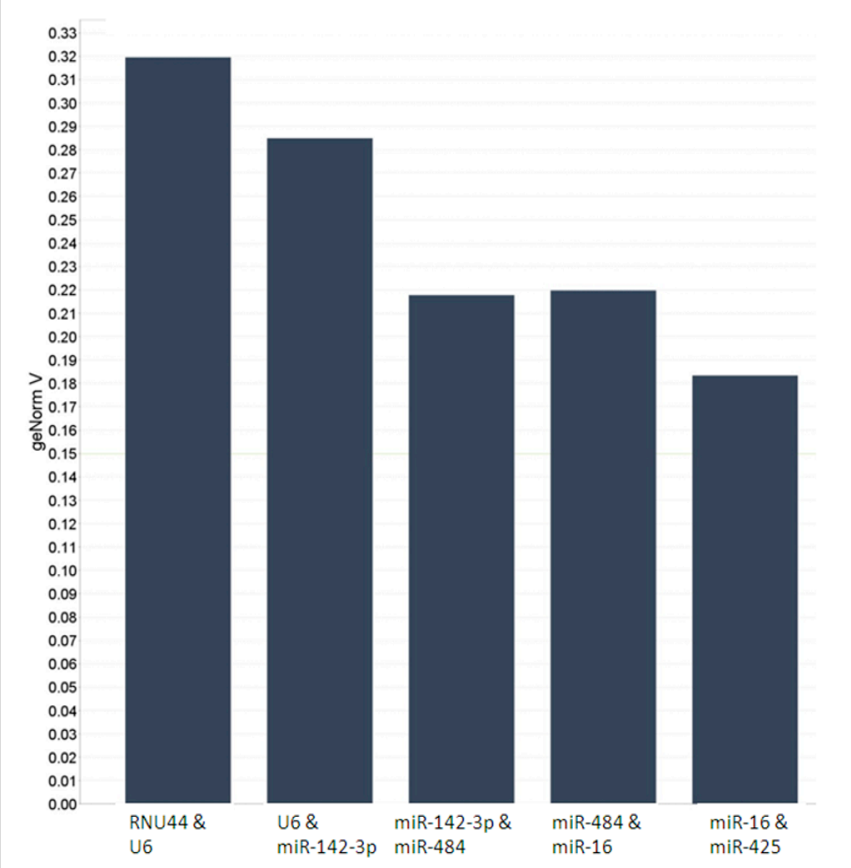
The quantity of each miRNA or snoRNA (quantification cycle) was determined by microarray for the cancer group and the control group. There was no significant difference (t-test) in candidate EC expression between the cancer group and the control group.

Figure 3.2 GeNorm analysis of average expression stability of candidate ECs



Ranking of candidate ECs according to average expression stability. The least stable candidate ECs with the highest stability measure (M) are on the left side of the graph, with the most stable ECs with the lowest M value on the right. *RNU48* and *RNU44* are the least stable ECs while *miR-425* and *miR-16* are the most stable candidate ECs.

Figure 3.3 Determination of the best combination of ECs for normalisation



Determination of optimum number of candidate ECs for normalisation. The GeNorm programme establishes the optimum combination of candidate ECs for normalisation, producing the lowest V variable. This factor is calculated using the variable 'V' as the pairwise variation (Vn/Vn + 1) between two sequential normalisation factors (NFs) (NF_n and NF_{n+1}). The combination of candidate ECs is deemed optimal when the V variable achieves the lowest value. The optimal combination was achieved by combining *miR-16* and *miR-425*.

The stability of 3 of the above miRNAs (*miR-425*, *miR-16* and *U6*) was further investigated by RQ-PCR in a larger cohort: *miR-425* was selected as it was identified by GME analysis and had the lowest geNorm M value. *MiR-16* had the next lowest geNorm M value and has been used to normalize PCR data in several cancer studies both in tissue and blood (92, 93, 98, 116). *U6* was selected, not on the basis of its apparent stability (it did not feature in GME analysis, and ranked comparatively low by geNorm), rather due to its wide use in the literature (95, 168).

3.4.2 Relative quantities of candidate EC genes

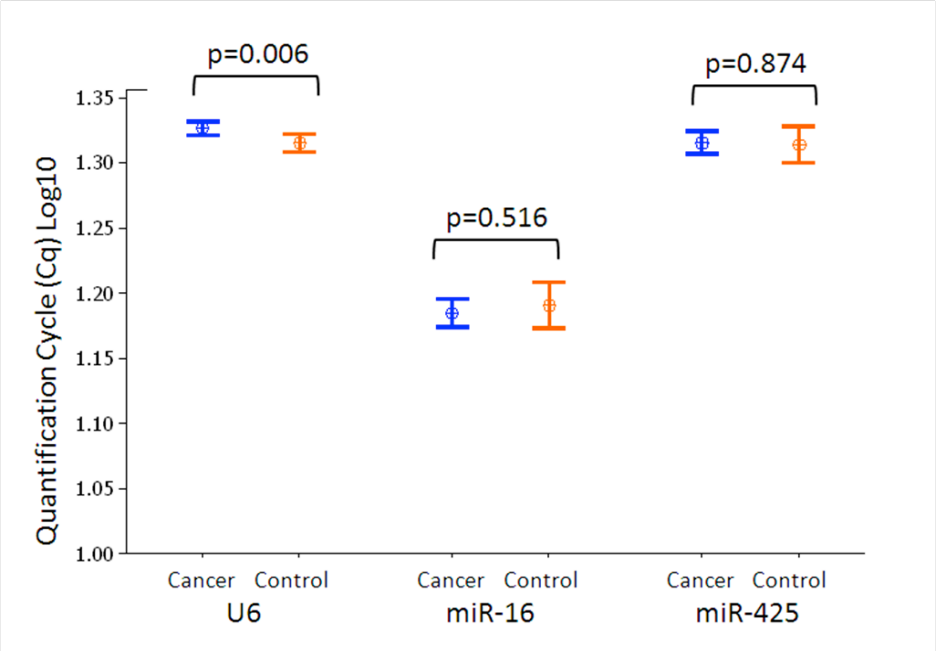
RQ-PCR was performed to validate the expression patterns of 3 candidate ECs in 60 blood samples, comprised of 40 samples from women with cancer and 20 from healthy controls (Table 3.1). All candidate ECs were expressed in abundance, with mean Ct values less than 25. *MiR-16* showed the highest expression, with mean Ct of 15.5 (range 13.5-18.7), followed by *miR-425*, mean C_T 20.7 (range 17.4-24.2) and then *U6*, mean Ct 21 (range 19.0-22.8), see Table 3.4.

Table 3.4 Cycle threshold (Ct) values for candidate ECs and target miRNAs in validation cohort

miRNA		**Mean Ct ± St Dev**	**Ct Range**	**Ct Min**	**Ct Max**
U6		21.042 ± 0.848	3.843	19.065	22.898
	Cancer	21.23 ± 0.854		19.568	22.898
	Control	20.661± 0.712		19.065	21.804
miR-16		15.460 ± 1.342	5.2	13.565	18.765
	Cancer	15.398 ± 1.346		13.565	18.765
	Control	15.584± 1.361		13.585	17.812
miR-425		20.740 ±1.415	6.746	17.459	24.206
	Cancer	20.772±1.416		18.100	24.206
	Control	20.676 ±1.447		17.459	23.395
miR-15b		16.607 ± 1.797	12.23	13.513	25.743
	Cancer	16.795 ±2.077		13.513	25.743
	Control	16.239 ±1.003		14.590	18.130
miR-93		17.206 ±1.249	6.04	15.038	21.175
	Cancer	17.085 ± 1.225		15.038	19.602
	Control	17.442 ± 1.293		15.771	21.175
miR-181a		23.775±1.255	5.521	21.740	27.261
	Cancer	23.934 ± 1.300		21.965	27.261
	Control	23.465 ± 1.131		21.74	25.963
miR-652		19.830 ± 1.720	7.21	16.480	23.697
	Cancer	20.562 ± 1.292		18.483	23.697
	Control	18.402 ± 1.565		16.480	22.232

The Ct values of each candidate EC were assessed for differential expression between cancer and control blood samples (Figure 3.4). *U6* was significantly more abundantly expressed in the control group (p=0.009). In this manner it was identified that there was no difference in expression of *miR-16* or *miR-425* between the cancer group and the controls, as would be expected for good candidate ECs.

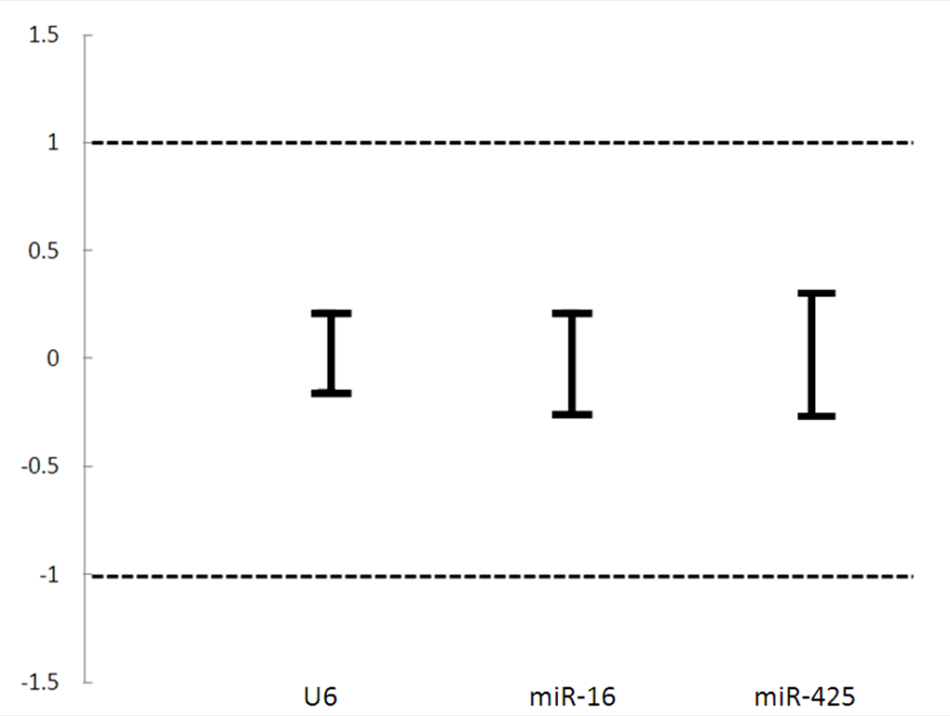
Figure 3.4 Relative quantity candidate ECs



Relative quantity of candidate EC miRNAs in blood of breast cancer patients (blue, n=40) and healthy controls (n=20) expressed as quantification cycle (Cq) values. Interval plots display the mean and 95% confidence interval. There was no significant difference (p>0.05, t-test) for *miR-16* and *miR-425.* However, *U6* was significantly more abundant in the control group (p=0.006).

Relative expression values of candidate ECs were log transformed and expressed as means with matching symmetrical confidence intervals (CI). Confidence intervals between -1 and +1 represented fold changes of ≤2, while those between -1.58 and +1.58 equated to fold changes of ≤3. A fold change cut off of 3 was applied as previously established (186). Confidence intervals with an upper border >1.58 signalled over-expression of a candidate EC cancer group. Confidence intervals with lower borders <1.58 indicated under-expression of the candidate EC in the control group (Figure 3.5). Equivalence testing was then performed to confirm that all three candidate ECs were equivalently expressed between the cancer group and the control group.

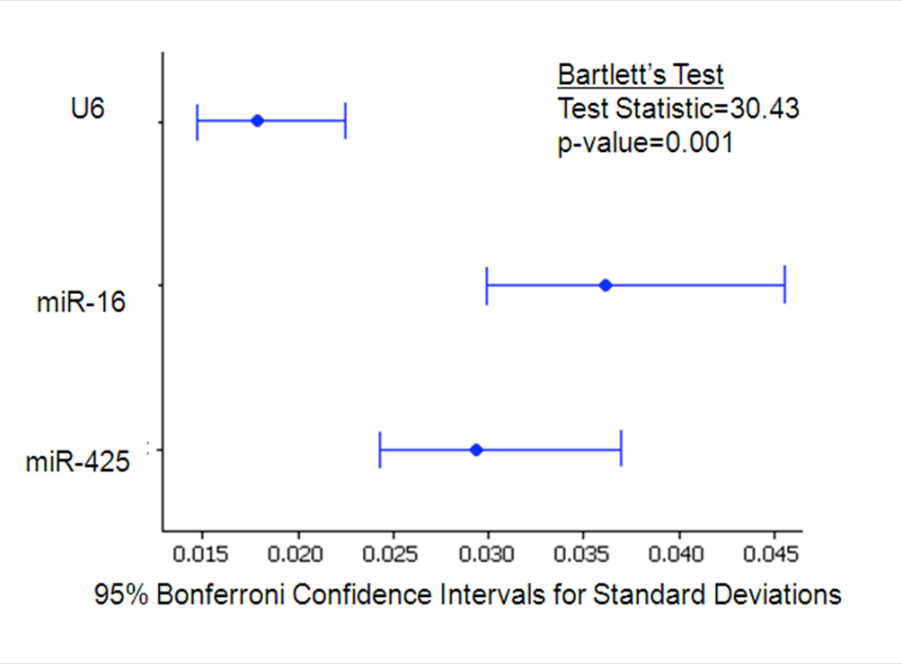
Figure 3.5 Equivalence test for candidate ECs



Each line represents the difference in logarithmic (log base2) expression between the cancer and control groups. The upper and lower bars of individual candidate ECs represents the upper and lower limits of symmetrical confidence intervals, respectively. Confidence intervals between -1 and +1 corresponded to fold changes of ≤2. No candidate EC displayed a fold change greater than 2. All three candidate ECs were equivalently expressed.

There was a significant difference in variance between ECs (Bartlett's test, $p<0.001$) indicating their differing stabilities, with *miR-16* showing the greatest variation (Figure 3.6).

Figure 3.6 Variation associated with each candidate EC



Bonferroni confidence intervals for standard deviations**.** There was a significant difference in variance (p<0.001, Bartletts's test) associated with each candidate EC, indicating differing stabilities.*MiR-16* showed greater variance than *miR-425* and *U6*.

3.4.3 Analysis of reference gene expression stability geNorm and NormFinder

The stability and variability of the candidate ECs was further assessed using NormFinder and geNorm as summarized in Table 3.5. Lower stability values indicate greater stability. GeNorm provided two values: a gene stability (geNorm M) value and a geNorm V value. The geNorm M value ranked candidate ECs according to their stability, from the most unstable (highest M value) to the most stable candidate (lowest M value). These values were generated on the basis of the average pairwise variation between all tested genes accompanied by stepwise exclusion of the least stable gene. GeNorm V values determined the optimum EC pairing for normalization, by defining the pairwise variation between two sequential normalization factors. GeNorm identified *miR-16* as the single most stably expressed miRNA, with a GeNorm M value of 1.191. NormFinder identified *miR-16* and *miR-425* as the best combination, with a stability value of 0.102. The single best EC as calculated by this algorithm was *miR-425,* followed by *miR-16* and *U6*. Consistent with the geNorm analysis on the microarray data *miR-16* and *miR-425* were identified as being the most appropriate ECs.

Table 3.5 GeNorm and NormFinder expression stability analysis

Rank	geNorm		NormFinder	
	Gene	**Stability**	**Gene**	**Stability (M)**
1	miR-16	1.191	*miR-425*	0.038
2	U6	1.232	*miR-16*	0.064
3	miR-425	1.251	U6	0.067

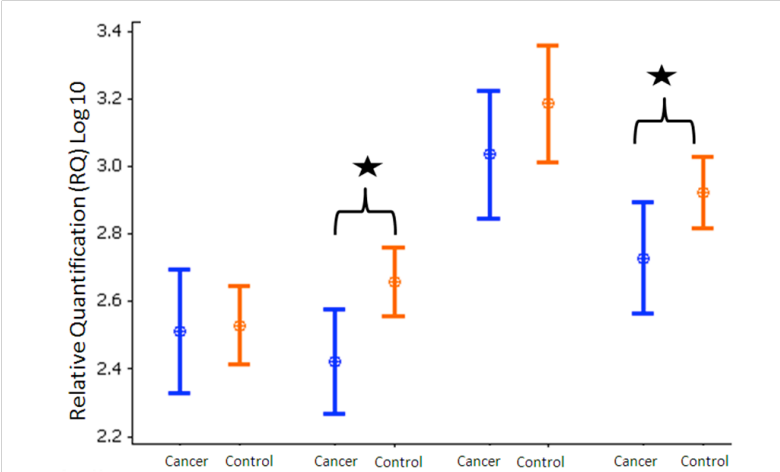
Ranking of candidate reference genes based on expression stability values calculated by NormFinder and geNorm

3.4.4 Effect of EC on relative expression of target miRNAs

To test their efficacy on target miRNA quantification, each of the candidate ECs was used to determine relative quantities of known breast cancer-associated miRNAs (Figure 3.7). *MiR-15b*, which has previously been shown to be underexpressed expressed in cancer is significantly under-expressed when *miR-16* (p=0.012) and the combination of *miR-16* and *miR-425* (p=0.048) are used as ECs. *MiR-93,* which was shown not to be dysregulated in breast cancer (Chapter 4) was overexpressed in the cancer group when *U6* (p=0.017) was used as an EC but was unaltered with other candidate ECs. *MiR-181a*, which was previously shown to be under-expressed in breast cancer (164)(Chapter 4) was under-expressed when *miR-16* (p=0.011) is used as the candidate EC. Of the four target miRNAs, the choice of EC did not influence the relative quantity of circulating *miR-652* between cancers and controls ($p < 0.001$) suggesting a highly significant differential expression of *miR-652* in breast cancer. Relative quantities of target miRNAs in cancer and control groups are shown in Figure 3.7.

Figure 3.7 Effect of EC choice on relative expression of target miRNAs
Interval plots depict mean and 95% confidence intervals for relative *miRNA* expression (Log_{10}) in the blood of women with breast cancer (blue) and healthy controls (orange) normalised to different candidate ECs with p-values indicated in the tables below each figure.

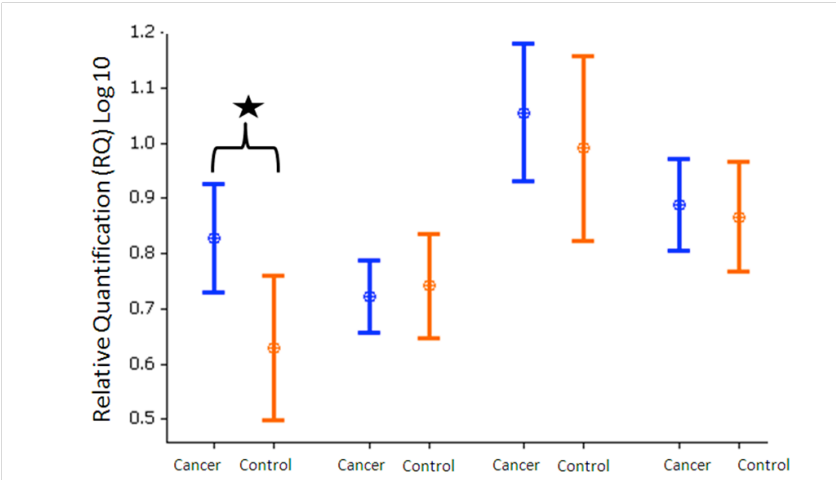
A. MiR-15b



EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	0.866	★0.012	0.232	★ 0.048

MiR-15b is underexpressed in the circulation of women with breast cancer when either *miR-16* alone (p=0.012) or a combination of *miR-16* and *miR-425* (p=0.048) is used as the endogenous control.

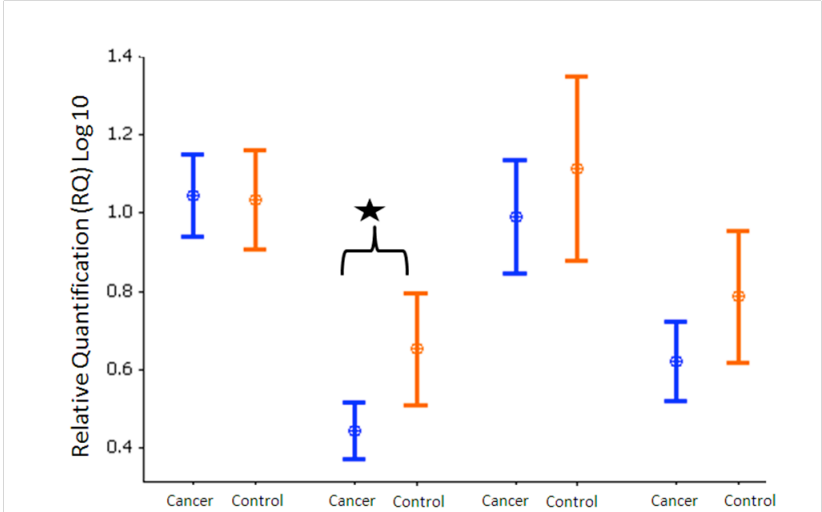
A. MiR-93



EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	★0.017	0.722	0.526	0.723

MiR-93 appears to be elevated in the circulation of women with breast cancer when *U6* is used as the candidate normaliser.

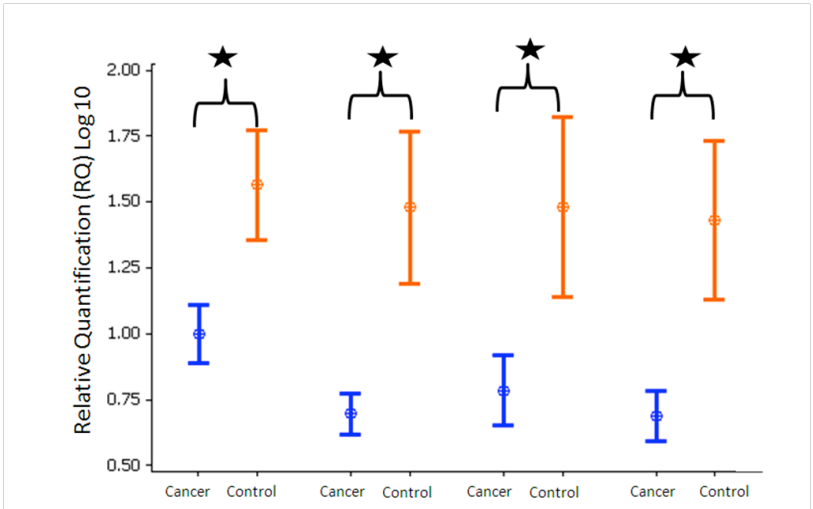
B. MiR-181a



EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	0.898	★0.011	0.361	0.091

MiR-181a is underexpressed with *miR-16* is the candidate normaliser (p=0.011).

C. MiR-652



EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	★0.001	★0.001	★0.001	★ 0.001

MiR-652 is under-expressed in the circulation of women with breast cancer, regardless of the choice of candidate EC (*U6*, *miR-425* or *miR-16*) indicating that it was highly differentially expressed in the blood of those with breast cancer.

These results highlight the importance of selecting appropriate and validated ECs. Despite the large sample size, true biological differences in miRNA expression were not detected when using less stable ECs for normalization.

3.5 Discussion

Altered miRNA expression is associated with most pathological disease processes, including carcinogenesis. Their ease of detection in biological fluids, including blood, makes them ideal candidates for exploitation as minimally invasive biomarkers. RQ-PCR is the most common technique for miRNA expression analysis. However, the high sensitivity of this approach means that accurate interpretation of RQ-PCR results depends heavily on the use of suitable, stably expressed ECs for data normalization in an effort to minimize non-biological variation between samples. Reference genes for mRNA studies have been well established but validated ECs for miRNA research are scarce. In addition, ECs for use in tissue miRNA related research may not be directly translated to other tissues or body fluids. Scrupulous miRNA data normalization may be more important than other functional RNA classes (132).

The first systematic assessment of candidate ECs for miRNA RQ-PCR was conducted by Davoren *et al* (92). This study which examined the expression stability of five miRNAs (*let-7a, miR-10b, miR-16*, *miR-21* and *miR-26b*) and 3 small nucleolar RNAs (*RNU19, RNU48* and *Z30*) was determined in normal, benign and malignant breast tissue. The best normalisation strategy for miRNA analysis in breast tissue was found to be a combination of *miR-16* and *let-7a*. There have been subsequent isolated reports of suitable ECs for specific disease states and specimen types, but the focus of this issue in the literature is vastly disproportionate to the number of accounts of altered miRNA expression in specific disease states (99, 129, 187, 188). Chang *et al* conducted a similar systematic approach to identify suitable ECs for application to colorectal cancer tissue (129). MiRNA profiling was performed on a small cohort of paired colorectal tumour tissues and normal tissue. Global mean expression analysis was performed to identify stably expressed candidate ECs. Six candidate miRNAs (*let-7a*, *miR-16*, *miR-26a*, *miR-345*, *miR-425* and *miR-454*) and 2 small nucleolar RNAs (*RNU48* and *Z30*) were chosen for further validation by RQ-PCR in a larger cohort of colorectal tissues. *MiR-16* and *miR-345* were identified as the best combination of reference miRNAs by both geNorm and NormFinder, with *miR-16* and *miR-345* being the single best normalisers identified by NormFinder and geNorm, respectively. Genovesi *et al* identified ECs for use in medulloblastoma studies involving TLDA cards, and recommended the combination of *miR-301a* and *miR-339-5p* for normalization of card A data, with a combination of *miR-425** and *RNU24* being used for Card B data analysis (187). Few studies have examined suitable ECs for use in circulating miRNA studies. Hu *et al* identified and

validated candidate miRNAs as ECs for serum miRNA expression studies in breast cancer (99). In this cohort, a combination of *miR-191* and *miR-484* provided the best normalization approach for target miRNA expression. Song *et al* focused on gastric cancer, examining 6 miRNAs (*let-7a, miR-16, miR-93, miR-103, miR-192, and miR-451*) and one small nucleolar RNA, *RNU6B* for suitability as candidate ECs (188). This study advocated the use of *miR-16* and *miR-93,* the most stably expressed candidate ECs, for normalization of miRNA expression in serum for gastric cancer.

The present study identified that the combined use of 2 miRNAs, (*miR-16* and *miR-425*) to normalize RQ-PCR data generated more reliable results than using either miRNA alone, or use of *U6*, which has been used by several authors to date. In the absence of a comprehensive analysis of reliable ECs for RQ-PCR data from blood samples, a microarray screen was performed at the outset. We profiled 20 blood samples (10 from women with breast cancer and 10 from healthy control women) for the expression of in excess of 370 miRNAs (including *U6* snoRNA). The dataset was analyzed using global mean expression (GME) to identify miRNAs with expression patterns close to the mean expression of the entire dataset. We selected *miR-425* from the GME analysis, and both *miR-16* and *U6* from the literature for further analysis by RQ-PCR in a validation cohort (n=60). Our initial validation step using raw Ct values of these 3 candidate ECs displayed that *U6* was more abundant in the control group, while there was no difference in *miR-16* or *miR-425* expression between the cancer or control group. Equivalent expression of candidate ECs between the cancer and control group was confirmed using a fold change cut off of ≤3, corresponding to confidence intervals between -1.58 and +1.58. We used both GeNorm and NormFinder algorithms which identified *miR-16* and *miR-425,* respectively, as the most stably expressed candidate ECs, with NormFinder suggesting their combination as the best combination.

As evident from the results presented in this study, use of an inappropriate EC for normalization can significantly alter the apparent expression of target miRNAs. Combination of *miR-16* and *miR-425* as EC detected significant dysregulation of *miR-15b* (p=0.048) and *miR-652* (p=0.001). *MiR-181a* has previously been shown to be under-expressed in breast cancer, but was shown in this study to be significantly under-expressed when *miR-16* alone was used as an EC. Normalization with the combination of *miR-16* and *miR-425* increased the p-value to 0.091. *MiR-93* has been shown to be stably expressed in

the blood of women with breast cancer compared to healthy controls. However, when *U6* was applied as the normalizer *miR-93* appeared to be upregulated in the cancer group. A combination of miRNAs for normalization augments the reliability of the data produced, and has been advocated by other studies (129, 172).

MiR-16 appears to be the most widely used EC for blood-related miRNA studies with application to breast, ovarian, pancreatic, gastric, prostate and renal cell cancer, melanoma and the haematological malignancies (93, 98, 116, 119, 164-167). Recent studies have reported on the origin of circulating *miR-16*, indicating red blood cell haemolysis as a major source of this miRNA in blood (189, 190). This may be more a concern in studies where cell-free blood fragments (serum/plasma) are the source of miRNAs. We utilized whole blood in a disease where it has been previously shown that patient red blood cell and haemoglobin levels are within the normal range in the majority of cases, particularly those with early stage disease (93). Therefore, as blood samples from both cancer and control patients are treated identically one would not anticipate this to have a direct effect on *miR-16* expression, as evidenced by our results where they was no difference in *miR-16* expression between the cancer and control groups. This issue may be more of a concern, when RNA extraction protocols not utilizing chaotrophic agents such as Trizol are used, implicating that individual sample treatment and storage in advance of RNA extraction would directly influence results. There are few reports of *miR-425* in the literature. This is reassuring as it denotes that *miR-425* may be a miRNA with little functional value in disease processes, an attractive trait of an EC. *U6* was selected for further validation by RQ-PCR as although not the most stably expressed EC based on GME or GeNorm analysis of the microarray data, it is commonly used for miRNA studies (95, 96, 117, 191). *U6* (*RNU6B*) is a small nucleolar RNA (snoRNA) that forms part of the *U6* small nuclear ribonucleoprotein, a component of the spliceosome responsible for splicing of pre-mRNA. The use of *U6* and other snoRNAs in miRNA related research is contentious (192). These larger molecules are likely to be less reliable than miRNA ECs as their expression is less stable than miRNA with studies showing more frequent degradation in serum samples (118, 193). This makes it difficult to draw conclusions pertaining to miRNA expression when snoRNAs are utilized as ECs. In this study we showed that *U6* was aberrantly expressed in the cancer group compared to the control group ($p=0.009$).

This study focuses on RQ-PCR data normalization using candidate ECs which is the most prevalent method. Two alternative normalization strategies for circulating miRNA expression have been proposed to date; Global Mean Expression (GME) and exogenous (spiked-in) miRNAs. GME was recently introduced by Metsdagh *et al* for use in high-throughput miRNA profiling. GME uses the average expression of all the miRNAs detected in a sample as the normaliser presuming that the mean miRNA expression of all miRNAs is constant when the same starting amount of total RNA is used, regardless of the sample type. This technique reduces technical variation and preserves biological variation and is very suited to large genome wide miRNA profiling (171). It is better suited to large expression profiling studies, with several such studies reporting its use (194-196). This technique is largely unsuited to biomarker studies as bias may be introduced in such studies when several of the target miRNAs being analysed show variation in expression (over-expression or under-expression) in one study group compared to another. Spiked-in non-human exogenous miRNAs, such as *cel-miR-39*, *cel-miR-54* and *cel-miR-238*, have also been used for normalization (197-199). This method presumes that by adding a known quantity of spiked in miRNA to an equal volume of serum/plasma/whole blood, a stable quantity of reference gene is obtained. However, this technique leaves room for technical and human error.

Accurate normalization strategies are crucial for miRNA related research, as detecting even small changes in miRNA expression can have major biological implications, as a single miRNA can target multiple mRNAs, even in the same pathway thus augmenting its effect (200). In truth, a single universal EC for use in all specimen types across all diseases, malignant or otherwise, is unlikely to exist. Suitable ECs need to be validated for use in specific disease states and specimen types. The surge of interest in identifying specific miRNAs as biomarkers for health and disease requires that an equal amount of attention is focused on the establishment of suitable ECs with which to normalize the data such that appropriate conclusions can be derived.

3.6 Conclusion

This study is of relevance in translational miRNA research for circulating miRNAs in breast cancer. It identifies a combination of two miRNAs, *miR-16* and *miR-425*, with application for use as ECs for normalization. Further investigation into suitable ECs for use in miRNA RQ-PCR studies is warranted.

Chapter 4

Identification and Validation of Oncologic miRNA Biomarkers for Luminal A Breast Cancer

4.1 Introduction

Breast cancer is a heterogeneous disease, with distinct tumour phenotypes reflecting a spectrum of underlying molecular alterations and initiating events (45). Analysis of gene expression patterns governing these events has resulted in the classification of breast tumours into subtypes broadly determined by expression of the oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2/*neu*). Targeted therapies including hormonal therapy for ER positive tumours and trastuzumab to inhibit HER2/*neu* signalling have become the major components of adjuvant breast cancer management. Consequently, when diagnosed and treated early, breast cancer is highly curable. Despite these advances, hematogenous spread of malignant cells from the primary tumour to distant organs with subsequent proliferation into metastases remains the leading cause of death for breast cancer patients (6). Further insight into the molecular mechanisms underlying tumorigenic transformation is clearly warranted for the identification of additional molecular predictors and disease biomarkers in the clinical management of breast cancer.

Much current cancer research is focused on the identification of circulating cancer-specific biomarkers for application to disease diagnostics, as well as predicting and monitoring response to disease and tumour recurrence. There are no reliable circulating biomarkers for breast cancer. Mammography is the most widespread screening tool, with a definitive diagnosis requiring an invasive tissue biopsy. This prevalent disease is in need of a minimally invasive biomarker which may be used in combination with radiological imaging to facilitate early subtype specific tumour diagnosis. Blood presents an excellent medium for biomarker discovery; it is minimally invasive and simple to obtain during routine clinical examination. Moreover, blood circulates throughout the body delivering nutrients and carrying proteins (including miRNAs), hormones and cells while eliminating waste substances, thereby reflecting the summation of physiological and pathological processes occurring in an individual at any one time.

Mi(cro)RNAs have shown much potential as cancer-specific biomarkers. MiRNAs regulate gene expression at the post-transcriptional level and are intimately linked with the cancer state. First, miRNA expression has a causal effect on tumourigenesis, acting as oncogenes and tumour suppressor genes and second, altered miRNA expression occurs as a result of the carcinogenic process. In breast cancer, altered tissue miRNA expression patterns have

been shown to correlate with molecular subtype and hormonal receptor status (109, 111). MiRNAs were originally studied in tissue, but several studies have demonstrated that tumour-specific miRNAs are detectable in the circulation (94, 119, 120). These studies allude to the promising role of circulating miRNAs as biomarkers for detection of disease. Furthermore, speculation that circulating miRNA profiles could reflect not only the tumour tissue-type, but also the intrinsic molecular subtype thus acting as a fluid biopsy would be particularly valuable in breast cancer where management, even immediately following diagnosis, is governed by hormonal and HER2/*neu* receptor status, largely conveying molecular subtype.

Luminal A, phenotypically characterized as hormone receptor positive and HER2/*neu* negative, is the most common subtype including over 70% of breast cancers. These tumours are frequently screen detected, node negative and therefore associated with a good prognosis. Recent advances such as the development of the Oncotype DX® test strive to prevent overtreatment of this common subtype by identifying women at high risk of recurrence for adjuvant chemotherapy.

4.2 Aims

The aims of this study were:

- To utilise microarray profiling to identify circulating miRNAs that are differentially expressed in women with Luminal A breast cancer (ER positive, PR positive, HER2/*neu* negative) in comparison to healthy controls
- To validate candidate miRNA expression in circulation and tissue using RQ-PCR
- To investigate candidate miRNA expression levels in association with common clinicopathological parameters
- To study their effectiveness as circulating diagnostic biomarkers in the clinical setting

4.3 Materials and Methods

4.3.1 Study cohort and sample collection

Blood samples were prospectively collected from 110 women; this included 54 consecutive patients with a new diagnosis of Luminal A breast cancer and 56 healthy control participants. All patients had histologically confirmed Luminal A breast cancer. Healthy control blood samples were collected from women residing in the same catchment area as the cancer cases. These women were interviewed by a clinician in advance of sample collection to ensure that there was no personal history of malignancy or current inflammatory or infectious condition. Venous non-fasting whole blood samples were collected in BD vacutainers ® containing 18mg dipotassium EDTA anticoagulant (BD-Plymouth). Microarray profiling was performed on RNA derived from blood on 10 of the above patients and 10 controls, the clinicopathological details of which are presented in Table 4.1. The remaining 44 cases and 46 controls were used to independently validate microarray findings. Clinicopathological details of the validation group are shown in Table 4.2. Tissue specimens both tumour (n=11) and tumour-associated normal (TAN, n=10) were prospectively collected from patients with Luminal A breast cancer at the time of surgical resection. Tissue samples were collected in RNAlater® RNA stabilization reagent (Qiagen, UK) prior to cryopreservation at -80^{o}C. Clinicopathological details of this cohort are included in Table 4.3.

Table 4.1 Clinicopathological patient data for blood samples analysed by microarray

Cases	Age (yrs)	Inv. T size (mm)	Whole T size (mm)	Histological Subtype	Nodal Status	Grade	UICC Stage	ER	PR	HER2 /neu	Intrinsic Subtype	Controls*	Age (yrs)
1	70	22	22	Inv. Muc.	-	2	1	+	+	-	Luminal A	1	81
2	52	15	15	Inv. Ductal	-	2	2	+	+	-	Luminal A	2	61
3	60	13	20	Inv. Ductal	-	2	2	+	+	-	Luminal A	3	82
4	50	22	22	Inv. Ductal	-	2	1	+	+	-	Luminal A	4	76
5	46	38	38	Inv. Ductal	+	1	2	+	+	-	Luminal A	5	74
6	59	120	120	Inv. Ductal	+	2	3	+	+	-	Luminal A	6	87
7	56	53	53	Inv. Lobular	+	2	2	+	+	-	Luminal A	7	94
8	55	61	61	Inv. Ductal	+	2	3	+	+	-	Luminal A	8	94
9	44	45	45	Inv. Ductal	-	2	2	+	+	-	Luminal A	9	96
10	75	50	60	Inv. Ductal	+	2	3	+	+	-	Luminal A	10	72

Yrs, Year; mm, Millimeters; Inv. T size, Invasive Tumour size; UICC, Stage of breast tumour according to the International Union Against Cancer staging criteria; ER, Oestrogen receptor; PR, Progesterone receptor: HER2/*neu*, Human epidermal growth factor receptor; -, negative; +, positive; NA not applicable. Luminal A subtype is phenotypically defined as ER positive, PR positive, HER2/*neu* negative.* All control subjects had no personal or family history of breast or ovarian cancer and were clinically well at the time of sampling.

Table 4.2 Clinicopathological patient data for blood in independent validation cohort

Luminal A Breast Tumours		**Number (%)** **44**
Mean age, years (range)		59.86 (±13.45)
Median Whole. T size (mm)		32.82 (±26.30)
	Missing data	11
Median Inv. T size (mm)		27.41 (±20.74)
	Missing data	22
Histological Subtype		
	Invasive ductal	35
	Invasive lobular	1
	Other	7
	Missing	1
Nodal status		
	Positive	22
	Negative	19
	Missing	3
Grade		
	1	7
	2	32
	3	4
	Missing	1
UICC stage		
	Stage 1	15
	Stage 2	14
	Stage 3	8
	Stage 4	2
	Missing	3
Oestrogen Receptor		
	Positive	44 (100%)
	Negative	0
Progesterone Receptor		
	Positive	44 (100%)
	Negative	0
HER2/*neu* Receptor		
	Positive	44 (100%)
	Negative	0
Intrinsic Subtype		
	Luminal A	44 (100%)
Controls		**46**
Mean Age, years (range)		44.21 (±20.61)

Table 4.3 Clinicopathological patient data for breast tumours

Luminal A Breast Tumours		**Number (%)** **11**
Mean age, years (range)		54.27 (±9.26)
Median Whole. T size (mm)		34.1 (±42.5)
Histological Subtype		
	Invasive ductal	11
Grade		
	1	1
	2	1
	3	8
	Missing	1
UICC stage		
	Stage 1	3
	Stage 2	8
Oestrogen Receptor		
	Positive	11 (100%)
	Negative	0
Progesterone Receptor		
	Positive	11(100%)
	Negative	0
HER2/*neu* Receptor		
	Positive	0
	Negative	11 (100%)
Intrinsic Subtype		
	Luminal A	11 (100%)

4.3.2 Ethics Statement

Ethical approval was granted by the Clinical Research Ethics Committee, Galway University Hospital. Written informed consent was obtained from all study participants.

4.3.3 RNA extraction

Total RNA was extracted from blood, normal breast tissue and breast tumour tissue as previously described in Section 2.3. In brief, RNA was extracted from 1ml of blood using Trizol as previously described (92). RNA was extracted from approximately 100ng tissue using the RNeasy MiniKit®.

4.3.4 RNA concentration and integrity

RNA concentration and integrity were examined by NanoDrop spectrophotometry (NanoDrop ND-1000 Technologies Inc., DE, USA) and Agilent Bioanalyzer RNA 6000 NanoChip Kit Series II (Agilent Technologies, Germany) analysis, respectively.

4.3.5 MiRNA microarray profiling

Expression profiling of circulating miRNAs was performed for 20 samples as described above using TaqMan human miRNA arrays and assays in accordance with the manufacturer's instructions and as outlined in Section 2.13 (TaqMan Low Density Array Human microRNA, Applied Biosystems, Foster City, CA, USA). In brief, total RNA was reverse transcribed using Megaplex primer pool A (Applied Biosystems) which contained sequence-specific primers for 381 specific miRNAs plus 3 controls (pool A). An additional panel of 384 miRNAs (381 miRNAs and 3 controls, pool B) was performed on a subset of 4 cancers and 4 controls. Real-time quantitative PCR was performed for 667 miRNAs, using A and B microfluidic cards, each containing primers and probes for 381 specific miRNAs plus 3 controls and thermal-cycled on an Applied Biosystems 7900HT instrument. MiRNA expression data are available from the National Centre for Biotechnology Gene Expression Omnibus (GEO) at accession number GSE46355.

4.3.6 Microarray data analysis

Within this study normalized miRNA array data were analyzed within a nonlinear ANN based data mining algorithm to identify those with altered expression in Luminal A breast cancer. This method comprised a feed-forward back-propagation algorithm utilizing a three layer architecture, a sigmoid transfer function, 2 hidden nodes and early stopping on unseen data full details are described by Lancashire *et al* (137)). Monte Carlo Cross validation was applied to the modelling approach to determine the performance of the miRNA probes on a randomly selected blind subset. This approach addressed issues with false discovery by preventing over fitting, driving the solution to one that has good predictability for a blind population.

The performance of single miRNA probes was determined by developing ANN models using the algorithm described above (each using a single probe intensity from the data), to classify between Luminal A breast cancer and healthy controls (Figure 2.11). This process was repeated for all of the probes on the array and their classification performance on blind data determined. In this way a rank order of miRNAs was determined. From this rank order the key miRNAs were taken forward for validation.

4.3.7 Validation by RQ-PCR

Quantification of individual miRNAs in both blood and tissue samples was determined by RQ-PCR using TaqMan miRNA assays (Applied Biosystems). Ten of the most differentially expressed miRNAs from the microarray screen were selected for validation. Following RNA isolation, 100ng of total RNA was reverse transcribed using stem-loop primers and MultiScribe reverse transcriptase. PCR reactions were performed in triplicate in final volumes of 10μl on 96 well plates. Each plate included an inter assay control (IAC) to account for run-to-run variation. Plates were run on a 7900HT instrument (Applied Biosystems) using standard thermal-cycling conditions.

Raw fluorescence (cycle threshold, C_T) data were subsequently calculated. High C_T values indicated low miRNA expression and vice versa. The threshold standard deviation for intra- and inter-assay replicates was 0.28. PCR amplification efficiencies (E) were calculated for each miRNA and Taqman miRNA assay using the formula E = (10-1/slope - 1) x 100, using the slope of the semi-log regression plot of Ct versus log input of cDNA (10-fold dilution series of five points). A threshold of 10% above or below 100% was adopted. C_T values were scaled to lowest expressing sample and normalized to *miR-16,* which has been shown to be stably expressed in breast cancer and was the most widely used endogenous control miRNA for breast cancer at the time of completing this study (92, 93). MiRNA expression was calculated by the comparative cycle threshold (ΔC_T) method, using qbase^{PLUS} ® software (Biogazelle, NV, Belgium).

4.3.8 Statistical analysis

Statistical analysis was performed using Minitab version 16.0 (Minitab Ltd, Coventry UK). The Kolmogorov-Smirnov test for normality was conducted. Data were log transformed (log_{10}) for analysis when non-normal distribution was identified. Significance and associations of circulating miRNA levels were determined using the Mann-Whitney U test, t-test, ANOVA, Spearman's Rho or Pearson correlation, as appropriate. Results with p-value less than 0.05 were deemed to be significant. Binary logistic regression analysis was used and receiver operating characteristic (ROC) curves were generated to evaluate the ability of chosen miRNAs to distinguish between cancer cases and controls. This was performed both individually and for combinations of miRNAs.

4.4 Results

4.4.1 Identification of dysregulated miRNAs in Luminal A breast cancer

The ANN data mining algorithm identified 76 miRNAs with detectable and altered expression in blood of patients with Luminal A breast cancer compared to healthy controls (Table 4.4).

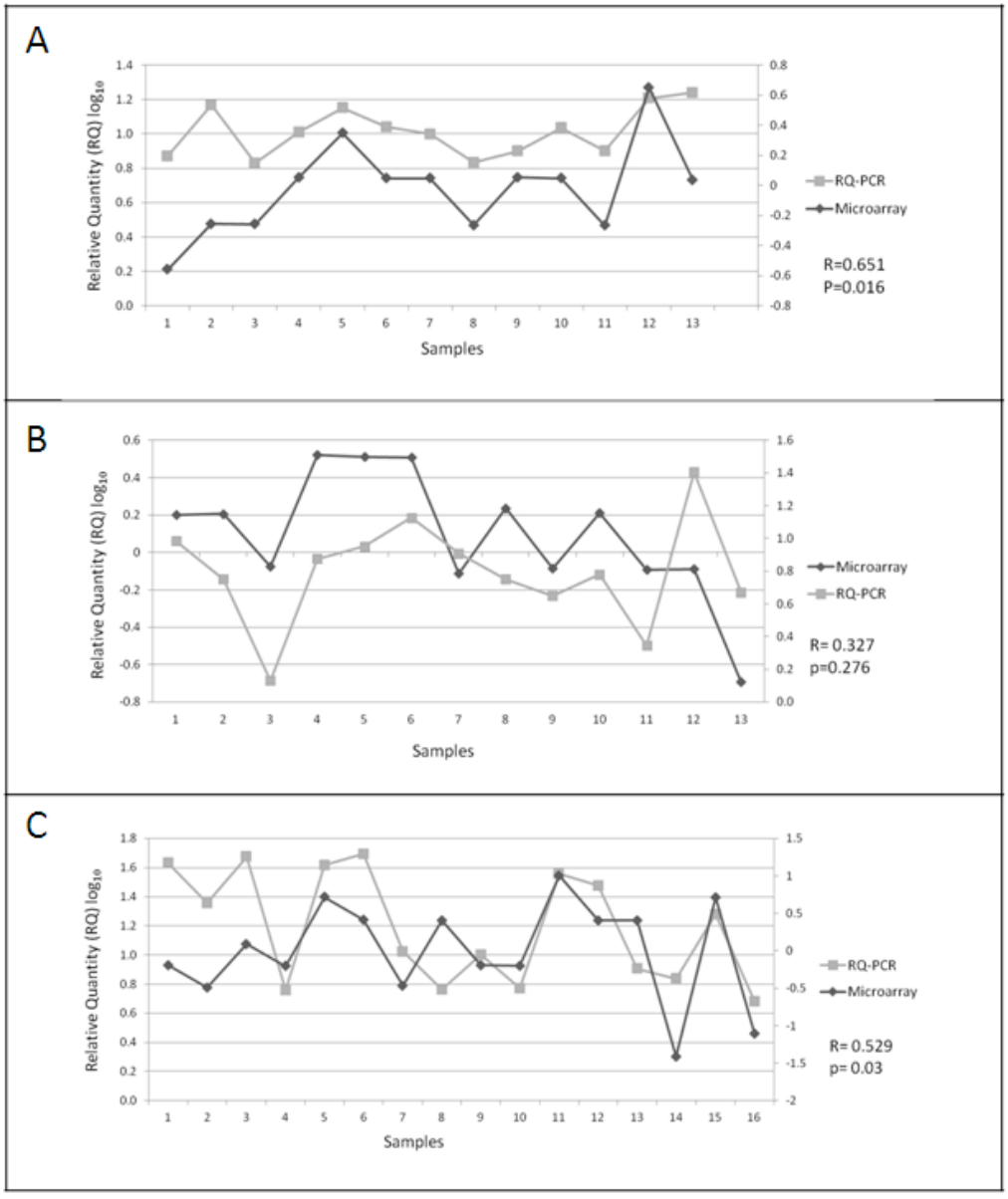
Table 4.4 MiRNAs with altered expression in Luminal A breast cancer

Rank	miRNA	Rank	miRNA
1	miR-181a	41	miR-345
2	miR-301a	42	let-7d
3	miR-182	43	miR-628-5p
4	miR-423-5p	44	let-7b
5	miR-19b	45	miR-16
6	miR-93	46	miR-17
7	miR-652	47	miR-331-3p
8	miR-29a	48	miR-886-5p
9	miR-486-5p	49	miR-501-5p
10	miR-223	50	miR-106b
11	miR-532-5p	51	RNU48
12	miR-30c	52	miR-146a
13	miR-103	53	miR-15b
14	miR-339-3p	54	miR-126
15	miR-183	55	miR-146-5p
16	miR-320	56	miR-374b
17	miR-29c	57	miR-19a
18	miR-210	58	miR-194
19	miR-425	59	miR-200c
20	miR-125a-5p	60	miR-374a
21	miR-196b	61	miR-186
22	miR-20b	62	miR-28-3p
23	miR-222	63	miR-342-3p
24	miR-24	64	miR-92a
25	miR-155	65	miR-145
26	miR-106a	66	miR-140-3p
27	miR-20a	67	miR-26a
28	miR-192	68	miR-142-3p
29	miR-532-3p	69	miR-150
30	miR-185	70	miR-451
31	miR-191	71	miR-26b
32	miR-744	72	miR-486-5p
33	let-7g	73	miR-574-3p
34	miR-30b	74	miR-139-5p
35	miR-324-3p	75	miR-195
36	miR-660	76	miR-484
37	miR-25		
38	miR-454		
39	let-7c		
40	let-7e		

4.4.2 Validation of microarray

To further evaluate the expression patterns of individual miRNAs derived from the microarray dataset, real-time quantitative PCR was performed. A subset of three candidate miRNAs was chosen for sample to sample expression analysis and in most cases revealed good correlation between the microarray profiling data and RQ-PCR validation (Figure 4.2).

Figure 4.2 Correlation between microarray and RQ-PCR data



Correlation (Pearson's) of miRNA expression levels between microarray (dark) and RQ-PCR (light) detected expression levels (A) *miR-29a* (B) *miR-181a* (C) *miR-182*

Expression of ten of the most deregulated miRNAs was confirmed in an independent cohort of blood from patients with luminal A breast cancer (n=44) and healthy controls (n=46). MiRNA expression levels were also measured in tumour tissue derived from patients with Luminal A breast tissue. The miRNAs selected for validation and results obtained are outlined in Table 4.5. Two miRNAs (*miR-181a* and *miR-652*) were found to be over-expressed in the microarray and were down-regulated in the circulation of women with Luminal A breast tumours in the validation group (p=0.004, and 0.009, respectively, Figure 4.3). Both *miR-181a* and *miR-652* miRNAs were also under-expressed in Luminal A tumour tissue compared to TAN (p=0.019 and p<0.001, respectively Figure 4.3). *MiR-29a* and *miR-223* were under-expressed in the circulation of those with Luminal A breast cancer compared to healthy controls, in both the array and the validation cohorts (p<0.001 and p=0.004, Figure 4.3).

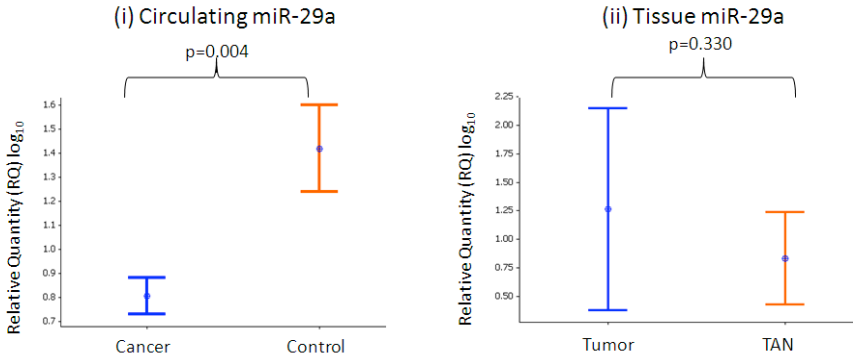
Table 4.5 Candidate miRNAs for validation by RQ-PCR

miRNA	Chromosomal Location	Sequence	Expression	p-value (Circulation, RQ-PCR) *	p-value (Tissue, RQ-PCR)*
miR-19b	Chromosome 13: 92001446-92005532 (+)	UGUGCAAAUCCAUGCAAAACUGA	Unchanged	0.775	NA
miR-29a	Chromosome 7: 130561506 - 130561569 [-]	UAGCACCAUCUGAAAUCGGUUA	**Down**	**0.001**	**0.330**
miR-93	Chromosome 7: 99691391 - 99691470 [-]	CAAAGUGCUGUUCGUGCAGGUAG	Unchanged	0.399	NA
miR-181a	Chromosome 1: 198828173 - 198828282 [-]	AACAUUCAACGCUGUCGGUGAGU	**Down**	**0.004**	**0.019**
miR-182	Chromosome 7: 129410223 - 129410332 [-]	UUUGGCAAUGGUAGAACUCACACU	Unchanged	0.355	NA
miR-223	Chromosome X: 65238712 - 65238821 [+]	UGUCAGUUUGUCAAAUACCCCA	**Down**	**0.004**	**NA**
miR-301a	Chromosome 17: 57228497 - 57228582 [-]	CAGUGCAAUAGUAUUGUCAAAGC	Unchanged	0.179	NA
miR-423-5p	Chromosome 17: 28444097 - 28444190 [+]	UGAGGGGCAGAGAGCGAGACUUU	Unchanged	0.519	NA
miR-486-5p	Chromosome 8: 41517959 - 41518026 [-]	UCCUGUACUGAGCUGCCCCGAG	Unchanged	0.333	NA
miR-652	Chromosome X: 109298557 - 109298654 [+]	AAUGGCGCCACUAGGGUUGUG	**Down**	**0.009**	**0.001**

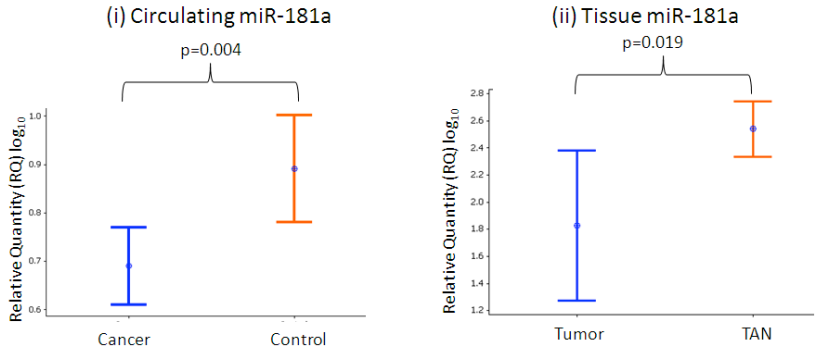
* p-value determined by t-test

Figure 4.3 Blood and tissue expression of *miR-29a, miR-181a* and *miR-652*

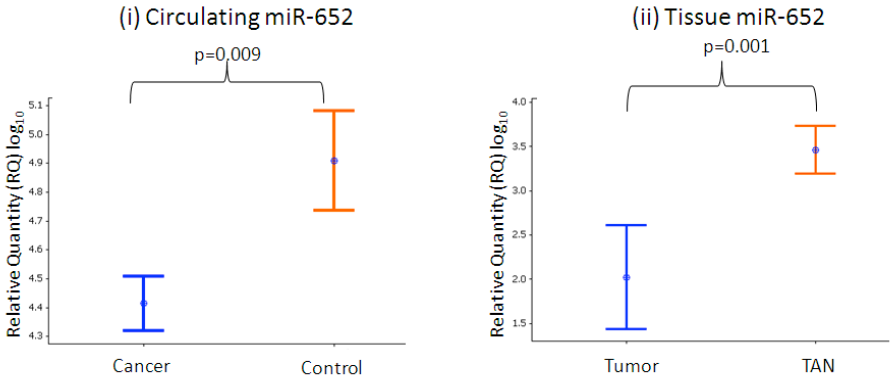
A. miR-29a



B. miR-181a



C. miR-652



Expression levels of (A) *miR-29a* (i) in the circulation of patients with Luminal A breast cancer versus healthy controls and (ii) *miR-29a* levels in tumour and TAN tissue; (B) *miR-181a* expression (i) in the circulation of cases and controls and (ii) and in tumour and TAN tissue; (C) *miR-652* expression in the circulation of cases and controls (i) and in tumour and TAN tissue (ii).

4.4.3 Relationship between miRNA expression and clinicopathological parameters

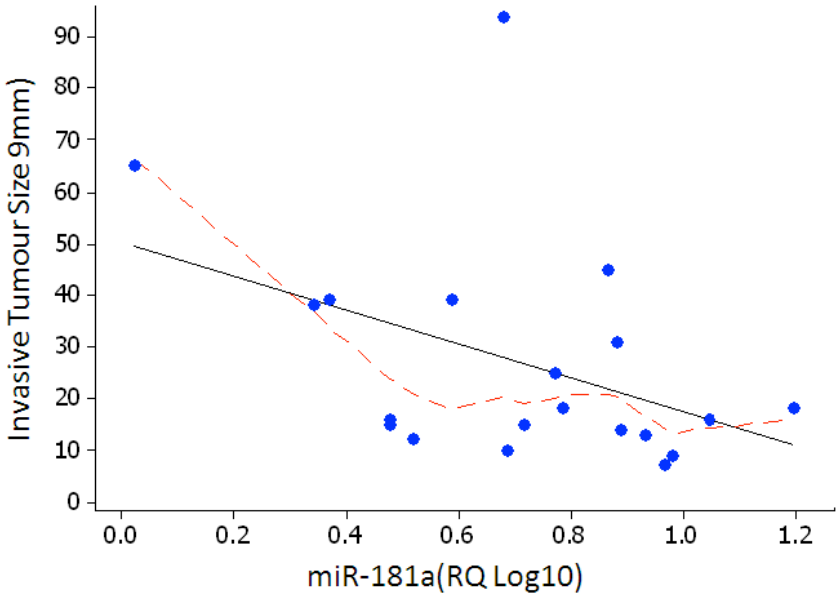
MiRNA (*miR-29a, miR-181a* and *miR-652*) expression data was compared with clinicopathological variables, namely grade, nodal status, tumour size and stage of disease. *MiR-29a*, *miR-181a* and *miR-652* were significantly down-regulated in the blood of patients compared to controls, irrespective of tumour grade, nodal status or stage of disease (Table 4.6). Altered expression in both early and late stage disease is an important biomarker characteristic. Interestingly, *miR-181a* was significantly down-regulated in the blood of patients with node positive disease compared to healthy controls (p=0.006) but not node negative disease (p=0.09). There was no difference in *miR-181a* expression between node positive or node negative disease. There was a negative correlation between *miR-181a* expression and invasive tumour size (Pearson correlation coefficient r=-429, p=0.059, Figure 4.4).

Table 4.6 miRNA expression and clinicopathological parameters

Clinicopathological Parameter	**miRNA**	**P-value***
Stage	miR-29a	0.737
	miR-181a	0.058
	miR-652	0.511
Grade	miR-29a	0.193
	miR-181a	0.924
	miR-652	0.998
Nodal Status	miR-29a	0.845
	miR-181a	0.257
	miR-652	0.845

This table demonstrates that although *miR-29a*, *miR-181a* and *miR-652* are under-expressed in women with Luminal A breast tumours, there is no significant difference in miRNA expression levels in the blood of women with breast cancer, regardless of stage of disease (1 to 4), grade of disease (1 to 3) or nodal status (positive or negative). This is an important biomarker trait, as it reflects miRNA expression alteration in early, as well as late stage disease.
*p-value using one-way ANOVA.

Figure 4.4 Correlation between circulating *miR-181a* expression and invasive tumour size



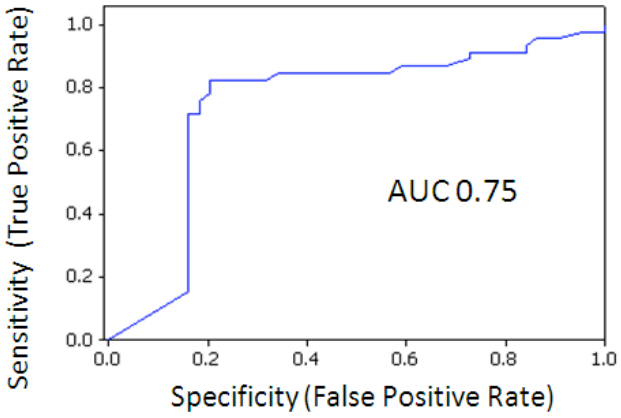
This figure shows a negative correlation between *miR-181a* expression and invasive tumour size (Pearson correlation coefficient r=-429, p=0.059).

4.4.4 Biomarker potential of miRNAs

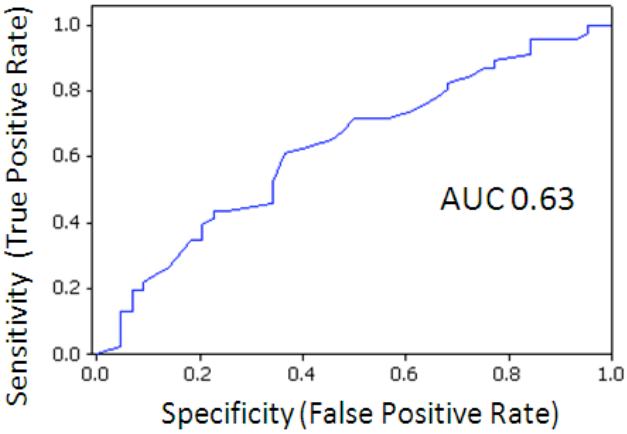
The evident dysregulation of *miR-29, miR-181a* and *miR-652* in the blood of women with Luminal A breast cancer, irrespective of tumour stage or grade, revealed a potential role for these miRNAs as circulating biomarkers for Luminal A breast cancer detection. We compared the area under the curve (AUC) produced from receiver operator characteristic (ROC) curve generation using binary logistic regression analysis for each individual miRNA (Figure 4.5)and miRNA combination profiles. The best AUC cut-off of 0.80 was generated from a combination of *miR-29a*, *miR-181a* and *miR-652*, providing a sensitivity and specificity of 77% and 74%, respectively (Figure 4.6). The addition of *miR-223* did not improve the sensitivity or specificity profile achieved.

Figure 4.5 Receiver Operator Characteristic (ROC) curve for each individual miRNA

A. miR-29a



B. miR-181a



C. miR-652

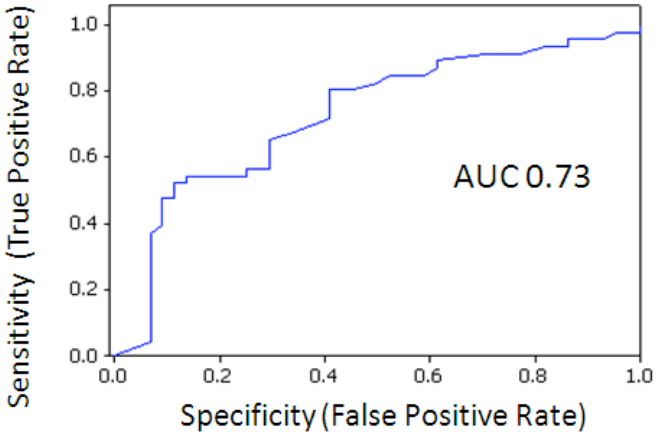
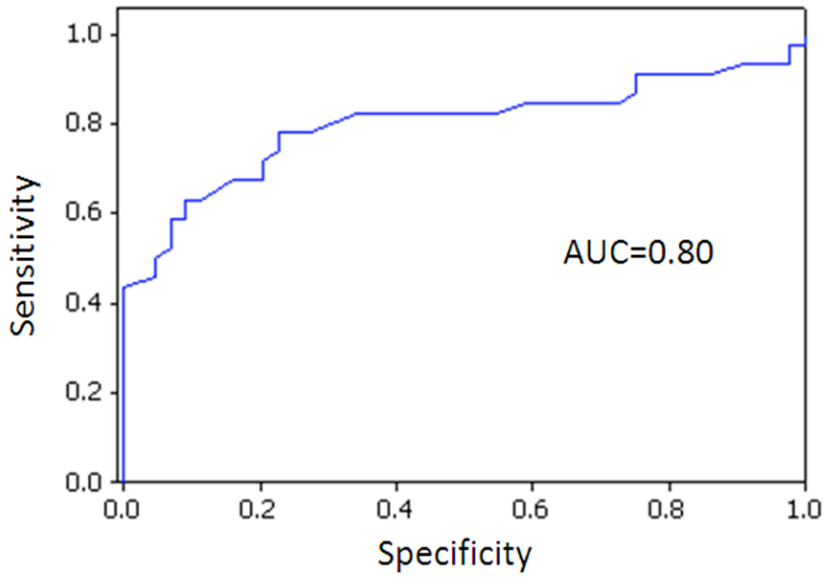


Figure 4.6 Receiver Operator Characteristic (ROC) curve for 3 miRNA combination (*miR-29a, miR-181a* and *miR-652*)



4.5 Discussion

Mammography is currently the gold standard screening tool for breast cancer diagnosis; however accurate diagnosis and intrinsic subtype confirmation requires histological evaluation from tissue obtained at breast biopsy, an invasive procedure. The identification of novel reliable minimally invasive breast cancer biomarkers would represent a significant development in the clinical management of this complex disease. The concept of a panel or profile of miRNAs for diagnostic purposes is a realistic approach, as to date no single miRNA has been reported with the qualities (sensitivity, specificity and reproducibility) for use in isolation. The 3 miRNAs identified in this study yielded a sensitivity and specificity of 77% and 74% respectively, and could be evaluated from blood collected during a simple blood test. Although not perfect, this sensitivity and specificity profile exceeds that of several currently used clinical biomarkers (201-204) and could be improved with the combination of mammography. There is no routinely used circulating biomarker for breast cancer detection. Carcinoma Antigen 15-3 (CA 15-3) and Carcinoembryonic Antigen (CEA) are circulating biomarkers. However their clinical application in breast cancer management is, if any, confined to detecting and monitoring disease recurrence and progression. These markers are merely elevated in 10% of stage 1 disease and 20% of stage 2 disease, precluding any usefulness in the diagnostic arena.

Early miRNA-related research mainly focused on tissue, with several reports of aberrant miRNA expression in breast cancer correlating with clinicopathological variables such as stage and hormone receptor status (108, 111, 205-208). Furthermore, individual miRNAs have been associated with metastatic potential of breast tumours (209). The rush to identify non-invasive diagnostic biomarkers for breast cancer has resulted in a surge of interest in circulating miRNAs. Several studies to date have evaluated miRNA expression in the blood of women with breast cancer (93, 210). Not all reports in the literature are directly comparable, as although circulating miRNAs are analyzed in each case, three alternative blood components have been used, namely whole blood, serum and plasma. Whole blood was analysed in this study as stability of miRNAs in EDTA-whole blood and the potential to profile miRNAs from this medium have repeatedly been demonstrated (93, 101, 119). In addition, given that circulating miRNA research is still in its infancy, it was chosen to utilize methods that could potentially be exploited in larger multi-centric trials by collecting whole blood stored in a refrigerator until transport rather than plasma or serum that requires prompt centrifugation, alloquotting and freezing.

It has been suggested that circulating miRNAs may reflect the presence of breast tumours but not the specific profiles of miRNAs within the breast tumours (211, 212). In the current study, we identified four miRNAs (*miR-29a*, *miR-181, miR-223* and *miR-652*) with dysregulated expression in the circulation of women with Luminal A breast cancer. *MiR-181a* and *miR-652* were down-regulated in Luminal A breast tumour tissue, while *miR-29a* was not. These findings support the hypothesis that circulating miRNA expression profiles may not act as a direct window on tumour activity and brings into question the mechanism by which they enter the blood stream, in addition to their functional role, if any, in the peripheral circulation. These processes remain poorly understood. MiRNAs can enter the peripheral circulation following selective secretion from tumour cells or circulating micro-vesicles (133). Other cells in the tumour microenvironment can also secrete miRNAs. Meanwhile another school of thought suggests that miRNAs may be detectable in the circulation as a consequence of passive leakage from apoptotic and necrotic cells (213). In reality it is likely that both of these theories are true, with accumulating evidence to support both plausible proposals.

Once in the circulation, miRNA transport is not uniform. Some miRNAs are encapsulated in microvesicles, apoptotic bodies, exosomes or high-density lipoprotein (HDL) particles

while others are in combination with proteins of the Argonaute (AGO) family (121, 123, 214). The protection conveyed by microparticles or in combination with AGO proteins explains the stability of miRNAs in nuclease rich and protease rich environments, such as the circulation, when compared to mRNA (215). The majority of circulating miRNAs, as much as 90-95%, are transported in combination with the AGO protein family (123, 215). The functional role of miRNAs in circulation has yet to be fully elucidated; are these tiny particles merely secreted as by-products of physiological and pathological processes or are they circulating messengers, with important intercellular and inter-organ cell to cell messaging capabilities? Some recent studies allude to the potential for exosomally-packaged miRNA to act as cell to cell signalling molecules, during viral infection, the immune response and most significantly cancer progression (216-218). However, despite these reports, it is likely that the majority of circulating extracellular miRNAs, particularly the AGO-transported form, have no functional role. Nonetheless, regardless of their source, their presence, relative stability and ease of detection can be exploited for biomarker means.

In this study ANN identified four specific miRNAs as being significantly altered in the circulation of women with Luminal A breast cancer. ANN data-mining algorithms have been shown to provide a robust solution to issues encountered within miRNA array data (111). They have been shown to cope with non-linearity, and complexity; whilst offering the ability to identify biomarkers of high biological relevance and good predictive sensitivity and specificity (137). *MiR-181a* has previously been reported as being significantly under-expressed in the serum of women with breast cancer compared to healthy controls (164). It has also been shown to be downregulated in tumour tissue of lung, oral, hepatocellular, and ovarian cancers (219-222). In addition, *miR-181a* was identified as a potential prognostic factor for colorectal and gastric cancer (223, 224). A recent study, using NGS-SOLiD sequencing followed by validation with RQ-PCR reported *miR-29a* as being over-expressed in the serum of women with breast cancer (174). This miRNA has been implicated in other cancers, predominantly colorectal where it may have a role in prognostication (225, 226). *MiR-223* has been reported in serum of patients with nasopharyngeal carcinoma and gastric cancer (227, 228). *In vitro* analysis revealed that *miR-223* was detected within exosomes and increased invasiveness of co-cultured cell lines (SKBR2 and MDA-MD-321) (218). In the present study, validation of *miR-223* expression was examined in fewer samples than were available for *miR-181a*, *miR-29a* and *miR-652*

validation (29 cancers, 40 controls), however we found it to be significantly lower in the circulation of cancer patients, *p*=0.004). There are no previous reports, to our knowledge, of a role for *miR-652* as a diagnostic biomarker for breast cancer.

Despite the rapidly evolving field of circulating miRNAs as oncologic biomarkers, there are still a number of challenges which must be overcome before miRNA profiling can be routinely incorporated into the diagnostic arena. Real time is the most common technique employed for miRNA quantification. Despite significant technological advances in PCR instrumentation, and levels of detection, there remains little consensus on assay design through to data analysis. In particular, there is a lack of concordance on protocols for data normalization.

Although these results are extremely promising, and substantiate the potential application of miRNAs as biomarkers for breast cancer, we recognize that this study has limitations. The sample size is relatively small; larger validation analyses, involving blinded samples are needed to confirm the clinical utility of the 3 miRNA panel for luminal A breast cancer detection. Such studies should ideally include blood samples from all breast tumour subtypes, namely Luminal B, HER2/*neu* over-expressing and basal subgroups, as well as from patients with benign breast disease. Future studies to evaluate the mechanism of action of these miRNAs, if any, in breast tumours and determine the exact processes by which *miR-29a, miR-181a, miR-223* and *miR-652* are shed into the circulation are also warranted.

The potential value of the miRNAs outlined in this study is not restricted to diagnostic biomarkers for breast cancer. The realm of miRNA-related therapeutic strategies is gaining increased momentum, particularly in hepatitis and hepatocellular carcinoma. MiRNAs with depleted expression levels may be restored to ‘normal’ levels by viral vector encoded miRNAs or miRNA mimetics. It seems plausible if these miRNAs have a functional role in the tumour microenvironment, tumourigenesis could potentially be halted or reversed by restoring their expression levels.

4.6 Conclusion

In conclusion, this study presents 76 miRNAs with differential expression in the circulation of women with Luminal A breast cancer compared to those who do not have breast cancer. A miRNA profile of three circulating tumour-associated miRNA biomarkers (*miR-29a, miR-181a* and *miR-652)* for breast cancer are identified which in combination provide a sensitivity and specificity profile which exceeds that of several current clinical biomarkers. A complementary test, for use in combination with mammography would prove extremely advantageous particularly in an era where swift diagnosis, expeditious commencement of appropriate adjuvant treatments and surgical resection have a role to play in ultimately improving patient outcomes. Further large prospective studies are required, to include all breast cancer subtypes and to elucidate the potential of miRNAs in the systemic circulation as subtype-specific diagnostic or therapeutic breast cancer markers.

Chapter 5

Differential Expression of *miR-15* Family Members in Breast Cancer

5.1 Introduction

Mi(cro)RNAs play critical roles in almost all biological processes finely tuning gene expression. Altered miRNA expression has been described in several disease states, including in the tissue and blood of those with breast cancer (93, 111). MiRNAs are synthesised in the nucleus and transported to the cytoplasm where they exert their effects, primarily of gene silencing, through translational inhibition or mRNA cleavage. The site and mechanism by which miRNAs manipulate gene expression largely depends on the degree of base-pair complementarity between the miRNA and its target mRNA. The seed sequence is the most important region on an miRNA molecule for this process, located between positions 2 and 8 from the 5' tail (55). The seed sequence governs binding, and therefore function: if there is perfect complementarity in the miRNA-mRNA interaction RNA cleavage takes place. However, more commonly in humans there is imperfect base pair matching resulting in translational repression.

MiRNA families are groups of miRNAs that have evolved from common ancestry and share a common seed sequence. As the seed sequence largely governs mRNA targeting, miRNAs with the same seed sequence are thought to have redundant functions (229). Much of the published miRNA studies have largely focused on individual miRNAs. However, with such significant similarity between family members it is plausible that this may confer some clinical and/or biological significance.

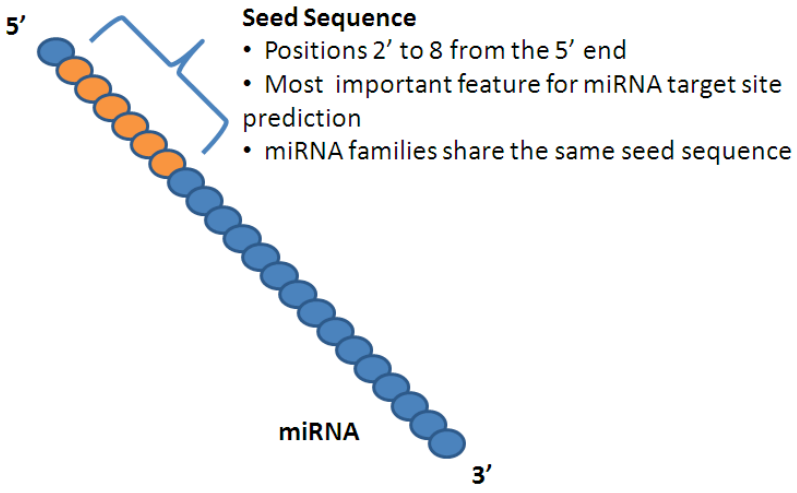
The role of a few miRNA families have been studied in cancer thus far. The *miR-200* family is comprised of five members, *miR-200a/b/c*, *miR-429* and *miR-141.* The entire *miR-200* family is up-regulated in endometrial adenocarcinoma (230). Four *miR-200* family members (namely *miR-200a, miR-200b, miR-200c* and *miR-141*) have been associated with epithelial to mesenchymal transition (EMT) in breast cancer (231). The *miR-34* family has been described as altered in several cancers including ovarian cancer where there is down-regulation of *miR-34b*/c* (232). A recent meta-analysis was conducted on *miR-183* family microRNAs (*miR-96, miR-182* and *miR-183*) in 18 cancers with only 10 of 50 independent studies on a variety of cancer types reporting on expression of all three family members (233). Different patterns of expression were reported depending on the tumour type, with colorectal and prostate cancer having the most consistent pattern of up-regulation.

MiR-195 has been implicated in breast cancer and several other types of cancers as demonstrated in Table 5.1 (93, 234-236). Heneghan *et al* reported increased expression in the blood and tissue of those with breast cancer compared to healthy controls, and patients with other malignancies (119, 237). *MiR-195* is clustered with *miR-497*, both of whom are members of the *miR-15* miRNA family. The *miR-15* family seed sequence consists of AGCAGCA from positions 2 to 8 from the five prime end of the miRNA strand and consists of highly conserved miRNAs (Figure 5.1). At the time of study conception, the *miR-15* miRNA family included *miR-15a, miR-15b, miR-16, miR-195, miR-424* and *miR-497* (Table 5.2, Figure 5.2). The number of miRNAs included in this family has expanded with time, with increasing annotations. Several members have been shown to be of interest in cancer however they have yet to be evaluated as a family in breast cancer.

Table 5.1 *MiR-15* miRNAs and their previous associations with cancer

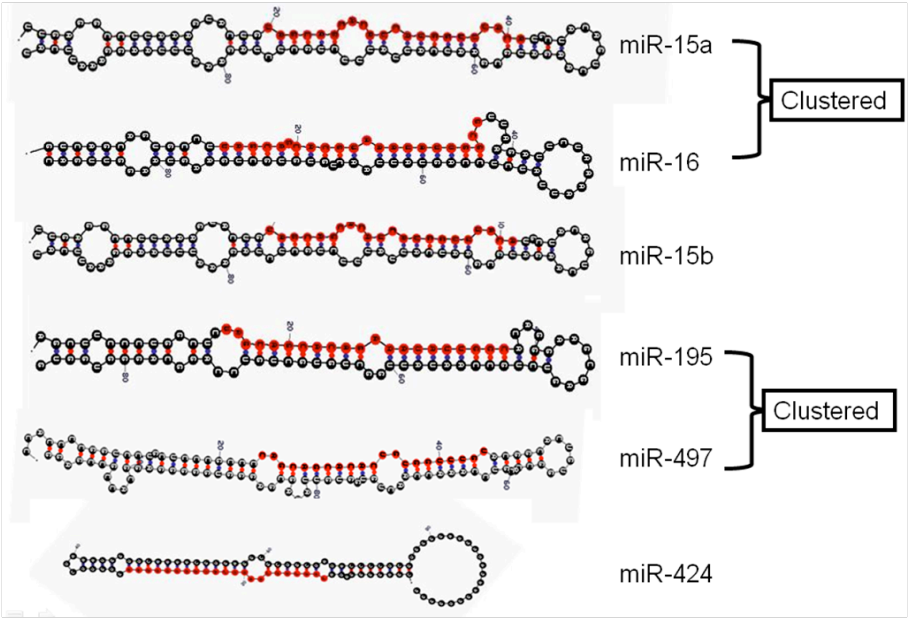
miRNA	Cancer Type	Ref.
miR-15a	Myelodysplastic Syndrome	(238)
	CLL	(239)
	Prostatic cancer	(240)
	Non small cell lung cancer	(241)
	Ovarian cancer	(242)
miR-15b	Hepatocellular carcinoma	(243)
	Colorectal carcinoma	(244)
	Ovarian cancer	(245)
miR-16	Oesophageal carcinoma	(246)
	Myelodysplastic Syndrome	(238)
	Renal cell carcinoma	(247)
	Non small cell lung cancer	(241)
	Acute lymphocytic leukaemia	(248)
	Ovarian cancer	(245)
	Prostate cancer	(240)
miR-195	Breast cancer	(93, 119, 191)
	Gastric cancer	(235, 249)
	Oesophageal carcinoma	(246)
	Bladder cancer	(250)
	Hepatocellular carcinoma	(141)
	CLL	(251)
miR-424	Colon cancer	(252)
	Osteosarcoma	(253)
	Cervical cancer	(254)
miR-497	Breast cancer	(191)

Figure 5.1 MiRNA seed sequence



A miRNA is small non-coding RNA molecule between 18 and 22 nucleotides in length. The seed sequence, located between positions 2 and 8 from the 5' end of the miRNA strand plays an important role in miRNA binding, and therefore function. A miRNA family is a group of miRNAs that share this common seed sequence.

Figure 5.2 *MiR-15* miRNA family



The *miR-15* miRNA family are a group of miRNAs that share the AGCAGCA seed sequence. The family is comprised of numerous miRNAs including *miR-15a, miR-16, miR-15b, miR-195, miR-497* and *miR-424.* This image displays the precursor stem loop miRNA (grey) including the mature miRNA strand (red).

Table 5.2 The *miR-15* family of miRNAs

miRNA	Seed Sequence	Genome Location
miR-195*	5'....uagcagcacagaaauauuggc....3'	17p13.1
miR-497*	5'....cagcagcacacugugguuugu....3'	17p13.1
miR-15b	5'....uagcagcacaucaugguuuaca....3'	3q25.33
miR-424	5'.... cagcagcaauucauguuuugaa....3'	Xq26.3
miR-16**	5'....uagcagcacguaaauauuggcg....3'	13q14.2
miR-15a**	5'....uagcagcacauaaugguuugug....3'	13q14.2

**miR-195* and *miR-497* are clustered on Chromosome 17
***miR-16* and *miR-15a* are clustered on Chromosome 13

5.2 Aims

The aims of this study were:

- To profile the expression patterns of the *miR-15* miRNAs (*miR-15a, miR-15b, miR-16, miR-195, miR-424* and *miR-497*) in the circulation and tissue of women with breast cancer
- To investigate their clinical utility as oncologic biomarkers for breast cancer
- To predict their role(s) by identifying putative mRNA targets

5.3 Materials and Methods

5.3.1 Study cohort and sample collection

Ethical approval (Galway Roscommon University Hospital Group) was obtained and blood samples were prospectively collected from consecutive women with a new diagnosis of breast cancer at our institution (n=67). Blood was also prospectively collected from healthy volunteers with no personal or family history of breast cancer residing in the same catchment area as the cancer subjects (n=56). All participants provided written informed consent. Non-fasting venous whole blood samples were collected in BD vacutainers® containing 18mg dipotassium EDTA anticoagulant. Clinicopathological details of the circulating miRNA cohort are shown in Table 2.3. Tissue specimens both tumour (n=20) and tumour-associated normal (TAN, n=10) were prospectively collected from patients with breast cancer at the time of surgical resection. Tissue samples were collected in RNAlater® RNA stabilization reagent prior to cryopreservation at -80^{o}C. Clinicopathological details of this cohort are included in Table 2.4.

There was a separate cohort of patients for which matched blood and tissue samples were available. Blood was collected pre-operatively while tissue specimens were obtained intra-operatively at the time of specimen resection. This cohort is outlined in Table 2.5.

5.3.2 RNA extraction

Extraction of total RNA from 1ml blood using Trizol (Life Technologies) was performed as described in Section 2.3. RNA was extracted from approximately 100ng tissue following homogenization as previously described in Section 2.3. RNA concentration and integrity were examined by NanoDrop spectrophotometry and Agilent Bioanalyzer RNA 6000 NanoChip Kit Series II analysis, respectively, as outlined in Section 2.4 and Section 2.5.

5.3.3 RQ-PCR for miR-15 family members

Quantification of individual *miR-15* miRNAs in both blood and tissue samples was determined by RQ-PCR using TaqMan miRNA assays. Following RNA extraction, 100 ng of total RNA was reverse transcribed using TaqMan stem-loop primers and MultiScribe reverse transcriptase (Applied Biosystems). PCR reactions were performed in triplicate in final volumes of 10 μL on 96 well plates. Each plate included an inter assay control (IAC) to account for run-to-run variation and a non-template control. Plates were run on a 7900HT instrument using standard fast thermal-cycling conditions.

Raw fluorescence (cycle threshold, C_T) data were subsequently calculated. C_T values were scaled to lowest expressing sample (scaled to minimum) and normalized to *miR-16,* which has been shown to be stably expressed in breast cancer and is the most widely used endogenous control miRNA for breast cancer thus far (86, 92-94). MiRNA expression was calculated qBase Plus software which employs the comparative cycle threshold (ΔC_T) method (135).

5.3.4 MiRNA target prediction

Identification and validation of genes targeted by miRNAs is a crucial step in furthering our understanding of the roles specific miRNAs play in controlling gene expression at the post-transcriptional level. In an effort to predict and understand interactions between miRNAs targeting several online computational algorithms have been devised, employing different approaches for target prediction (Table 2.15). These algorithms provide a practical means of narrowing the funnel of potential mRNA targets to validate in scientific experiments.

Bioinformatic analyses was performed to identify putative miRNA-mRNA duplexes by employing three independent target prediction algorithms, namely TargetScan, PicTar and Diana Lab. A list of potential miRNA-mRNA duplexes was produced for further validation.

5.3.5 Predicted target mRNA expression

These candidate miRNA-mRNA duplexes were validated by measuring the mRNA expression levels of individual target genes and correlating with miRNA expression in the same tissue specimen. To achieve this, a dataset derived from a whole genome expression microarray previously conducted in the department was utilised. The experiment was performed on RNA derived from 30 breast tumours, which was simultaneously hybridised to probes representing in excess of 27,000 individual human genes, on an Applied Biosystems platform. Global normalisation of the microarray dataset was achieved using Bioconductor R[21] . MiRNA expression was determined on a cohort of the same tissue specimens (Table 5.3).

[21] http://www.bioconductor.org/

Table 5.3 Clinicopathological data for breast tumours used for mRNA target analysis

Breast Tumours		**Number**
Number		**18**
Mean age, years (range)		52.50 (±7.09)
Median Whole. T size (mm)		22.7 (±7.3)
Histological Subtype		
	Invasive ductal carcinoma	18
Grade		
	1	2
	2	2
	3	12
	Missing	2
UICC stage		
	Stage 1	6
	Stage 2	12
	Stage 3	0
Intrinsic Subtype		
	Luminal A	9
	Luminal B	0
	HER2/*neu* over-expressing	4
	Basal	5
Menopausal Status		
	Pre	6
	Post	12
Nodal Status		
	Positive	0
	Negative	18

5.3.6 Statistical Analysis

Statistical analysis was performed using Minitab version 16.0. Data were visually examined and the Kolmogorov-Smirnov test for normality was conducted. Data were log transformed (log_{10}) for analysis when non-normal distribution was identified. Logged data was also assessed for normality. Significance and associations of circulating and tissue miRNA levels and mRNA targets were determined using the Mann-Whitney U test, t-test, ANOVA, Spearman's Rho or Pearson correlation, as deemed appropriate. P-values less than 0.05 were deemed to be significant. Binary logistic regression analysis was applied and receiver operating characteristic (ROC) curves were generated to determine the ability of chosen miRNAs to differentiate between cancer cases and controls. This was performed both individually and for combinations of miRNAs.

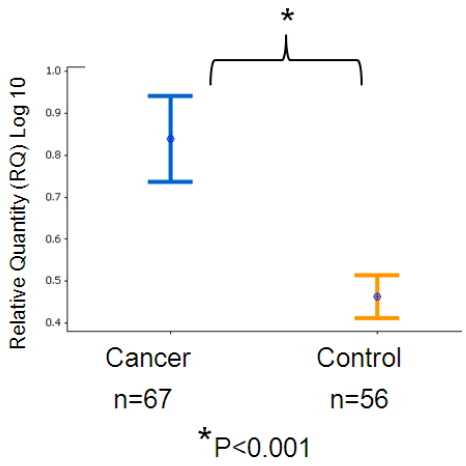
5.4 Results

5.4.1 Expression profiles of miR-15 miRNAs in blood and tissue

MiR-15 miRNAs (*miR-15a, miR-15b, miR-16, miR-195, miR-424* and *miR-497*) were detectable in the blood and tissue of women with breast cancer. We investigated the expression profile of *miR-15* miRNAs in the blood of consecutive women with a new diagnosis of breast cancer (n=67) and compared against that of healthy controls (n=56). We also evaluated miRNA expression in tumour specimens (n=20) and compared against tumour associated normal (TAN, n=10). The results are presented in Figure 5.3, 5.4 and 5.5.

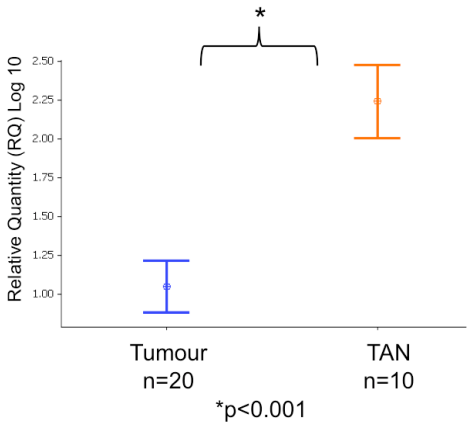
MiR-15a was significantly under-expressed in the blood of women with breast cancer ($p<0.001$) with no alteration in expression between tumour and TAN. *MiR-15b* was significantly underexpressed in both the blood and tissue of women with breast cancer ($p=0.013$ and $p=0.015$, respectively). Consistent with previous findings from our group on a separate cohort of patient samples, *miR-195* was significantly overexpressed in blood of women with breast cancer compared to those who did not have breast cancer ($p<0.001$, Figure 5.3). However, in this cohort of patients, *miR-195* was underexpressed in breast tumour tissue compared to tumour associated normal (TAN) ($p<0.001$). There was no alteration in *miR-424* expression in circulation; however it was overexpressed in the tumours of women with breast cancer in our study. *MiR-497* was underexpressed in both the blood and tissue of women with breast cancer compared to healthy controls ($p=0.046$ and $p=0.033$, respectively).

Figure 5.3 Circulating *miR-195* expression



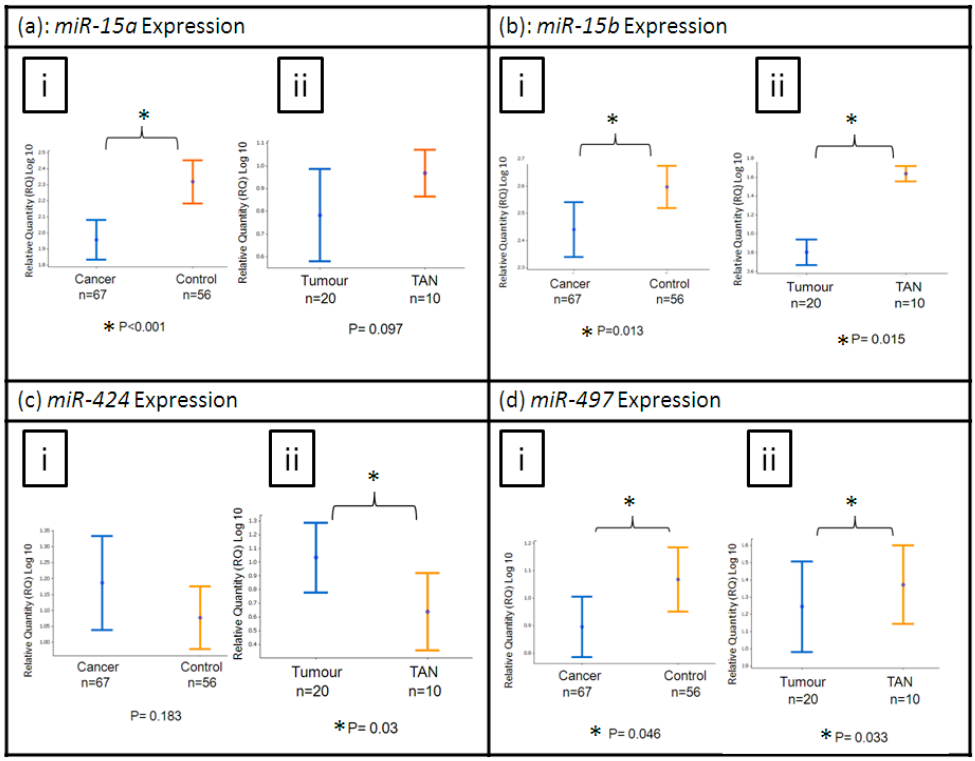
MiR-195 expression in relative quantity on the Log_{10} scale in blood of women with breast cancer (blue) and normal controls (orange). *MiR-195* was significantly over-expressed in the cancer group ($p<0.001$) compared to the control group.

Figure 5.4 Tissue *miR-195* expression



MiR-195 expression in relative quantity on the Log_{10} scale in breast tumour tissue (blue) and tumour associated normal (orange). *MiR-195* was significantly under-expressed in the cancer group ($p<0.001$) compared to the control group.

Figure 5.5 Relative expression of *miR-15* family miRNAs in blood and tissue



Blood miRNA expression (i) is on the left side for each miRNA while tissue miRNA expression (ii) is on the right side for each miRNA. For blood miRNA expression, the cancer group is in blue while the control group is in orange. For tissue miRNA expression, tumour miRNA expression is in blue while tumour associated normal (TAN) miRNA expression is in orange.

5.4.2 *Expression level of miRNAs and breast cancer clinicopathological details*

Blood miRNA expression was compared against certain clinicopathological variables, namely stage, grade and nodal status (Table 5.4). There was no significant difference in *miR-15* family expression across tumour grade or stage of disease. Similarly there was no difference in miRNA expression between node positive or negative tumours. Altered miRNA expression in the cancer group, irrespective of stage of disease is an interesting property, suggesting a potential role as circulating biomarkers for breast cancer.

Table 5.4 MiRNA expression and clinicopathological parameters

Clinicopathological Parameter	miRNA	P-value
Stage*	miR-15a	0.931
	miR-15b	0.288
	miR-195	0.540
	miR-424	0.084
	miR-497	0.648
Grade*	miR-15a	0.858
	miR-15b	0.366
	miR-195	0.962
	miR-424	0.419
	miR-497	0.121
Nodal Status**	miR-15a	0.686
	miR-15b	0.347
	miR-195	0.170
	miR-424	0.128
	miR-497	0.482

There was no significant difference in miRNA expression across different clinicopathological parameters.
* p-value determined by one-way ANOVA
** p-value determined using 2-sample *t*-test

5.4.3 Biomarker potential of miR-15 miRNAs

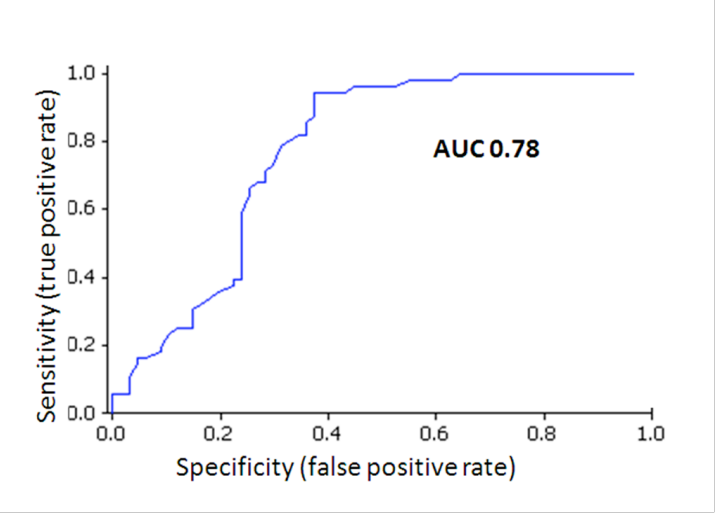
The altered expression of several of the *miR-15* miRNAs in the blood of women with breast cancer when compared to healthy controls regardless of stage of disease, unveiled putative roles for these miRNAs as circulating biomarkers for breast cancer. Binary logistic regression analysis was performed, for individual miRNAs, and miRNAs in combination, to generate receiver operator characteristic (ROC) permitting comparisons against resulting area under the curve (AUC), sensitivity and specificity (Table 5.5). The highest AUC of 0.89 was generated from a combination of *miR-15a*, *miR-195* and *miR-497,* correlating with a sensitivity and specificity of 82% and 76.5%, respectively (Figure 5.7).

Table 5.5 AUC, sensitivity and specificity profiles of *miR-15* family miRNAs in isolation and in combination

miRNA	AUC	Sensitivity (%)	Specificity (%)
miR-15a	0.71	85.1	48.2
miR-15b	0.64	74.6	48.2
miR-195	0.78	71.6	71.4
miR-424	0.55	71.6	17.9
miR-497	0.61	76.1	35.7
miR-15a and miR-195	0.88	80.6	76.8
miR-195 and miR-497	0.82	68.7	76.8
miR-15a, miR-195 and miR-497	0.89	82.0	76.5

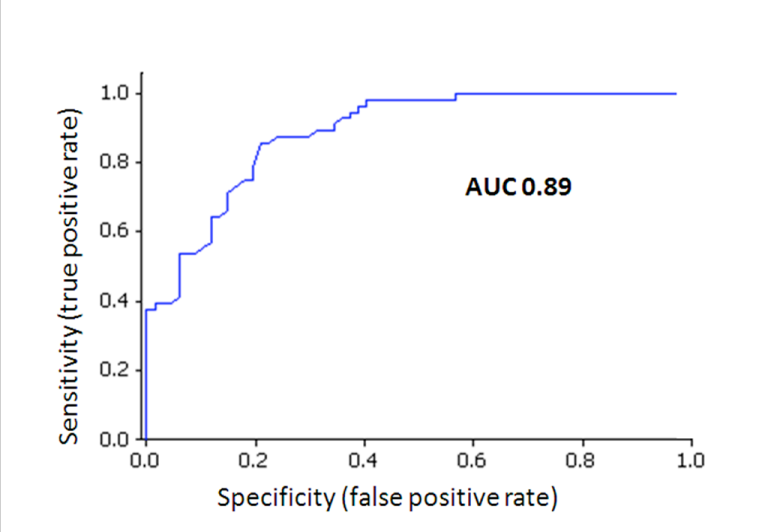
AUC, Area Under the Curve

Figure 5.6 Receiver operator curve for *miR-195* alone



This ROC curve, created using binary logistics regression, demonstrates the performance of *miR-195* in discriminating between women with breast cancer and those who do not have breast cancer. The Area Under the Curve (AUC) is accuracy by which this miRNA profile differentiates between these two groups. An AUC of 0.78 provides a sensitivity of 71.6% and a specificity of 71.4%.

Figure 5.7 Receiver operator curve for a 3-miRNA profile including *miR-15a, miR-195* and *miR-497*

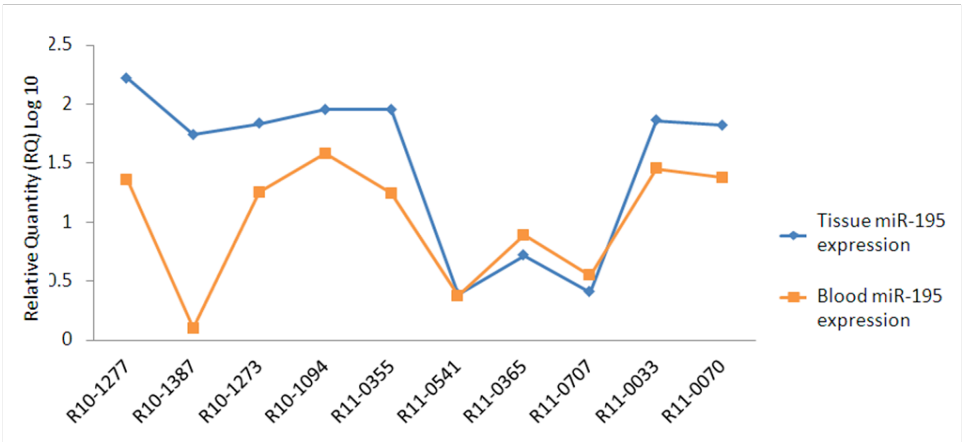


This ROC curve, created using binary logistics regression, demonstrates the performance of *miR-15* miRNAs (*miR-15a, miR-195* and *miR-497*) in discriminating between women with breast cancer and those who did not have breast cancer. The area under the curve (AUC) depicts the accuracy by which this trio of miRNAs differentiated between cancers and controls. An AUC of 0.89 provided a sensitivity of 82% and a specificity of 76.5%.

5.4.4 Matched tissue and blood miRNA profiles

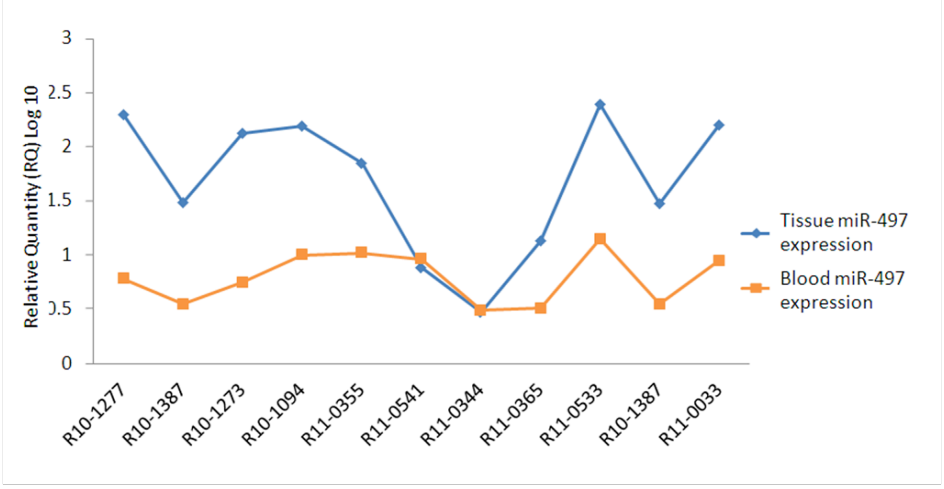
Given the expression profiles of the *miR-15* miRNAs, and the interesting similar altered expression patterns at both the tumour and blood level for certain miRNAs (namely *miR-195* and *miR-497*), we wished to determine if, for specific miRNAs of interest, circulating miRNA expression reflected that of the tumour. Pre-operative blood specimens and intra-operative tumour specimens were collected from the same patient (n=15). There was a significant correlation between tissue and blood *miR-195* (p=0.043, R=0.64, Figure 5.8) and *miR-497* (p=0.047, R= 0.611, Figure 5.9) expression.

Figure 5.8 *MiR-195* expression in matched blood and tissue samples



MiR-195 expression in matched blood (orange) and tissue (blue) samples. There was a significant strong correlation between blood and tissue *miR-195* expression (p=0.043, R=0.642).

Figure 5.9 *MiR-497* expression in matched blood and tissue samples



MiR-497 expression in matched blood (orange) and tissue (blue) samples. There was a significant correlation between blood and tissue *miR-497* expression (p=0.047, R=0.611).

5.4.5 Target prediction

In silico target prediction was then performed in order to elucidate the functional roles, if any, of *miR-15* miRNAs in cancer. Three online target prediction algorithms were used exploited, namely PicTar, TargetScan and DianaLAB. The predicted miRNA-mRNA duplexes are summarized in Table 5.6.

Table 5.6 Predicted miRNA-mRNA duplexes by TargetScan, PicTar and DianaLAB

mRNA	TargetScan	PicTar	DianaLab
Method	**Seed Complementary**	**Thermodynamics**	**Experimentally Validated Targets**
	TargetScan Score	**PicTar Score**	**Previously validated**
BCL2	-0.03	2.08	Yes
CCND1	-0.22	5.57	Yes
UNC80	-0.78	No	No
CD80	No	no	No
VEGFa	-0.44	no	Yes
BCL2L2	-0.35	11.01	No
ARL2	-0.65	19.30	No
LUZP1	-0.20	14.44	No
KIF1B	-0.53	12.67	No
FGF2	-1.54	12.53	No
MAP2K1	-0.35	2.91	No

The target prediction output indicating predicted miRNA-mRNA duplexes from each of these resources were examined and cross validated to identify common predicted mRNA targets with putative roles in carcinogenesis. The seed sequence is the most important component of the miRNA molecule in determining binding and target mRNA identification. As all *miR-15* miRNAs share a common seed sequence, any predicted mRNA target could potentially act as a target for any of the *miR-15* family.

5.4.6 Target mRNA expression

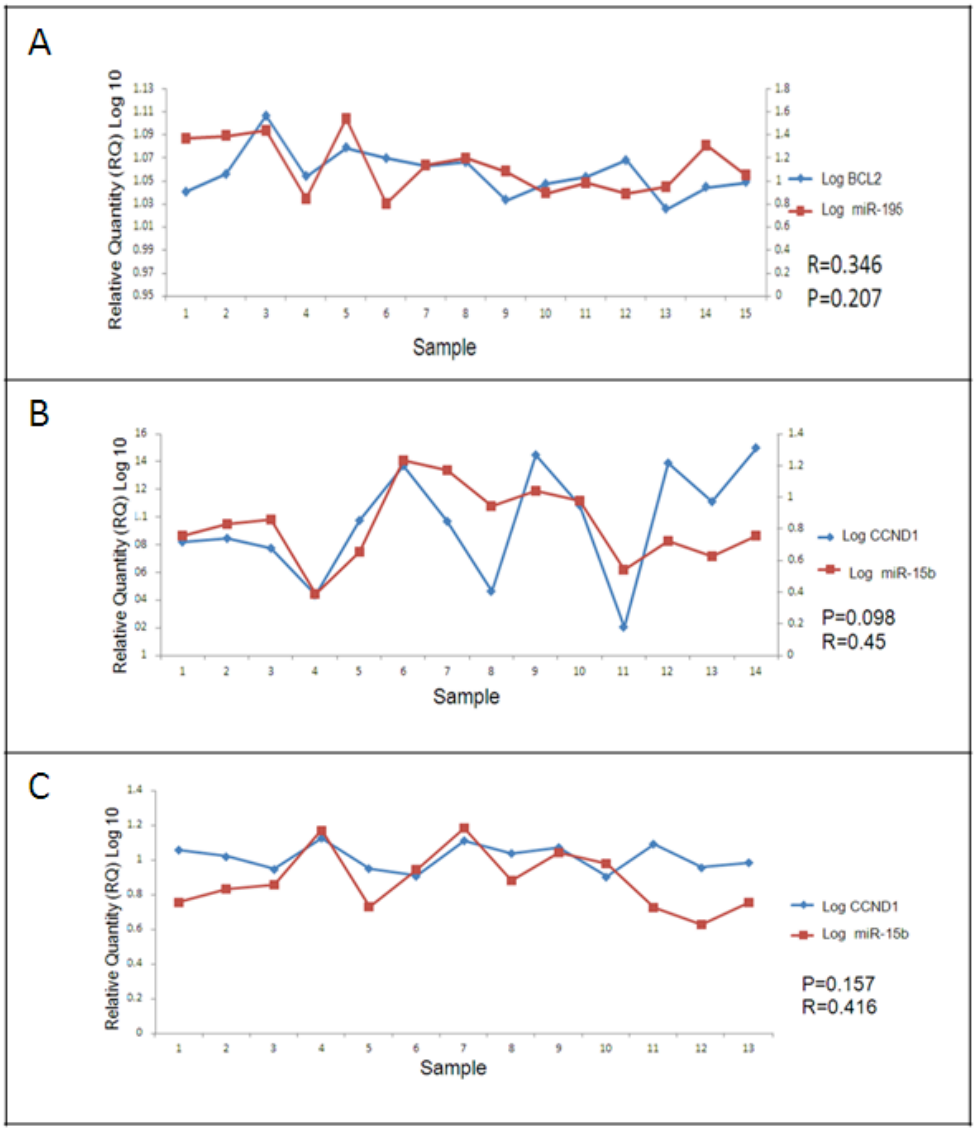
MiRNA and target mRNA expression was determined on a cohort of breast tumours presented in Table 5.3 for three of the predicted mRNA targets. Despite previous validation of two of the miRNA-mRNA interactions, no significant inverse correlations (as would be expected as a consequence of miRNA binding resulting in mRNA cleavage) were found (Table 5.7). Interestingly, direct correlations were observed between *miR-195* and BCL2 expression (R=0.34), *miR-15b* and CCND1 expression (R=0.46) and *miR-15b* and FGF2

(R=0.41). These correlations were moderate in strength (0.25<R>0.75) and were not statistically significant. These results are presented in Figure 5.10.

Table 5.7 Correlation between miRNA and mRNA expression

Clinicopathological Parameter	miRNA	P-value	Pearson Correlation Coefficient
BCL2	miR-15a	0.407	0.215
	miR-15b	0.769	-0.077
	miR-195	0.207	0.346
	miR-424	0.668	0.116
	miR-497	0.273	0.314
CCND1	miR-15a	0.724	-0.093
	miR-15b	0.098	0.460
	miR-195	0.561	0.170
	miR-424	0.158	-0.370
	miR-497	0.826	-0.060
FGF2	miR-15a	0.856	0.048
	miR-15b	0.157	0.416
	miR-195	0.137	0.389
	miR-424	0.833	0.057
	miR-497	0.691	0.104

Figure 5.10 Correlations between *miR-15* family miRNAs and predicted targets



The correlation between (A) *miR-195* expression and BCL2 expression in the same breast tissue (Pearson correlation R=0.346) (B) *miR-15b* and CCND1 expression in the same breast tissue (Pearson correlation R=0.45) (C) between *miR-15b* and FGF2 expression in the same breast tissue (Pearson correlation R=0.416).

5.5 Discussion

This observational study is the first to report on the expression of a miRNA family in the circulation of women with breast cancer. The results corroborate the putative role for breast cancer associated miRNAs to act as circulating biomarkers. The data demonstrates that *miR-15* miRNAs were aberrantly expressed in both blood and tissue of women with breast cancer. Those miRNAs with altered expression (under or over-expression) in the

circulation were uniformly dysregulated in the breast cancer state regardless of tumour grade, stage of disease or nodal status. This study shows that, in a select number of cases, circulating *miR-195* and *miR-497* level reflected those of the tumour unveiling the exciting possibility that circulating miRNAs may shed light on underlying tumour biology. The combination of *miR-15a, miR-195* and *miR-497* yielded a sensitivity and specificity for discrimination between women with breast cancer and healthy controls of 82% and 76.5%, respectively. Although not perfect, this profile exceeds that of several commonly used clinical biomarkers (Table 5.8). Finally, in an effort to elucidate the functional role, if any of the *miR-15* miRNAs *in silico* analysis was performed to identify predicted miRNA-mRNA duplex interactions. MiRNA and predicted target mRNA expression levels were determined on three putative targets (CCND1, FGF2 and BCL2). The expression patterns of genuine miRNA-mRNA interactions would be expected to correlate inversely. No such expression patterns were observed, however a number of moderate direct correlations were noted.

Table 5.8 Sensitivity and specificity of commonly used circulating clinical biomarkers

Biomarker	Condition	Sensitivity	Specificity	Ref.
Troponin	Acute MI	94%	84%	(255)
3 miRNA Profile	***Breast Cancer***	***82%***	***76.5%***	NA
CA-125	Ovarian Ca	50%	78%	(256)
PSA	Prostate Ca	21%	91%	(257)
D-dimers	DVT	93%	50%	(258)
AFP	HCC	60%	90%	(259)
B-hCG	Ectopic Pregnancy	36%	63-71%	(260)

Breast cancer is a prevalent disease accounting for the most commonly diagnosed cancer in women, with a worldwide incidence of over 1,300,000 (3). When diagnosed and treated early breast cancer is a curable disease. While the incidence of breast cancer has increased over the past few decades, mortality from breast cancer has fluctuated less resulting in a divergence between incidence and mortality (260). Breast cancer mortality primarily results from metastases, disease spread to distant organs. Breast cancer is a disease in need of a novel group of biomarkers to facilitate both earlier diagnosis and prediction and monitoring of response to adjuvant therapies. Like several other solid cancers, breast cancer is a heterogeneous disease reflecting a multitude of individual molecular alterations likely to be responsible in the initiation and progression of carcinogenesis. Most breast tumours can be broadly classified into one of four intrinsic subtypes, with a plethora of subgroups likely to

exist within each of these subtypes (41, 45). Our increasing understanding od the complex nature of cancer has lead to a shift in the way we treat these disease. The days of uniform diagnostic and therapeutic approaches have been superseded by an era of personalised or individualised medicine which is advancing in parallel with our comprehension of molecular biology. MiRNAs are a relatively new discovery, and have become a focus of translational research.

MiRNAs are fragments of the genome once thought to be 'junk' RNA. It is now known that these short strands of RNA have important regulatory functions, influencing almost every aspect of the cell cycle, despite having no direct role in protein translation. MiRNAs function at the post translational level and exhibit their effects by either translational repression or mRNA cleavage. Despite the epigenetic nature of their role, the importance of miRNAs in both health and disease cannot be underestimated. For the most part, miRNAs are thought to finely tune gene expression. However, it has been shown that several miRNAs may have more potent roles. Ventura *et al* demonstrated that deficiency of specific miRNAs within the *miR-17-92* cluster has major implications for phenotype in a murine model of knock-out mice, with mice deficient in *miR-17* suffering from lung hypoplasia and a ventricular septal defect resulting in death shortly after birth (261). The important functional role of specific miRNAs has also been corroborated *in vivo*, with the description of two conditions in humans believed to be as a consequence of miRNA depletion; the first is a non-syndromic progressive hearing loss and the second has been described as Feingold syndrome (262, 263). Studies of this nature are extremely informative; miRNAs can be exploited as circulating biomarkers but it is important to remember that several have an important functional role and are not mere by-products of biological and pathological processes.

Despite the abundance of studies on miRNAs in cancer, few studies have profiled a miRNA family in breast cancer, with no previous reports of miRNA family expression in circulation (231, 264). The findings of this study are interesting, despite the same seed sequence, and therefore several overlapping putative mRNA targets, the *miR-15* family members exhibit fluctuations/differences in circulating expression. This could be explained by several theories. Firstly, it is possible that although still present and detectable, some members of this family could be functionally redundant, a concept which has been elegantly proven in a plant model for the *miR-164* family (265). If this holds true, some *miR-15* family members

may be more biologically important than others. This is noteworthy, especially in the setting of the inverse circulating expression pattern of *miR-195* and *miR-497* reported in this study, despite their co-location on Chromosome 17. Secondly, varied *miR-15* family expression could be explained by a difference in the mechanisms by which these miRNAs arrive in the circulation. The means by which miRNAs enter the blood stream remains poorly understood. Some miRNAs are selectively secreted from tumour epithelial cells or even circulating micro-vesicles (133). It is plausible that any cell in the tumour microenvironment could secrete miRNAs in this manner. Other miRNAs may appear in the circulation as a consequence of passive leakage from cells undergoing apoptosis and necrosis (213). The shedding mechanism of each *miR-15* family has yet to be fully elucidated. Another theoretical explanation for this finding could be the origin of specific members of the *miR-15* family, some of these miRNAs could emanate from the tumour microenvironment itself; however other miRNAs may play a role in the immune (host) response to cancer, as demonstrated for *miR-155* by Dudda *et al* (266).

Altered expression of the *miR-15* miRNA family has been described for other cancers however very few studies have been conducted for circulating miRNA expression. Seminal miRNA studies by Calin *et al*, identified *miR-15a* and *miR-16*, co-located at Chromosome 13q14.3, as being lost or downregulated in the majority of Chronic Lymphocytic Leukaemia (CLL) and was the first evidence that miRNAs are involved in human carcinogenesis (267). Indeed this miRNA pair has been shown to be dysregulated in other cancer tissues (240, 268). *MiR-15b* was one of the first miRNAs reported as being detectable in the circulation (133). Alterations in *miR-15b* expression in the circulation have been reported for hepatocellular carcinoma (269). Its distorted expression has been reported frequently at the tissue level (244, 270). Although less common, *miR-424* has also been implicated in cancer but with no reports in the circulation. *MiR-424* is overexpressed in colorectal cancer tissue and has been shown to have a role in cancer-associated pathways (252-254).

MiR-195 and *miR-497* are co-located on chromosome 17. In the present study, we have shown that *miR-195* is overexpressed while *miR-497* is underexpressed. This study validates the work of Heneghan *et al* which showed overexpression of *miR-195* in the circulation of an independent cohort of women with breast cancer (93). Since then, *miR-195* has also been described as a diagnostic biomarker for gastric cancer (235). *MiR-195* plays a functional role in a number of cancer related pathological processes and has also been

exploited as a modulator of response to adjuvant chemotherapy in colon cancer (234, 271-279). *MiR-497* was found to be underexpressed in the peripheral circulation and tissue of women with breast cancer in this study. Li et al were the first to report on *miR-497* in breast cancer and found that it was underexpressed in breast cancer cell lines (191). Since then it has been reported as a having a tumour suppressor role in other solid cancers (234, 280). However this study is the first that examines circulating *miR-497* expression in cancer patients.

A strength of this study lies in the availability of blood and tissue specimens from patients with breast cancer and healthy controls. Despite the surge of interest in miRNA-related cancer research, few studies have reported on the relationship between tumour and circulating miRNA expression level in cancer patients (281). From a circulating diagnostic biomarker perspective the most important feature is consistently reproducible variations in miRNA expression level, regardless of the source of the specific miRNA or panel of miRNAs. However, if the capacity of circulating miRNAs is to transcend from simple diagnostics (Cancer: yes/no) to a more informative platform that could function almost as a fluid biopsy (breast tumour subtype prediction, for example), an understanding of the relationship between tumour and tissue miRNA expression level is crucial. In this study, we report that circulating *miR-195* and *miR-497* levels reflect that of the tumour suggesting that circulating miRNAs could act as the molecular fingerprint of the tumour phenotype.

We exploited online computational algorithms in an effort to elucidate potential miRNA-mRNA interactions. Target prediction is a complicated task as there are potentially hundreds of targets for each miRNA, with estimates of miRNAs governing in excess of 30% of protein coding genes (58). In addition, one mRNA could be targeted by multiple miRNAs or have multiple sites for the same miRNA (143). An additional layer of complexity is added to miRNA target prediction due to our limited knowledge of the rules governing interactions between miRNA-mRNA duplexes. These algorithms provide a practical cost effective means of narrowing the funnel of potential mRNA targets to validate in scientific experiments. Predicting miRNA-mRNA interactions is complex and explains why algorithms based on this feature alone would be inadequate. The differing results produced by alternative algorithms can be explained by the additional features taken into account, including the degree of stringency governing complementarity between the mRNA and the miRNA, free energy, where the target site is positioned (5'UTR, 3'UTR, coding sequence),

mRNA target site conservation and other additional attributes (144). Multiple target sites on an mRNA for the same miRNA may enhance the degree of downregulation, with some algorithms also taking this into consideration (145).

Three mRNA targets were selected for analysis in this study for their known involvement in cancer; BCL2, CCND1 and FGF2. Bcl-2 (B-cell lymphoma 2) is encoded by the BCL-2 gene and is the cardinal member of the Bcl-2 protein family which functions in the complex regulation of apoptosis (282). Bcl-2 has been implicated in a number of cancers. In health, Bcl-2 works to repress apoptosis. However, damage to the Bcl-2 gene and subsequent overexpression, which occurs in several cancers, can impede apoptosis in mutated cells, resulting in cancer initiation, and potentially metastases. Bcl-2 has been validated as a target for a number of *miR-15* family members. *MiR-15a* and *miR-16* have been shown to target Bcl-2 in Chronic Lymphocytic Leukaemia (CLL) (140). *MiR-15b* has been validated as targeting Bcl-2 in gastric cancer (139). Liu *et al* reported that *miR-195* was downregulated in colorectal cancer, with an anti-apoptotic effect (279). Luciferase reporter plasmids containing the Bcl-2 3'UTR were constructed and exploited *in vitro* to demonstrate that *miR-195* directly targets Bcl-2. Furthermore, they revealed that the pro-apoptotic effect of *miR-195* is mediated through targeting of Bcl-2. This relationship between *miR-195* and Bcl-2 has also been demonstrated in breast cancer (283).

CCND1, cyclin-D1 or G1/S-specific cyclin-D1 is a protein encoded by the CCND1 gene forming a member of the cyclin family which is highly conserved (284). Cyclin proteins typically regulate cyclin-dependent kinases (CDKs) which play critical roles in the cell cycle. Cyclin-D1 exerts its effects during G1/S transition, functioning as a subunit of CDK4 or CDK6. CCND1 mutations and overexpression with resulting effects on the cell cycle has been observed in several cancers and may be a contributing factor in tumourigenesis. This oncogene is amplified in 15% of primary breast cancers and overexpressed in 30-50% of breast cancers (285). *MiR-195* was shown to suppress tumourigenesis with regulatory role in cell cycle (G1/S) transition in HCC by targeting multiple molecules such as cyclin D1, CDK6 and E2F3 (141). *MiR-16* and *miR-424* have been shown to directly target CCND1 in HCC (142).

FGF2 (Basic Fibroblast Growth Factor or FGF-ß) is found in cellular basement membranes and in the subendothelial extracellular matrix of blood vessels. It binds heparin and has a

broad range of function including angiogenesis and mitosis resulting in involvement in development (limb and nervous system), wound healing and tumour growth. At the time of writing, there were no reported interactions between the *miR-15* family and FGF2. However, FGF2 was identified as a putative mRNA target using the aforementioned computational algorithms and subsequently was selected for further evaluation given its implication in various cancer related pathways.

No inverse correlations between *miR-15* family miRNA expression and predicted target expression were detected. Inverse correlations would be expected as miRNAs typically regulate gene expression by mRNA cleavage or translational repression, thus reducing the amount of mRNA detected. In recent times, there have been reports of miRNAs with the capacity to increase target mRNA expression (69, 71). Experimental validation of miRNA-mRNA target interactions is essential to help discriminate miRNAs with biological relevance.

We recognise that this study has limitations. Phylogeny for this group of miRNAs is incomplete and an exhaustive description of its members is not possible and will continue to grow as further miRNAs are identified. These miRNAs were chosen for analysis at the time of study conception; however this family also includes *miR-103, miR-107, miR-503, miR-646* and several others depending on the source used and the date accessed. Although the *miR-15* miRNAs presented in this study share the same seed sequence and thus belong to the same miRNA family, several further classifications are likely to exist, including subfamilies and superfamilies. Some have been described while others will evolve as further information becomes available (142, 286, 287). The *miR-15* superfamily contains those miRNAs described in this study, in addition to *miR-322* and *miR-457*, and is believed to have evolved from the common ancestor of all vertebrates (288-290).

5.6 Conclusion

This study contributes to the literature on miRNAs in cancer. It is the first report of a miRNA family in the circulation of those with breast cancer. *MiR-15* family miRNAs are aberrantly expressed in the circulation of women with breast cancer compared to controls. The numerous remarkable properties of the *miR-15* miRNAs and their altered expression patterns and potential role in breast cancer make them promising biomarkers and therapeutic targets in the future.

Chapter 6

Analysis of miRNA Expression in the Tumour Microenvironment

6.1 Introduction

MiRNA quantification by RQ-PCR provides precise information about the relative quantity of miRNAs, but lacks the ability to provide critical spatial information on the location of miRNAs within the tumour microenvironment. Using RQ-PCR alone, it is not possible to determine the responsible component for the altered miRNA expression; whether it is the cancer epithelial cells themselves or the surrounding non-malignant cells comprised of fibroblasts, myofibroblasts, adipocytes, non-tumour epithelial cells, immune cells and endothelial cells. The understanding of the intimate dynamic communicating relationship and crosstalk between tumour cells and the surrounding microenvironment during carcinogenesis has advanced in recent years. It is now accepted that seemingly normal surrounding cells contribute towards the neoplastic microenvironment and may be as important as the cancer cells themselves (291). The source of dysregulated miRNAs in circulation may not necessarily be the tumour epithelial cells, but may be another cell type in this neoplastic niche.

MiR-195 and *miR-497* are members of the *miR-15* family and are clustered on chromosome 17 (17p13.1). *MiR-195* has been shown in Section 5.4.1 and in previous studies to be over-expressed in blood of women with breast cancer compared to healthy controls (93). *MiR-195* has been shown in Section 5.4.1 to be underexpressed in a small cohort of breast tumour tissue compared to tumour associated normal. Li *et al* also reported *miR-195* to be downregulated in breast cancer cell lines (191). Using matched tissue and blood specimens from the same patient, circulating *miR-195* expression levels were shown to reflect that of the tumour tissue in Section 5.4.4.

Interestingly *miR-497*, which is co-located with *miR-195,* exhibits an inverse expression pattern in circulation, with underexpression in blood of women with breast cancer compared to healthy controls. Li *et al* discovered that *miR-497* is downregulated in breast cancer cell lines. This work is the first to report on *miR-497* in circulation of women with breast cancer. Similar to *miR-195* expression, circulating *miR-497* expression levels are shown to reflect that of the tumour tissue in Section 5.4.4.

This supports the hypothesis that circulating miRNAs could be exploited to act as a window on tumour activity. However, the breast tumour specimens collected at the time of surgical resection contain a breast tumour with an assortment of different cell types found in the

tumour microenvironment. No information can be extrapolated regarding the cellular origin of these circulating miRNAs. The specific origin of *miR-195* and *miR-497* is not of critical importance in exploiting these miRNAs as biomarkers for breast cancer. However, this becomes more critical in elucidating the role these miRNAs are playing in carcinogenesis and also in determining whether miRNA expression manipulation could be explored as a viable therapeutic avenue for breast cancer. *In situ* hybridisation (ISH) is a technique which enables the direct assessment of miRNA expression in vivo, providing localisation of miRNAs in the tumour microenvironment (103). ISH uses locked nucleic acid (LNA) based oligonucleotides to provide sensitive and specific miRNA localisation.

The application of miRNAs as biomarkers of disease exploits the dysregulation of a specific miRNA or a panel of miRNAs in the disease state. There are numerous studies reporting on altered miRNA expression in blood and tissue of women with breast cancer, with comparatively few studies reporting on the mechanism/pathway resulting in their subsequent dysregulation. Epigenetics refers to the inheritance of gene expression alterations with no change in the DNA sequence (104). There is a growing body of evidence that suggests that epigenetic and genetic variation may be responsible for altered expression of a number of miRNAs (105, 292). DNA methylation is a type of epigenetic modification which causes phenotypic alterations as a consequence of gene expression repression. Hypermethylation of promoter DNA has been shown to silence the expression of some specific miRNAs and has been implicated in tumourigenesis (106). DNA methylation in mammals predominantly occurs at the C-5 position of complementary CpG by DNA methyltransferases (107). DNA methylation of CpG islands was found to be at promoter regions of tumour suppressors miRNAs, with under-expression in the cancer state. If a miRNA is under epigenetic regulation by methylation, the addition of a de-methylation agent would increase the target miRNA expression. Once the miRNAs of interest, *miR-195* and *miR-497*, were localised in the tumour microenvironment, the potential influence of such epigenetic modification in modulating expression of *miR-195* and *miR-497* was assessed to determine if methylation could play a role in altering the expression of these miRNAs in carcinogenesis.

6.2 Aims

The aims of this study were:

- To determine where in the cellular localisation of *miR-195* and *miR-497* using *in situ* hybridisation (ISH)
- To assess *miR-195* and *miR-497* expression in different breast cancer cell lines
- To evaluate if epigenetic modulation by methylation altered *miR-195* and *miR-497* expression *in vitro*

6.3 Materials and Methods

6.3.1 Study groups

6.3.1.1 ISH cohort

Following ethical approval and written informed consent, a cohort of 10 Luminal A breast cancer patients for whom both whole blood samples and paraffin tissue blocks were available were selected for inclusion in the *in situ* hybridisation study. This patient cohort is that included in microarray cohort outlined in Chapter 4. The clinicopathological details of this group are outlined in Table 2.7.

Blood samples were collected pre-operatively in 10ml vacuette EDTA K3E blood bottles (Grenier Bio-one) by peripheral venous sampling. Paraffin embedded tissue blocks of the breast tumours were retrieved for these patients in collaboration with the Department of Histopathology, NUI Galway.

6.3.1.2 Cell lines

The cell lines used in this study were obtained from the American Type Culture Collection (ATCC). Their characteristics are outlined in Table 2.14. Cells were cultured as outlined in Section 2.15. Cells were cultured for 5 days prior to harvesting.

6.3.2 RNA extraction from whole blood

RNA isolation from whole blood was performed using Trizol, a phenol-chloroform based extraction technique as described fully in Section 2.3. In brief, total RNA was extracted from whole blood samples by phase separation with the addition of Trizol (3 mL Trizol/I mL whole blood) supplemented with 200µL 1-bromo-4-methoxybenzene (BAN, Molecular Research Centre). Total RNA was then precipitated using isopropanol and followed by a

75% ethanol wash. RNA pellets were then re-solubilised in a total of 60 µL nuclease free water.

6.3.3 RNA extraction from cell pellets

Total RNA was extracted from cell lines using the RNeasy MiniKit® (Life Technologies), the details of which are described in Section 2.3. A cell pellet was prepared as described in Section 2.16.6 in advance of RNA extraction and frozen at -80°C until extraction. Samples were thawed gently on ice and extracted in 1mL Trizol using the RNeasy Mini Kit® and manufacturer's protocol was used.

6.3.4 Analysis of RNA concentration

The quantity of extracted RNA was estimated using NanoDrop spectrophotometry (NanoDrop Technologies) as described in Section 2.4. The quality of extracted RNA was assessed by the Agilent Bioanalyzer as presented in Section 2.5.

6.3.5 Analysis of miR-195 and miR-497 expression by RQ-PCR

Extracted RNA was reverse transcribed using primers specific to *miR-195* and *miR-497* (Table 6.1) as described in Section 2.6. In both instances, 100ng of RNA was reverse transcribed using Multiscribe and *miR-195*/*miR-497* specific RT primers. RQ-PCR was then performed using target specific Taqman® probes (Life Technologies) on the 7900 HT Fast Real-Time PCR System (Life Technologies) as detailed in Section 2.7. All samples were repeated in triplicate with interassay controls. For blood, *miR-16* was used as the endogenous control. *U6* snRNA was used as the endogenous control for the cell line study. MiRNA expression levels were determined using qBase PLUS (Biogazelle) and Minitab V16.0 (Minitab Ltd.).

Table 6.1 *MiR-195* and *miR-497* stem loop primers

miRNA	Mature miRNA sequence	Length (nt)	Stem loop primer sequence
miR-195	UAGCAGCACAGAAAUAUUGGC	21	AGCUUCCCUGGCUCUAGCAGCACAGAAAUAUUGGCACAGGGAAGCGAGUCUGCCAAUAUUGGCUGUGCUGCUCCAGGCAGGGUGGUG
miR-497	CAAACCACACUGUGGUGUUAGA	22	CCACCCCGGUCCUGCUCCCGCCCCAGCAGCACACUGUGGUUUGUACGGCACUGUGGCCACGUCCAAACCACACUGUGGUGUUAGAGCGAGGGUGGGGGAGGCACCGCCGAGG

6.3.6 In situ hybridisation of miR-195 and miR-497

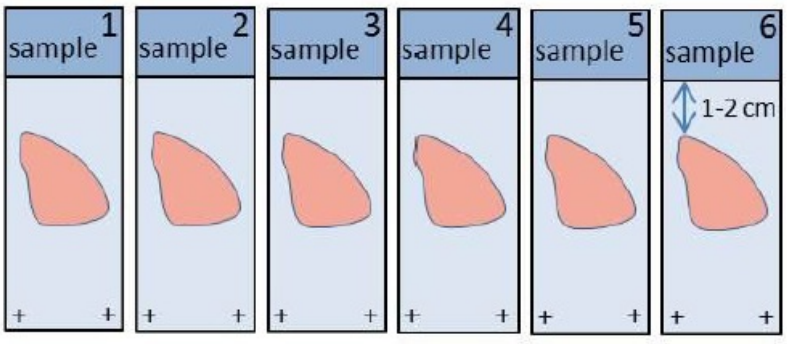
In order to localise miRNA expression *in vivo* and confirm miRNA expression levels in breast tumours, *in situ* hybridisation (ISH) analysis was performed for *miR-195* and *miR-497* on formalin fixed, paraffin embedded (FFPE) sections of breast tumours using locked nucleic acid (LNA) probes (Exiqon). The breast tumour sections were reviewed by a histopathologist to confirm presence of tumour tissue. The clinicopathological parameters of the patients included in this study are presented in Table 2.7.

6.3.7 Cutting FFPE sections

Before handling the tissue sections, the entire workstation was cleansed with RNase ZAP. The FFPE blocks were then placed on ice. Slides were prepared and labelled. Two water baths were prepared; one standard water bath containing RNase-free ddH_2O water (between 40 and 50°C) and another RNase-free coplin jar with room temperature RNase free ddH_2O water.

When cutting the FFPE sections, new disposable blades were used for each block. The cutting angle was set to 15°. The outer sections were trimmed from the block and 6 µm sections were cut and placed into a sterile Ziehl-Neehlson jar with room temperature sterilized ddH_20, to allow any folding to be reversed. The slides were then transferred to the heated water bath, where folds were stretched out before being mounted immediately on electrostatic treated slides (Superfrost Plus®). The slides were allowed to dry at room temperature for 2 hours prior to storage at 4°C in a dry box containing silica gel.

Figure 6.1 Preparation of FFPE tissue specimens for ISH analysis



The tissue sections are mounted on RNase-free Superfrost PLUS electrostatic treated slides with a 1 to 2 cm margin from the sides on the slide.

6.3.8 LNA probes

DNA oligonucleotides with 30 to 42% locked nucleic acid (LNA) substitutions (138) for *miR-195* and *miR-497* (Exiqon) were utilised, as described in Table 6.2 below. A probe specific for *miR-126-3p* was used as a positive control and a 21-mer scrambled probe with a randomly generated sequence for which there is no known complementary human sequence target was included as a negative control. All LNA-oligos for this study were double-FAM labelled, meaning 6-carboxyfluorescein (FAM) was attached at both the 5' and 3' end.

Figure 6.2 LNA structure

The ribose ring of an LNA is connected by a methylene bridge (orange) between the 2'-O and the 4'-C atoms which 'locks' the ribose ring in a conformation that allows for Watson-Crick base pairing. Subsequent incorporation into an RNA or DNA oligonucleotide LNA makes pairing with the complementary nucleotide more rapid and produces a more stable duplex[22].

Table 6.2 Probe design for ISH analysis

miRNA	Anti-parallel	RNA T_m(^oC)	LNA*(%)	Nucleotides
miR-195 (5p)	CAATATTTCTGTGCTGCTA	84	42	19
miR-497 (5p)	ACAAACCACAGTGTGCTGCT	86	30	20
miR-126-3p	CATTATTACTCACGGTACGA	84	40	20
miR-scramble	TGTAACACGTCTATACGCCCA	87	33	21

*the precise positions of the LNAs in the oligos are not disclosed by Exiqon.

[22] http://www.exiqon.com/ls/PublishingImages/Figures/LNA-molecule.htm

6.3.9 ISH analysis

A chromogenic ISH assay was utilised for miRNA detection. Chromogenic ISH takes advantage of a chromogenic precipitate generated during the enzymatic step based on Alkaline Phosphatase (AP) that converts soluble 4-nitroblue tetrazolium (NBT) and 5-brom-4-chloro-3'-indolylphosphate (BCIP) into a water and alcohol insoluble dark blue NBT-BCIP formazan precipitate. Slides are often counter stained with nuclear fast red (NFR) that gives a good contrast to the blue ISH signal when using bright field microscopy. Chromogenic ISH is a well-described and sensitive procedure, suited to miRNA studies as it allows assessment of the ISH signal and tissue morphology simultaneously.

FFPE sections (6 µm) were deparaffinised by placing the slides in xylene for 15 minutes, and then hydrated through five minute incubations in ethanol solutions (99% 3 coplin jars, 96% 2 coplin jars, 70% 2 coplin jars) ending up in PBS (2 coplin jars) before being mounted onto flow through slide chambers and placed in an automated hybridization instrument (Tecan Freedom Evo, Tecan).

In the Tecan instrument, the slides were pre-digested by treatment with proteinase K reagent (15µg/ml) and incubated at 37°C for 8 minutes. Prehybridization was then performed with the addition of 50 µl Exiqon hybridization buffer (Exiqon) and incubated at 62°C for 15 minutes. This was followed by addition of the double-FAM LNA probe diluted in Exiqon hybridization buffer. The slides were stringently washed with saline sodium citrate (SSC) buffers: 5x SCC, 1x SCC and 0.2x SCC at 62°C over 33 minutes. A blocking step was then performed with the addition of blocking solution to each slide and incubated for 15 minutes at room temperature. Alkaline phosphatase-conjugated anti-FAM (prepared by diluting in blocking solution) was then added and incubated at 30°C for 30 minutes. Enzymatic development was then performed with the addition of NBT and BCIP substrate (Roche) at 30°C for 60 minutes. An optional step was the application of nuclear fast red counterstain (Vector Laboratories). Between 200 and 300 µl of nuclear red counterstain was added to slides undergoing this step and incubated at 25°C for 1 minute.

Finally, slides were placed in staining racks and rinsed in tap water for 10 minutes before undergoing a series of one minute dehydration washes in ethanol: 70% ethanol (2 coplin jars), 96% ethanol (2 coplin jars) and 99% ethanol (2 coplin jars). Slides were then mounted

(Eukitt mounting medium), and allowed to settle overnight before analysis with light microscopy the following day.

6.3.10 Image Generation

A Visiopharm integrated microscope and software module (Visiopharm) was used for image production and analysis. This system consists of a Leica DM 6000B microscope (Leica) which is equipped with a stage and slide loader (Ludl) and a camera (Olympus DP72 CCD). Overview images were obtained for each slide using low magnification (1.25 x objective). Representative x20 images were obtained from all slides using a 20x objective. For *miR-195*, the following was observed; blue areas denoting *miR-195* staining, red areas consistent with red nuclear counterstaining and purple areas representing areas of overlap between red and blue staining.

6.3.11 Treatment of cells with 5-azacytidine

MCF-7 cells were selected as the cell line of choice for this study as represents an ER positive invasive ductal carcinoma (Luminal A) and it is the most commonly used cell line in the world. The addition of methyl groups to cytosine DNA nucleotides is catalysed by DNA methyltransferase. 5-aza-2'-deoxycytidine is a de-methylating agent that acts by inhibition of DNA methyltransferase. Cells (MCF-7) were cultured 24 hours in advance of treatment and treated with 5-aza-2'-deoxycytidine (5-Aza-dC) as described in Section 2.15.10. There were three treatment groups, namely the control group (untreated), the 5nM 5-Aza-dC group and the 10mM-Aza-cD group. After 5 days of exposure to the de-methylating agent, the cells were harvested as described in section 2.14.5.

6.3.12 Statistical analysis

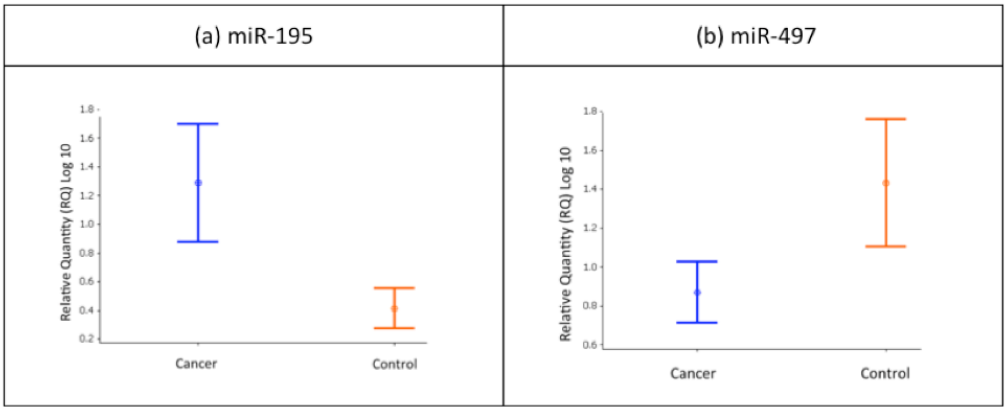
Data was analysed using qBase PLUS (Biogazelle) and Minitab V16 (Minitab Ltd.). MiRNA expression data was transformed to the log scale prior to analysis if un-normalised data was observed. Following log transformation, the 2 sample t-test was used for intra-group comparisons of normalised data. A p value of less than 0.05 was deemed significant.

6.4Results

6.4.1 Circulating miR-195 and miR-497 expression

In situ hybridisation (ISH) was performed on sections from tumours of women with high circulating *miR-195* expression levels and low *miR-497* expression. Circulating *miR-195* and *miR-497* expression of the patients included in the ISH cohort are presented in Figure 6.3 below.

Figure 6.3 *MiR-195* and *miR-497* expression in blood of women for ISH analysis



MiR-195 is overexpressed in the blood of women with breast cancer (n=10) included in the ISH cohort compared to the control group (n=10). *MiR-497* is underexpressed in the blood of women with breast cancer in this ISH cohort (n=10) compared to those who do not have breast cancer (n=10).

6.4.2 Localisation of miR-195 and miR-497 in breast tumour tissue by in situ hybridisation

A high affinity locked nucleic acid (LNA) containing DNA oligos labelled at both the 5' and the 3' ends with FAM (double carboxyfluorescein (FAM)-labelled)was employed for specific identification of *miR-195* and *miR-497* in breast tissue sections. Experimental conditions were optimised during the first two experiments (Experiment 1 and Experiment 2), including probe concentrations and hybridisation temperature.

Under optimised conditions for *miR-195* (Experiment 3), a signal with the double FAM-labelled probe with insignificant background staining was observed, in conjunction with a signal for the positive control probe (*miR-126-3p*) and no signal for the negative control, the double FAM labelled miR-scramble probe.

6.4.2.1 Experiment 1

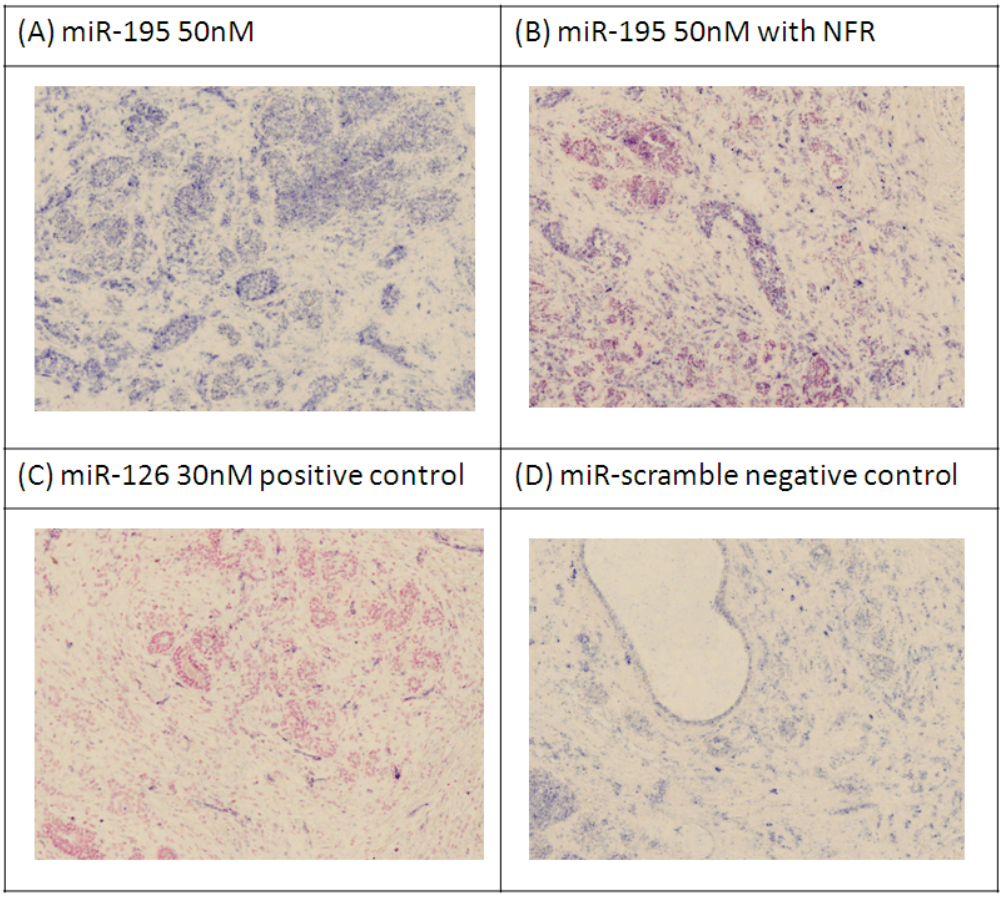
In the first experiment, the LNA probes were tested at standard conditions (Table 6.3) applying increased probe concentration (50mM) and prolonged chromogen substrate incubation, both with and without red nuclear counterstain. An intense ISH signal was detected with the *miR-195* probe. Therefore, the probe concentration was reduced for subsequent experiments. The *miR-497* probe signal was that of unspecific hybridisation. The positive control, *miR-126*, gave an intense signal in the vessels. The negative control, miR-scramble, resulted in high background staining in some samples.

Table 6.3 Experiment 1 properties

Probe Name	Proteinase K (µg/mL)	T_{hyb} (°C)	Probe Conc (nM)	NBT-BCIP (Min)
miR-195	25	57	50	90’
miR-497	25	57	50	90’
miR-126	25	57	30	90’
miR-scr	25	57	50	90’

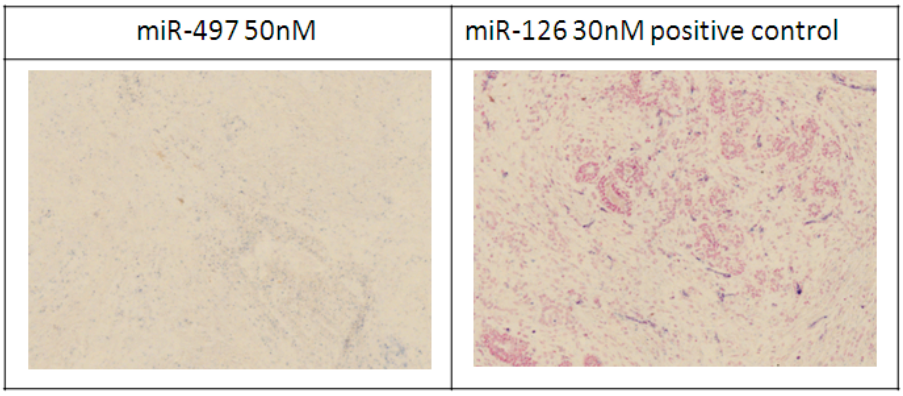
T_{hyb} hybridisation temperature; Probe conc, probe concentration; NBT-BCIP, 4-nitroblue tetrazolium (NBT) and 5-brom-4-chloro-3’-indolylphosphate (BCIP) substrate; Min, minutes

Figure 6.4 *MiR-195* localisation for sample R10-0033 in Experiment 1



An intense ISH signal was detected with the *miR-195* probe both in isolation (panel A) and in the presence of nuclear fast red counterstain (panel B) making it difficult to draw conclusions at this probe concentration. Both the positive and negative controls functioned satisfactorily with *miR-126* staining noted in (C) and no staining above background noted in (D).

Figure 6.5 *MiR-497* staining for sample R10-0033 in Experiment 1



The *miR-497* signal detected in all samples throughout this experiment resembled that of unspecific hybridization. This image shows the *miR-497* signal achieved in comparison to the positive control, *miR-126* for sample R10-0033.

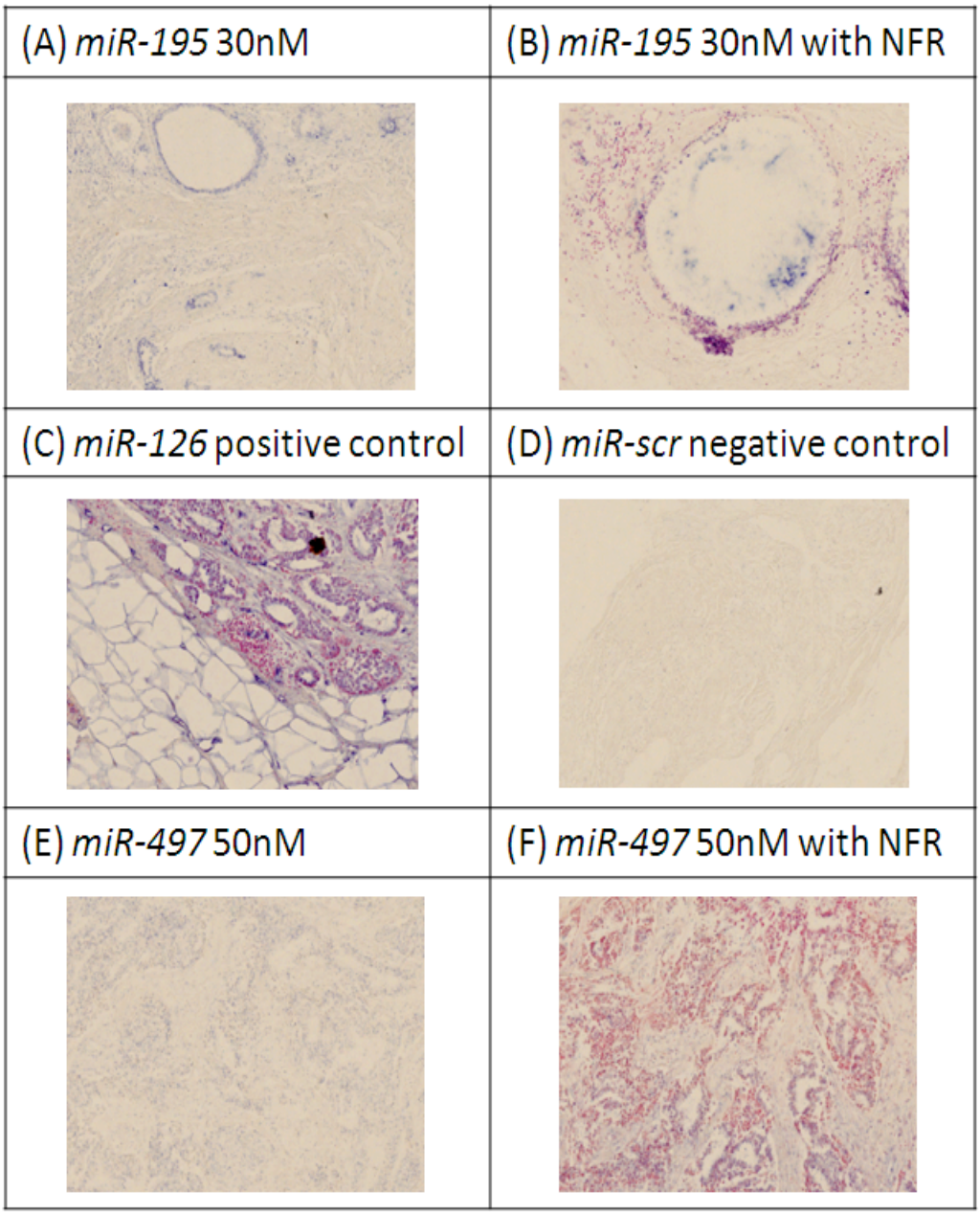
6.4.2.2 Experiment 2

In the second experiment (Table 6.4), the *miR-195* probe was tested at lower concentration (30mM) and prolonged chromogen substrate incubation both with and without nuclear red counterstain in an effort to achieve better signal-to-noise for *miR-195*. It is not clear why *miR-497* was not detectable, but it is likely expressed at a level below the detection limit of the assay. The *miR-497* probe resulted in background staining in a similar fashion to the negative control, *miR-scramble*.

Table 6.4 Experiment 2 properties

Probe Name	Proteinase K (µg/mL)	T_{hyb} (°C)	Probe Conc (nM)	NBT-BCIP (Min)
miR-195	25	57	30	90’
miR-497	25	57	50	90’
miR-126	25	57	30	90’
miR-scr	25	57	30	90’

Figure 6.6 MiRNA localisation for sample R10-1144 in Experiment 2



An ISH signal was detected with the *miR-195* probe both in isolation (panel A) and in the presence of nuclear fast red (NFR) counterstain (panel B), however some background staining persists. Both the positive and negative controls functioned satisfactorily with *miR-126* staining noted in (C) and no staining above background noted in (D). As in experiment 1, there was no signal above background for *miR-497*, as demonstrated in (E) and (F).

6.4.2.3 Experiment 3

In the final experiment (Table 6.5), the conditions were optimised and it is from this experiment that the most accurate interpretation could be performed. The *miR-195* probe signal was compared against the scramble probe at standard conditions applying the lowered probe concentration as described above, and reducing the chromagen substrate incubation. *MiR-497* was not included in the final experiment as conditions for accurate detection could not be optimised.

Table 6.5 Experiment 3 properties

Probe Name	Proteinase K (µg/mL)	T_{hyb} (°C)	Probe Conc (nM)	NBT-BCIP (Min)
miR-195	15	57	30	60’
miR-126	15	57	30	60’
miR-scr	15	57	30	60’

The intense *miR-126* ISH signal was obtained in virtually all samples and provides a good indicator of experiment performance. Under optimised conditions, we observed strong signal with the double-FAM labelled *miR-195* probe with little or no background stain and no signal with the double-FAM labelled negative control, *miR-scramble*. The positive control, *miR-126* was observed in the endothelial cells of vessels, as expected. The *miR-195* signal was detected in most cases (9 of 10) and is observed in both stromal and epithelial tumour cells. Stromal cells produced the most marked ISH signal, however, this varied in prevalence from case to case and locally within the section. The ISH signal in epithelial tumour cells was more consistent in staining intensity across the various cases and sections.

Figure 6.7 (A) *MiR-195* localisation by ISH compared to *miR-126*, the positive control.

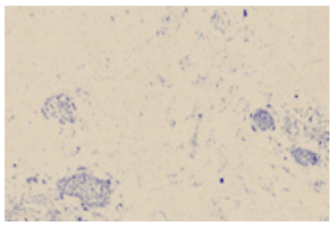
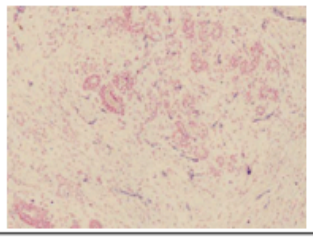
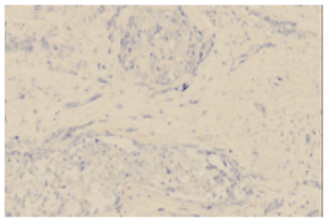
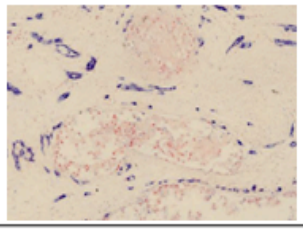
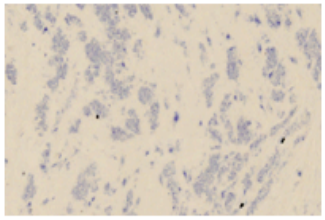
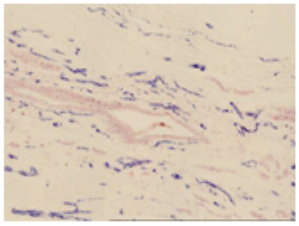
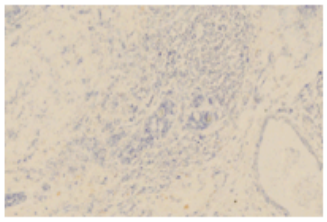
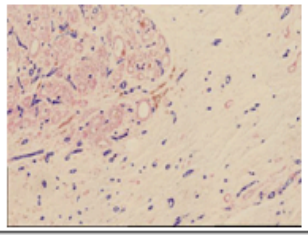
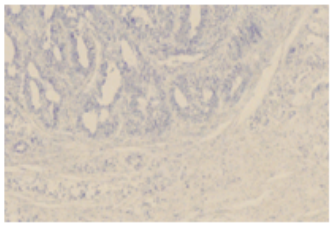
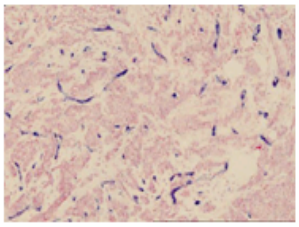
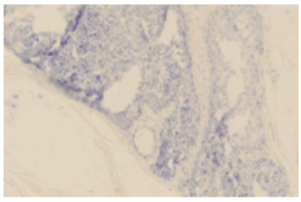
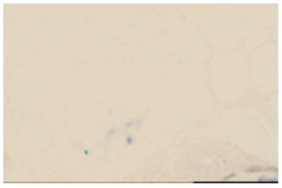
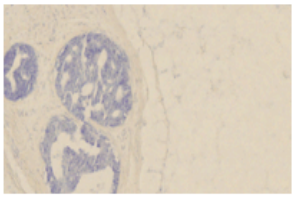
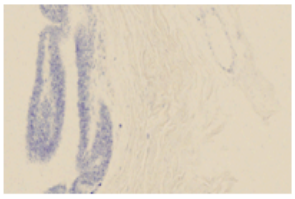
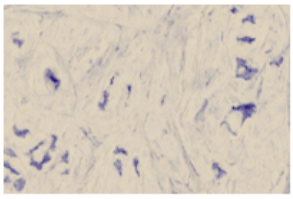
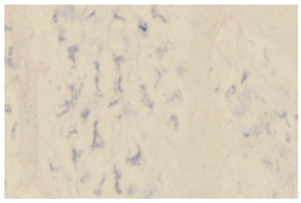
Sample	*miR-195*	*miR-126*
01098/11 R10-0033		
01499/11 R10-1689		
05512/11 R10-1387		
06949/11 R10-1526		
19222/10 R10-1094		

Figure 6.7 (B) *MiR-195* expression in samples 6 to 10 compared to the negative control, *miR-scramble*

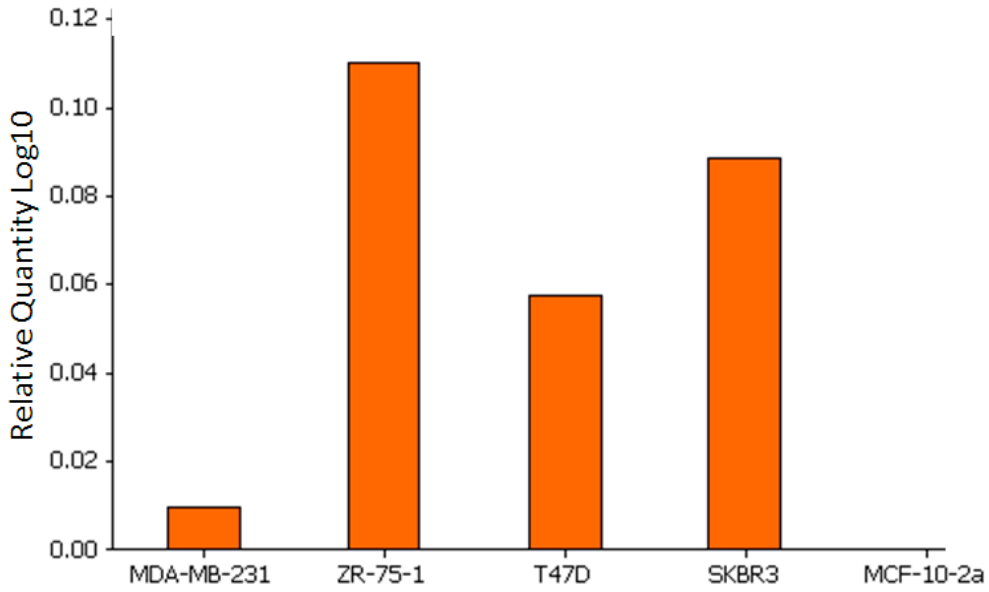
Sample	*miR-195*	*miR-scramble*
20088/10 R10-1062		
20959/10 R10-1144		
21091/10 R10-1251		
21362/10 R10-1277		
23844/10 R10-1412		

The most accurate interpretation of *miR-195* localisation by ISH can be drawn from the final experiment, Experiment 3. This figure demonstrates the *miR-195* probe signal on ten tumour sections, each from a different patient sample. *MiR-195* expression is compared to the positive control in part (A) of the figure and compared to the negative control in part (B) of the figure. All tumours in this experiment are Luminal A (ER+, PR+ and HER2/*neu*-). *MiR-195* and *miR-scramble* ISH examples are shown with no counter stain, whereas *miR-126* stained section were counterstained with nuclear fast red. As illustrated by the images on the right of (A), *miR-126* is the positive control (staining blue) specific for endothelial cells. This demonstrates the vasculature within the breast tumour and stroma. On the right side of (B), *miR-scr*, the negative control, demonstrates low background signal. ISH images for *miR-195* are the images on the left for each sample. The *miR-195* signal was present for nine of the ten samples (not detected in R10-1251). *MiR-195* was detected in both the stromal and tumour epithelial cells. In most cases the most intense ISH signal was present in the stromal cells.

6.4.3 MiR-195 and miR-497 expression in cell lines

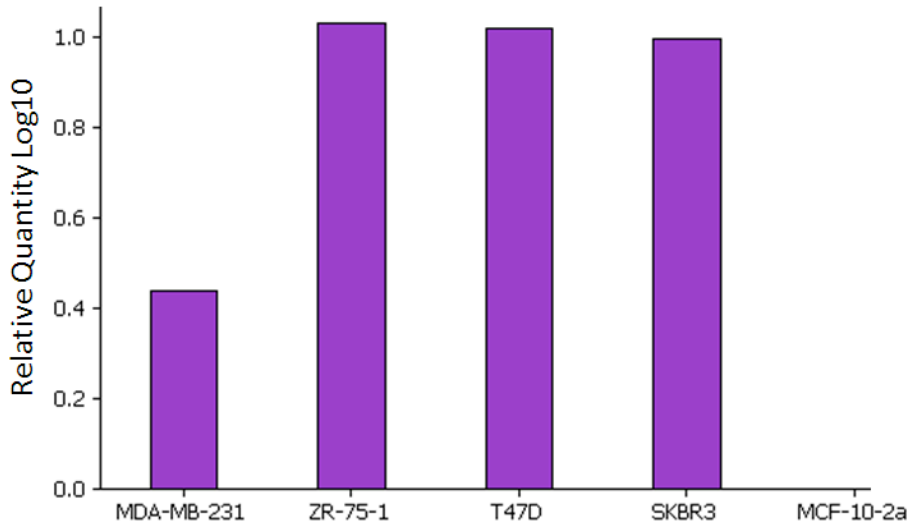
Given that *miR-195* was detected in the tumour epithelial cells by ISH, the expression of *miR-195* and *miR-497* was then analysed in cell lines, to determine if different or similar miRNA expression patterns was observed in all cell lines, regardless of their representative subtype. As with other miRNA expression data, these results are presented relative to the lowest expresser, explaining the absent expression data for the MCF-10-2a cell line.

Figure 6.8 *MiR-195* expression in cell lines



MiR-195 was detectable in all cell lines, including MCF-10-2a, the lowest expresser, which represents fibrocystic disease. The MDA-MB-231 cell line (basal subtype) displayed the lowest *miR-195* expression. While the hormone receptor positive and HER2/*neu* overexpressors (ZR-75-1, T47D and SKBR3 cell lines) display higher rates of expression. The ZR-75 cell line displayed the highest *miR-195* expression.

Figure 6.9 *MiR-497* expression in cell lines

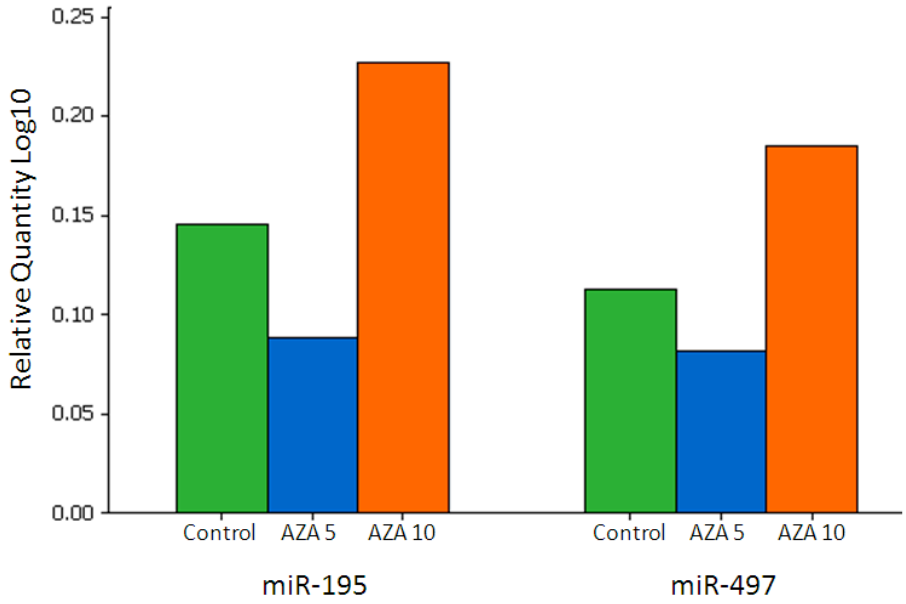


MiR-497 was detectable in all cell lines, including MCF-10-2a, the lowest expresser, which represents fibrocystic disease and was used to scale for normalising. The MDA-MB-231 cell line (basal subtype) displayed the lowest *miR-497* expression. While the hormone receptor positive and HER2/*neu* overexpressing (ZR-75-1, T47D and SKBR3) cell lines display higher and similar rates of *miR-497* expression.

6.4.4 Epigenetic modulation of miR-195 and miR-497 expression in vitro

Given the numerous and interesting data presented throughout this thesis on *miR-195* and *miR-497*, we wished to determine if these miRNAs were subject to epigenetic modulation by methylation. Cells were treated with two different doses of azacytidine (5mM and 10mM) and harvested after 5 days, along with a control group. The cells treated with 10mM azacytidine (AZA 10) showed an overexpression of both *miR-195* and *miR-497* compared to the cells treated with 5mM azacytidine and the control group.

Figure 6.10 *MiR-195* and *miR-497* expression in azacytidine treated cells



The cells treated with 10mM azacytidine (AZA 10, orange) display the highest expression of *miR-195* and *miR-497*, compared to both the control (green) and AZA 5 (blue) groups. Overexpression following treatment with azacytidine suggests that these miRNAs are under epigenetic regulation by methylation. Subsequent demethylation (with azacytidine) increases their expression.

6.5Discussion

This study provides an insight into the cellular location and function of *miR-195* and *miR-497* within the tumour microenvironment. Using ISH, *miR-195* was shown to be present in the tumour microenvironment and was localised to both stromal and tumour epithelial cells. Despite a variety of experimental conditions, *miR-497* could not be detected. *MiR-195* and *miR-497* expression was then analysed in a variety of cell lines, in an attempt to determine if molecular tumour subtypes displayed equivalent expression of these miRNAs, or whether differences in its expression are more marked in some cell lines rather than others, potentially reflecting diverse molecular pathways underpinning each molecular subtype. Both miRNAs were detectable in all cell lines included in the study. Hormone receptor and HER2/*neu* receptor positive subtypes displayed relatively higher expression of both *miR-195* and *miR-497*. Interestingly, cells reflecting the basal subtype, MDA-MB-231 cell line, displayed the lowest *miR-195* and *miR-497* expression. Finally, the potential for epigenetic modulation by methylation to alter *miR-195* and *miR-497* expression was

evaluated. Cells treated with demethylating azacytidine (10mM) displayed an increased expression of *miR-195* and *miR-497* compared to the control group suggesting that expression is affected by epigenetic modulation by methylation.

It is interesting that *miR-497* was not detectable by ISH in this study. It cannot be concluded that *miR-497* was not present in the tumour microenvironment of these samples. In addition to normal breast tissue and breast tumour presented in this study, *miR-497* ISH was performed on ovarian, tonsil and uterine tissue to determine if a signal could be generated although without reward. There are no reports to date of *miR-497* ISH being successfully performed on an alternative tissue type, in health, development or disease states. One possible explanation is that *miR-497* was present in small quantities, and was therefore below the threshold of detection using ISH. This is a plausible interpretation as we have demonstrated that *miR-497* was underexpressed in the circulation of the patients whose tumour samples were used for this study compared to that of healthy controls. In addition, even in the healthy control state, *miR-497* was present in lower quantities compared to *miR-195*, with mean raw Ct values for this cancer cohort of 29.35 (range 28.19 to 30.17) for *miR-497* compared to 19.43 (range 18.18 to 20.9) for *miR-195*. MiRNA ISH is still a relatively recent technology and there is still a lot to be determined about the accessibility for the probes to bind the miRNAs. *MiR-195* was readily detectable in breast tumour tissue. The miRNA ISH signal obtained using ISH has previously been reported to be specific. Neilsen et al showed that as few as 3 base pair mismatches for *miR-21* resulted in no specific ISH signal being observed (293). However, it cannot be excluded that a cross-reaction of the *miR-195* probe with *miR-16-2-3p* occurred, as there is only 1 nucleotide different. Nonetheless, it is interesting that despite being co-located on chromosome 17; *miR-195* and *miR-497* miRNAs display an apparent inverse expression pattern at both the circulation and tissue level.

MiR-195 and *miR-497* were detected in breast cancer cell lines by RQ-PCR in this study. These miRNAs were also shown to be detectable in systemic circulation in Chapter 5. This raises questions regarding the source of these circulating miRNAs, and the mechanism by which they are shed into the systemic circulation. There are several mechanisms by which miRNAs are potentially shed from tumour epithelial cells and stromal cells into the tumour microenvironment and subsequently transported the systemic circulation. MiRNAs may be selectively packaged into exosomes, small lipoprotein vesicles originating from the

endosome, which are released from the cell by fusion with the cell membrane (121). Microvesicles are larger than exosomes and also package miRNAs for exportation and are formed by simple blebbing of the plasma membrane. MiRNAs may also be passively released or secreted from the cell free from encapsulation by lipoprotein bodies. It has also been demonstrated that a large proportion of circulating miRNAs are not encompassed in lipoprotein particles, but rather bound to protein complexes such as nucleophosmin 1(NPM1) and Argonaute2 (Ago2) (122, 123). It has been shown that the *Let-7* family is selectively secreted in exosomes from gastric cancer cell lines and can be detected in culture media (294). The fact that specific miRNAs are selectively secreted from cells implies that they may have some functional relevance, potentially acting locally to influence neighbouring cells or at a distant site, functioning in a manner similar to conventional hormones. Valadi *et al* pioneered this concept in 2007 by showing that exosomal miRNAs were evident in murine mast cell lines (MC-9), a human mast cell line (HMC-1) and primary bone marrow derived mast cells (121). However, the mechanisms by which these circulating miRNAs are deposited into the blood, delivered to and consumed by recipient cells has yet to be fully elucidated and remains an exciting avenue for further research.

Altered miRNA expression in cancer has been described here and indeed throughout the literature. Alterations in miRNA expression during development, throughout normal biological processes and in disease states are tissue specific and tightly regulated. However, relatively little is known on the exact mechanisms governing this altered expression. Epigenetic regulation by methylation denotes the methylation of cytosine at the C5 position in CpG dinucleotides and plays an important role in governing gene expression (295). As miRNA genes are largely transcribed in the same manner as protein coding genes by RNA polymerase, it stands to reason that the mechanisms of epigenetic regulation of protein coding genes, including methylation, also apply. There are two important constituents in the methylation process: methyl-CpG binding domain proteins (MBDs) and DNA methyltransferases (DNMTs). Methylation directly inhibits transcription by preventing the binding of transcription factors, and indirectly by recruiting MBDs. DNA methylation usually occurs at repetitive sequences (296). However in cancer, DNA hypermethylation largely occurs at CpG islands which remain unmethylated in healthy cells (297, 298). Global changes in methylation patterns are frequently found during tumourigenesis, both hypo- and hyper-methylation. Hypermethylation of tumour suppressor miRNAs has been

reported in cancer, including *miR-9-1, miR-34b, miR-34c, miR-137, miR-193a, miR-203* and *miR-342* (299, 300). Hypomethylation of miRNA was reported in lung adenocarcinoma, with hypomethylation of the *let-7-3a* locus producing an elevation of expression of this oncogenic miRNA (301). Epigenetic regulation by methylation at CpG islands has been shown to be a plausible and functionally relevant method of miRNA regulation (301-303). Over half of all annotated miRNAs genes possess CpG islands and could therefore be subject to this method of regulation (304). In addition to methylation, miRNAs also appear to be regulated by histone modification, the other major form of epigenetics (305, 306). These variations induce resistant (although transient) changes in gene expression and are therefore functionally equivalent to genetic mutations.

As well as being affected by epigenetic modifications, miRNAs themselves may act as epigenetic modifiers. A recent publication by Song *et al* in Cell implicates *miR-22* as a potent epigenetic modulator of EMT and a promoter of metastasis (307).

In addition to epigenetic alterations at the transcriptional level, altered miRNA expression in pathologic conditions may occur as a result of altered miRNA processing during miRNA biogenesis. This may occur at any step throughout the biogenesis process. Relative quantities of Drosha and DGCR8 must be closely regulated for accurate miRNA processing (308).There are several recently published accounts of altered processing of miRNAs by Drosha resulting in reduced mature miRNA expression (309-311).

Li *et al* also reported on methylation of *miR-195* and *miR-497* in breast cancer (191). Firstly, this group compared the methylation status of CpG islands in breast cancer tissue compared to normal breast tissue and reported that CpG islands are specifically methylated in human breast cancer. Next, this group sought to specifically determine the methylation status of the CpG islands in the nucleotide sequence upstream of *miR-195* and *miR-497* promoter regions on Chromosome 17. Bisulphite sequencing analysis of genomic DNA isolated from normal breast tissue, breast tumour tissue and breast cancer cell lines was performed and displayed heavy methylation of certain CpGs in this region in both breast cancer tumour tissue and cell lines (specifically MCF-7 and ZR7530). In contrast, methylation of only a few scattered CpGs was found in normal breast tissue. Li *et al* then progressed to demonstrate that treatment with azacytidine increased the expression of both *miR-195* and *miR-497*, as was the case in this study.

Modification of gene expression by capitalising on the knowledge of epigenetics and its role on miRNA expression has been identified as a novel treatment strategy for cancer. The DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine is used to treat myelodysplastic syndrome resulting in improved survival (312). Histone deacetylase (HDAC) inhibitors have also undergone clinical trials in haematological malignancies, with vorinostat and romidepsin being approved by the Food and Drug Administration (313, 314). Reintroducing the expression of epigenetically silenced genes is a promising cancer treatment approach and methods modulating miRNA expression have shown promising results (315, 316). *MiR-29b* has been identified as a potent hypomethylating agent in non-invasive breast cancer cell lines (317).

6.6 Conclusion

In conclusion, this study demonstrates that *miR-195* is detectable within the tumour microenvironment, and can be specifically localised to stromal and tumour epithelial cells. Conclusive statements cannot be made on *miR-497* expression. Both *miR-195* and *miR-497* are detectable in breast cancer epithelial cell lines. The expression of these miRNAs in vitro is affected by epigenetic modification, and aberrant methylation could be responsible for their altered expression in the breast cancer state.

Chapter 7
Circulating miRNAs: Novel Breast Cancer Biomarkers and their Use for Guiding and Monitoring Response to Chemotherapy

7.1 Introduction

The understanding of breast cancer has been revolutionised over the past decade. Breast cancer is no longer considered a single disease entity but rather a heterogeneous disease with four main molecular subtypes each conveying a distinct tumour phenotype with discrete prognoses and response to treatment. However, despite recent advances in the understanding of the molecular mechanisms underpinning breast cancer aetiology and development, management of this heterogeneous disease has altered little. This is particularly true in relation to the provision of adjuvant chemotherapy. Current practice recommends that women with lymph node involvement receive adjuvant chemotherapy, in addition to hormonal or biological therapies if appropriate (318). The decision to commence such a regimen should not be taken lightly as each of these treatment modalities has an associated cluster of adverse effects. Undoubtedly, it is critical to identify the patient group that will benefit from adjuvant therapies. However, it is equally as important to have the ability to select those that have achieved surgical cure of their disease, and as such would derive no benefit from additional adjuvant therapies.

Neoadjuvant chemotherapy (primary systemic chemotherapy) has become an increasingly popular for early operable breast cancer (319, 320). The National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol-B18 was conducted to determine whether four cycles of pre-operative doxorubicin/cyclophosphamide improved disease free survival (DFS) and overall survival (OS) when compared to the same regimen administered post-operatively. The initial results from the NSABP-B18 published in 1998 demonstrated that neoadjuvant chemotherapy is as effective as post-operative chemotherapy and is appropriate for stage I and II disease, permitting less radical surgery (320). The conclusions of this study also state that it 'can be used to study breast cancer biology'. After nine years of follow up, the NSABP-B18 demonstrated that neoadjuvant chemotherapy offers equivalent DFS and OS to adjuvant chemotherapy with an increased rate of BCS at nine years of follow up (51). Standard neoadjuvant chemotherapy regimens involve six to eight cycles (fortnightly) of combination chemotherapy regimens, commonly adriamycin and cyclophosphamide followed by docetaxel or paclitaxel, with the addition of trastuzumab for HER2/*neu* overexpressors (52, 319). The results of the European Organisation for Research and Treatment of Cancer trial 10902 also advocated the use of neoadjuvant chemotherapy (321). In this study patients were randomly assigned to receive four cycles of fluorouracil, epirubicin and cyclophosphamide either pre- or post-operatively. After a median follow-up

of 56 months, there was no significant difference between the two groups in terms of OS, progression free survival (PFS) or time to locoregional recurrence (LRR). Twenty three percent of patients in this study were down-staged by NAC. The benefit of the addition of a taxol was demonstrated in the NSABP-B27 trial (322). Patients were randomly assigned to preoperative doxorubicin/cyclophosphamide (AC) alone, preoperative AC followed by docetaxel or preoperative AC followed by surgery and then docetaxel. Patients in the pre-operative taxel group displayed slightly improved DFS and decreased incidence of local recurrences (322).

Early experience with this treatment have demonstrated that response to therapy is not uniform; some patients respond better than others, with the range of response reported to be as varied as 4 to 34% with response rates as high as 50% in HER2/*neu* overexpressing subsets (50-52). Yet some patients derive no benefit at all, and essentially suffer the treatment side effects in vain. The complete pathological response (pCR) rate has been used as a primary endpoint in several NAC trials and has been shown to be a good prognostic factor for women with breast cancer (51, 323-325). Interestingly, the US FDA issued a guidance protocol for the industry on the use of pCR as an endpoint for clinical trials to accelerate drug approval for NAC in early stage breast cancer[23]. However, there is currently no clinical test (tissue- or circulation-based) that can scrupulously predict responders from non-responders, or that can be exploited to monitor treatment response in real time. Subsequently, a number of women are administered toxic treatment without any promise of deriving any benefit. With the increasing patient awareness of the toxicity of chemotherapy and era of individualised, patient-centred cancer management plans there is a need to develop an accurate, acceptable clinical test that would identify patients who are most likely to obtain pCR from neoadjuvant treatment, and thus spare a proportion of patients from the toxic effects of treatments from which they would derive no benefit.

The All Ireland Cooperative Research Cooperative Group (ICORG)[24] is a not-for-profit registered charity established in 1996 by a group of hospital consultants working with cancer management. Its purpose was to generate research opportunities for Irish cancer patients by making Ireland a more attractive location for international clinical and pharmaceutical research. Membership is open to individuals in the Republic of Ireland and

[23] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM305501.pdf
[24] http://www.icorg.ie

Northern Ireland working in the oncology field, including Oncologists, Radiation Oncologists, Surgeons, Haematologists and Research Specialists including Oncology Nurse Specialists and Translational Scientists. Since inception, ICORG has expanded to include more than 95% of Irish consultants treating cancer as members. It has opened more than 260 research protocols and has facilitated access to research treatments for over 9000 Irish cancer patients. ICORG has trials in numerous areas of cancer research including breast, lung, neurological, gastrointestinal, genitourinary, gynaecology, haematology and melanoma.

MiRNAs appear to be ideal biomarker candidates due to their inherent characteristics: they are easily detected in blood, are relatively stable and are aberrantly expressed in the breast cancer state. Expression profiling of miRNAs provides insight into the stages of development of disease states, and provides a means to devise disease-specific signatures for both diagnosis and prognostication. Previous work by our group has shown that specific miRNA signatures in tissue correlate with intrinsic breast cancer subtype (111). Furthermore, early studies have demonstrated that miRNAs may be able to discriminate between the cancer and cancer-free state*; MiR-195* was shown to be elevated in women with breast cancer with expression levels returning to that of healthy controls two weeks post-operatively (93). It is therefore realistic to suggest that miRNA signatures could not only reflect outcomes but also predict outcomes. Similarly it is plausible to suggest that specific miRNA signatures for each breast cancer subtype (Luminal A, Luminal B, HER2/*neu* overexpressing and Basal subtypes) could predict patients who will respond to conventional chemotherapeutic agents, monoclonal antibodies and hormonal therapies. Early studies have demonstrated that. A clinical trial in collaboration with ICORG was therefore initiated to expand on the original findings of *miR-195*, and other miRNAs, and to determine their clinical and translational relevance.

7.2 Aims

This aims of this study were as follows:

- To establish a multicentre prospective clinical trial to recruit patients undergoing neoadjuvant chemotherapy for newly diagnosed breast cancer
- To assess fluctuations in circulating miRNA expression levels over the course of neoadjuvant chemotherapy

- To investigate the relationship between circulating miRNA profiles and patients' intrinsic subtype of breast cancer
- To assess the relationship between miRNA expression profiles and response to treatment
- To assess the relationship between miRNA expression levels and other existing clinicopathological parameters, specifically nodal status, ER status and HER2/*neu* receptor status.

7.3 Materials and methods

7.3.1 *Patient selection*

Patients planning to undergo neoadjuvant chemotherapy in Galway University Hospital or the affiliated study site (St James' Hospital) were invited to participate in this prospective cohort study provided they met the following inclusion and exclusion criteria:

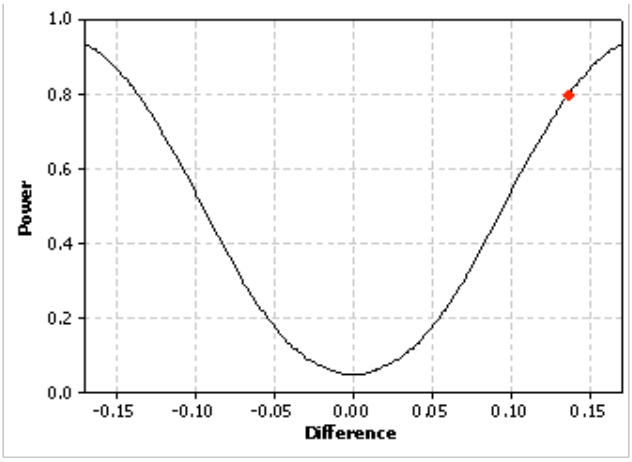
1. All patients with a new diagnosis of breast cancer, who were destined to undergo neoadjuvant chemotherapy
2. Patients must have been aged 18 years or over
3. Patients must have been able to provide written informed consent

All patients who did not satisfy the exclusion criteria above were not invited to participate in the study.

7.3.2 *Sample size*

The power calculation to determine the sample size was conducted using the initial *miR-195* data (93). A total of 122 patients would need to be recruited to achieve 80% power in detecting differences in miRNA levels as small as 0.136 RQ units (Figure 7.1).

Figure 7.1 Power calculation



7.3.3 *Study cohort*

The following describes the recruitment, processing and analysis of the first 30 patients enrolled into the ICORG 10-11 Clinical trial. As the average length of time to follow a patient through all study timepoints (diagnosis through neoadjuvant chemotherapy treatment and post-operative follow-up) was 12-18 months, it was prohibitive for all 122 patients to be included in this study group. It was decided to perform a preliminary analysis of the first 30 patients across all 5 of their time-points. Thirty patients were enrolled (Table 2.6 and 7.1).

Table 7.1 Individual patient clinicopathological details

Patient	Intrinsic Subtype	Age	ER Status	PR Status	HER2 Status	Grade	Histological Type	Nodal Status	Size (mm)
1	Luminal A	50	Positive	Negative	Negative	2	Invasive Ductal	Negative	45
2	Luminal A	46	Positive	Positive	Negative	2	Invasive Ductal	*	50
3	Luminal A	59	Positive	Positive	Negative	2	Invasive Ductal	Positive	68
4	Luminal B	44	Positive	Negative	Positive	2	Invasive Ductal	Positive	70
5	Luminal B	56	Positive	Positive	Positive	2	Invasive Ductal	Positive	31
6	Basal	44	Negative	Negative	Negative	3	Invasive Ductal	Positive	35
7	Luminal A	57	Positive	Negative	Negative	2	Invasive Ductal	Negative	*
8	Luminal B	44	Positive	Positive	Positive	3	Invasive Ductal	Positive	*
9	HER2	50	Negative	Negative	Positive	3	Invasive Ductal	Positive	33
10	Luminal A	50	Positive	Positive	Negative	3	Invasive Ductal	Positive	40
11	Luminal A	50	Positive	Negative	Negative	3	Invasive Ductal	Negative	36
12	Luminal A	36	Positive	Positive	Negative	2	Invasive Ductal	Positive	29
13	Luminal A	47	Positive	Positive	Negative	2	Invasive Ductal	Positive	16
14	Luminal A	28	Positive	Positive	Negative	3	Invasive Ductal	Positive	20
15	Basal	69	Negative	Negative	Negative	1	Invasive Ductal	Positive	30
16	Luminal A	71	Positive	Positive	Negative	2	Invasive Ductal	Positive	100
17	HER2	38	Negative	Negative	Positive	3	Invasive Ductal	Positive	*
18	Basal	56	Negative	Negative	Negative	3	Invasive Ductal	Positive	16
19	Luminal A	50	Positive	Positive	Negative	2	Invasive Ductal	Positive	21
20	Basal	42	Negative	Negative	Negative	3	Invasive Ductal	Negative	34
21	Basal	60	Negative	Negative	Negative	3	Invasive Ductal	Positive	20
22	Luminal A	58	Positive	Negative	Negative	2	Invasive Lobular	Positive	40
23	Luminal A	36	Positive	Positive	Negative	2	Invasive Ductal	Positive	62
24	Luminal B	42	Positive	Positive	Positive	3	Invasive Ductal	Positive	*
25	Basal	55	Negative	Negative	Negative	3	Invasive Ductal	Positive	25
26	Luminal A	51	Positive	Positive	Negative	3	Invasive Ductal	Positive	70
27	HER2	35	Negative	Negative	Positive	2	Invasive Ductal	Positive	32
28	Luminal A	62	Positive	Positive	Negative	2	Invasive Ductal	Negative	*
29	Luminal A	64	Positive	Positive	Negative	2	Invasive Ductal	Positive	20
30	Luminal A	60	Positive	Negative	Positive	3	Invasive Ductal	Positive	35

ER status, PR status and HER2 status at diagnosis, Size (mm) at diagnosis as determined by radiological imaging with mammography, MRI or ultrasound

7.3.4 Patient enrolment procedure

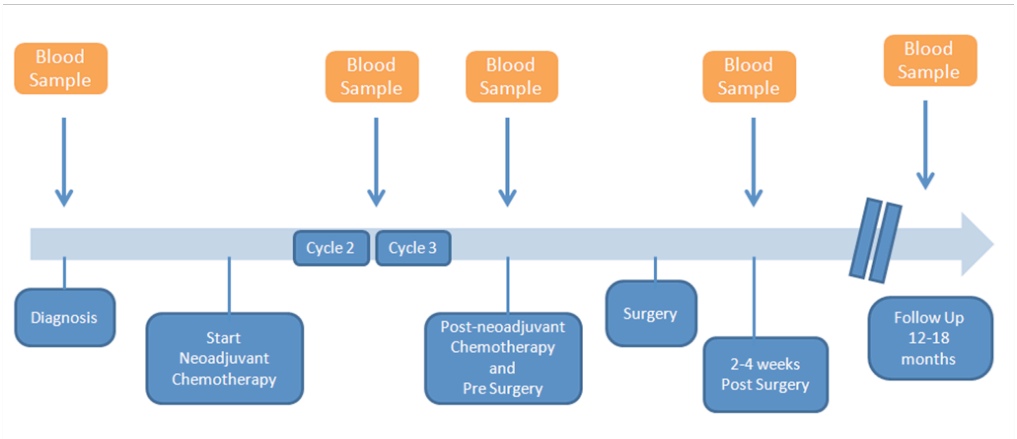
Once written informed consent was received from the patient, a Patient Registration Form was completed and sent to ICORG (Appendix 2.1). A study number was assigned to the patient and sent to the study site. In order to maintain patient privacy, all Case Report Forms (CRFs, Appendix 2.2, 2.3 and 2.4), study reports and communications identified the patient by initials and the assigned patient number.

As per EU directive and ICH-GCP guidelines, patients could withdraw voluntarily from the study at any time and their samples would be destroyed (Off Study form, Appendix 2.5).

7.3.5 Blood sample collection

One 4-8ml bottle of whole blood (EDTA) was taken from each patient as described above at the designated time-points below (Figure 7.2). Whole blood samples were stored at 4°C until transfer to the laboratory in the Discipline of Surgery at NUI Galway. Once received in the laboratory, blood samples were again refrigerated until processing.

Figure 7.2 Timepoints for blood sampling



A total of five peripheral blood samples were obtained from each patient enrolled into the trial. The first sample was collected following diagnosis. The second sample was drawn midway through the neoadjuvant chemotherapy regimen (before cycle 3 if undergoing a 4 cycle regimen, before cycle 5 if undergoing an 8 cycle regimen). The third sample was collected following completion of neoadjuvant chemotherapy and in advance of surgical resection. If the patient was undergoing surgical resection in GUH, a tissue sample was also obtained intraoperatively. Between two and four weeks postoperatively the fourth blood sample was collected. The final blood specimen was drawn at annual follow up, between 12 and 18 months after diagnosis and enrolment onto the study.

7.3.6 Candidate miRNAs

A panel of 7 miRNAs and 2 endogenous controls were selected for expression profiling over the course of neoadjuvant chemotherapy (Table 7.2).

Table 7.2 Candidate miRNAs and their previous associations with breast cancer

Target miRNAs	Previous association with breast cancer	Reference
miR-195	Part of a miRNA signature associated with hormone receptor status Significantly elevated in blood of breast cancer compared to other cancers (prostate, renal, colon and melanoma) and healthy controls	(93, 111, 237)
miR-497	Underexpressed in breast cancer cell lines	(191)
miR-21	Overexpressed in breast tumour tissue compared to normal breast tissue	(86)
miR-29a	Overexpressed in serum of women with breast cancer Identified in Chapter 4 as being elevated in blood of women with Luminal A breast cancer	(174)
miR-223	Reported by Yang *et al* to increase invasiveness in breast cancer cell lines Identified in Chapter 4 as being reduced in blood of women with Luminal A breast cancer	(218)
miR-181a	Under-expressed in serum of women with breast cancer Identified in Chapter 4 as being reduced in blood of women with Luminal A breast cancer	(164)
miR-652	Identified in Chapter 4 as being reduced in blood of women with Luminal A breast cancer	NA
Endogenous Controls		
miR-16	Reliable endogenous control combination for analysis of miRNA expression by RQ-PCR in blood	Chapter 3
miR-425		

7.3.7 Sample processing

7.3.7.1 RNA Isolation from whole blood

Total RNA was extracted from 1ml blood using Trizol (Life Technologies), as previously described (Section 2.3).

7.3.7.2 Analysis of RNA concentration and integrity

RNA concentration was determined by Nanodrop Spectrophotometry (Section 2.4). The small (miRNA) element of all RNA in solution was determined using the '33' wavelength

coefficient. Each 1 mL of whole blood yielded approximately 60 µL total RNA which was transferred to secure tubes prior to storage at -80°C.

7.3.7.3 Reverse transcription

RNA samples were reverse transcribed using Taqman RT primers specific to each miRNA target. Reactions were prepared and run as described in Section 2.6. In brief, 100 ng of total RNA per sample was reverse transcribed using MultiScribe reverse transcriptase in total reaction volumes of 15 µL.

7.3.7.4 RQ-PCR

Real time relative quantification polymerase chain reaction (RQ-PCR) was carried out using Taqman miRNA primers and probes as described in Section 2.7. PCR reactions were carried out in final volumes of 10 µL using an ABI 7900 HT Fast Real Time PCR instrument. In short, reactions were performed in triplicate and consisted of 0.7 µL of the reverse transcription product, 5 µL TaqMan Universal PCR Fast Mastermix (no UNG), 0.2 µM TaqMan primer-probe mix. An interassay control (IAC) was included on each plate. The threshold standard deviation for intra-assay and inter-assay replicates was <0.28. The PCR amplication efficiency for each Taqman miRNA assay was determined and converted to a percentage. The relative quantity of miRNA expression was calculated using the comparative cycle threshold (ΔΔCt) method, normalised to *miR-16* and *miR-425,* and scaled to the lowest-expressing sample.

7.3.8 Data reporting

Clinicopathological data was collected using Case Report Forms (CRFs) which were specifically designed for the study (Appendix 2). The CRFs were filed locally with scanned copies sent to ICORG for data centralisation. Data was then inputted Microsoft Excel.

7.3.9 Statistical analysis

Statistical analyses were performed using Minitab version 16.0 (Minitab Ltd). The Kolmogorov-Smirnov test for normality was conducted. Data were log transformed (log_{10}) for analysis when non-normal distribution was identified. The significance of differing miRNA expression between groups was determined using the ANOVA (one-way), ANOVA (General Linear Model), Mann-Whitney U test, unpaired 2 sample t-test, or paired t-test, as appropriate. Results with p-value less than 0.05 were deemed to be significant. The

Pearson correlation coefficient was used to assess the strength of a relationship between miRNA expression in different groups.

Table 7.3 Interpretation of the Pearson correlation coefficient

Pearson Coefficient (R)	Interpretation
0	No linear relationship
+ (-) 0.0 to 0.3	Weak positive (negative) linear relationship
+ (-) 0.3 to 0.7	Moderate positive (negative) linear relationship
+ (-) 0.7 to 1.0	Strong positive (negative) linear relationship
+ (-) 1	Perfect positive (negative) linear relationship

7.4 Results

7.4.1 Relationship between circulating miRNA expression and neoadjuvant chemotherapy

Clinical details, histological details and serial blood samples were prospectively collected from 30 women undergoing neoadjuvant chemotherapy at Galway University Hospital. RNA was extracted and miRNA expression profiles were generated for 2 miRNAs (*miR-195* and *miR-497*) in a cohort of thirty patients (n=121 samples), with a further 5 miRNAs being assessed in a 15 patient cohort (*miR-21, miR-29a, miR-181a, miR-223* and *miR-652*, n=61 samples). The patients included in this cohort were the first 15 patients for whom all sample timepoints were available. A blood sample from every timepoint for each patient was not always available, explaining 121 samples (of a potential 150) for the thirty patient cohort and 61 samples (of a potential 75) for the fifteen patient cohort (Table 7.4). Each miRNA profile was studied with respect to individual patient, response to treatment, molecular subtype, nodal status, oestrogen receptor status and HER2/*neu* receptor status. The results are presented according to individual miRNA for the sake of clarity.

Table 7.4 Samples available for miRNA analysis

→Timepoint ↓Patient	Diagnosis	Peri-NAC	Post NAC	Post Surgery	Annual
1	X	X	*	*	X
2	X	X	X	X	X
3	X	X	X	X	X
4	X	X	X	X	X
5	X	X	X	X	X
6	X	*	X	*	*
7	X	X	X	X	X
8	X	X	*	X	X
9	X	X	X	X	X
10	X	X	*	X	X
11	X	*	X	*	X
12	X	X	X	X	*
13	X	X	*	*	X
14	X	*	X	X	X
15	X	X	X	X	X
16	X	X	X	*	X
17	X	X	X	*	X
18	X	X	*	*	*
19	X	X	X	X	X
20	X	X	X	X	*
21	X	X	X	X	X
22	X	X	X	X	*
23	X	X	X	X	*
24	X	*	X	X	*
25	X	*	X	*	X
26	X	X	X	X	X
27	X	X	X	X	*
28	X	X	*	X	*
29	X	*	*	*	*
30	X	X	X	X	X

X sample available; * sample missing

7.4.2 MiRNA yield

There was sufficient miRNA yield from each sample. There was no significant difference in mean total miRNA yield (ng/µl) between the timepoints (ANOVA, p=0.216, Table 7.5).

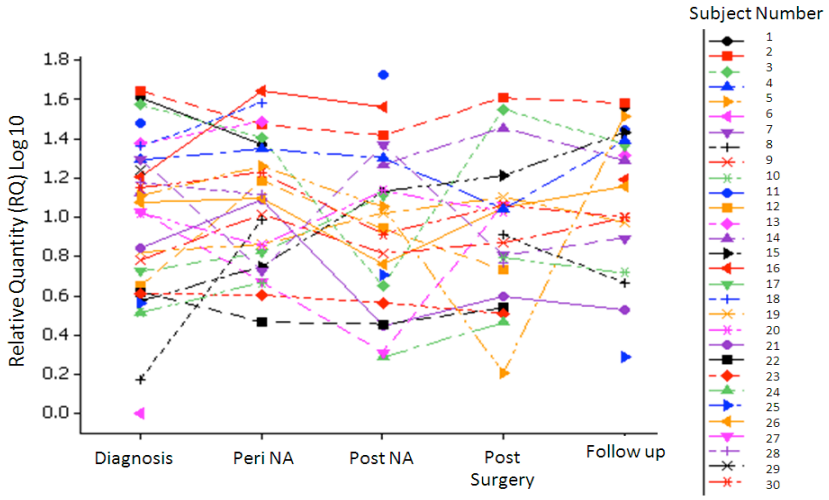
Table 7.5 MiRNA yield from each timepoint

Timepoint	Mean miRNA Yield (ng/µl)
Diagnosis	167.0 (± 87.8)
Peri NAC	231.1 (± 193.3)
Post NAC	185.2 (± 127.8)
Post Surgery	157.0 (± 73.8)
Follow up	158.9 (± 97.9)

7.4.3 MiR-195

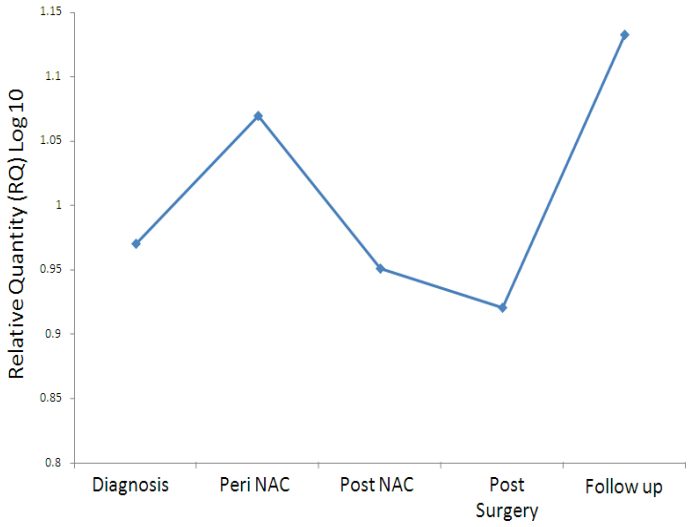
MiR-195 was detectable at all study timepoints in all samples (Figure 7.3). The mean *miR-195* expression profile was generated to examine the overall trend over the course of NAC. Figure 7.4 outlines that although not significant, there was a trend towards a reduction in *miR-195* expression from diagnosis to post-surgery, with a subsequent increase in expression at annual follow up (p=0.329, ANOVA). The correlation between *miR-195* expression at specific timepoints was then assessed (Figure 7.5).

Figure 7.3 *MiR-195* expression in each individual patient



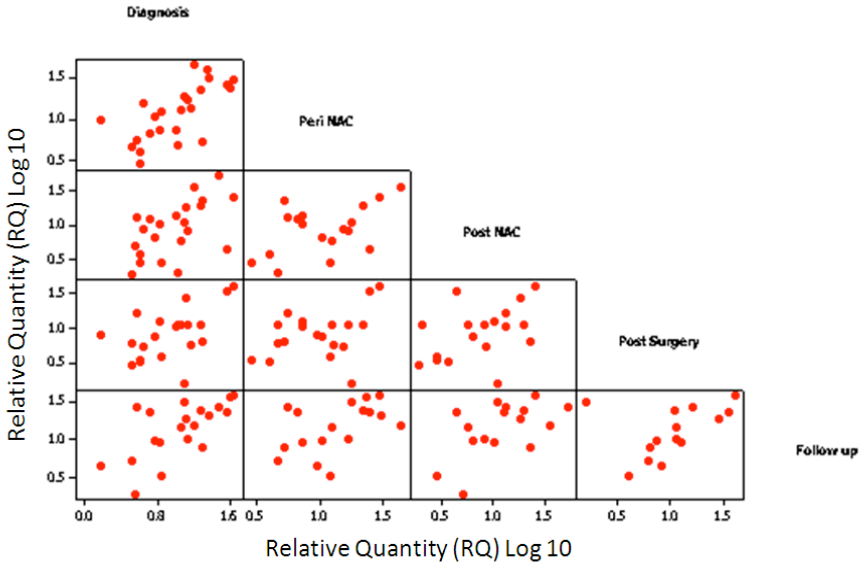
MiR-195 expression profile for individual patients (n=30) over the course of NAC, a total of 118 blood samples.

Figure 7.4 Mean *miR-195* expression for all patients



Mean *miR-195* expression for each timepoint. Although not significant, there was a trend towards a reduction in *miR-195* expression from diagnosis to post-surgery, with a subsequent increase in expression at annual follow up (p=0.329, ANOVA).

Figure 7.5 Correlation between *miR-195* expression and study timepoints



The matrix plot above illustrates the correlation between *miR-195* at each timepoint plotted against each other timepoint. Several positive correlations were noted as presented in Table 7.6 below. For example, there was a positive correlation (R=0.68) between *miR-195* at diagnosis and at the peri NAC timepoint; Patients with increased levels of *miR-195* at diagnosis also had high *miR-195* peri-NAC. Pearson's correlation coefficient (R) between 0.3 and 0.7 denotes a moderate positive correlation.

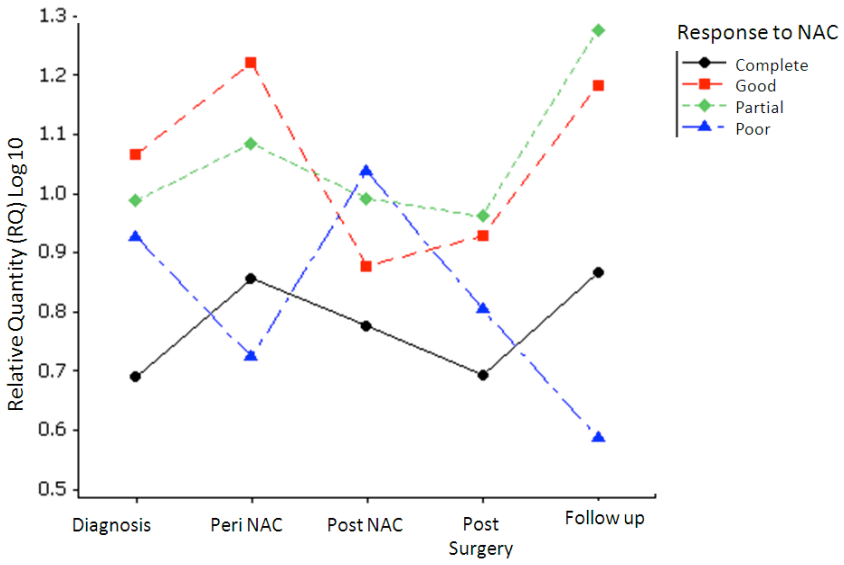
Table 7.6 Correlation between *miR-195* expression and study timepoints

Timepoint	Diagnosis		Peri NAC		Post NAC		Post Surgery		Follow up	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Diagnosis	NA	NA	0.68	0.001*	0.56	0.006*	0.51	0.017*	0.66	0.001*
Peri NAC			NA	NA	0.48	0.042*	0.38	0.109	0.49	0.045*
Post NAC					NA	NA	0.43	0.073	0.56	0.024*
Post Surgery							NA	NA	0.41	0.143
Follow up									NA	NA

*P value <0.05

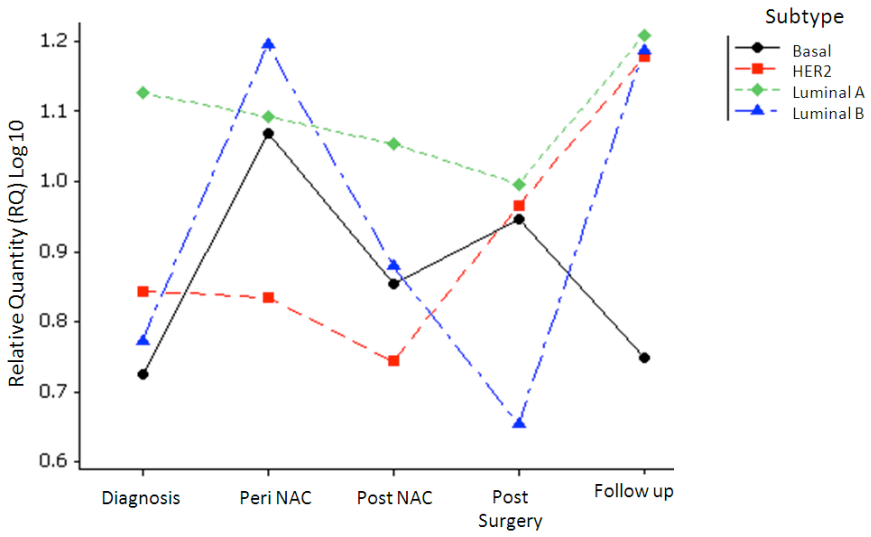
The relationships between *miR-195* expression, response to NAC and molecular subtype was then determined (Figure 7.6 and 7.7) Response to NAC was classified according to the Miller and Payne method and broadly divided into complete (pCR), good, partial or poor response groups (326). The statistical analyses are presented in Table 7.7.

Figure 7.6 *MiR-195* expression and response to NAC



Mean *miR-195* expression for each of the response groups is plotted above. The complete response group had relatively low overall *miR-195* expression while the partial and poor response group had comparatively higher *miR-195* expression levels. There was no significant difference in *miR-195* expression between the groups at any timepoint (Table 7.7).

Figure 7.7 *MiR-195* expression and molecular subtype



Mean *miR-195* expression for each of the subtypes is presented above. Circulating *miR-195* levels were higher in the Luminal A group and lower in the HER2/*neu* group during the first three timepoints, with all groups having comparatively higher levels of *miR-195* at follow up. There was no significant difference in *miR-195* expression between subtypes at any timepoint (Table 7.7)

Table 7.7 *MiR-195* expression across timepoints for molecular subtypes and response groups

Timepoint	Subtype	Response
Diagnosis	0.128	0.656
Peri NAC	0.604	0.357
Post NAC	0.624	0.875
Post Surgery	0.455	0.808
Follow up	0.281	0.031*

Differences in *miR-195* expression between subgroups at each timepoint were assessed for significance using the ANOVA. There was no significant difference at any timepoint between molecular subtypes (Luminal A, Luminal B, HER2/*neu* or Basal). There was a significant difference in *miR-195* expression at follow up between treatment response groups ((complete, good, partial or poor, ANOVA, p=0.031).

Finally the relationship between *miR-195* expression and nodal status, HER2/*neu* status and ER status was assessed (Table 7.8).

Table 7.8 Difference in *miR-195* expression with clinicopathological parameters

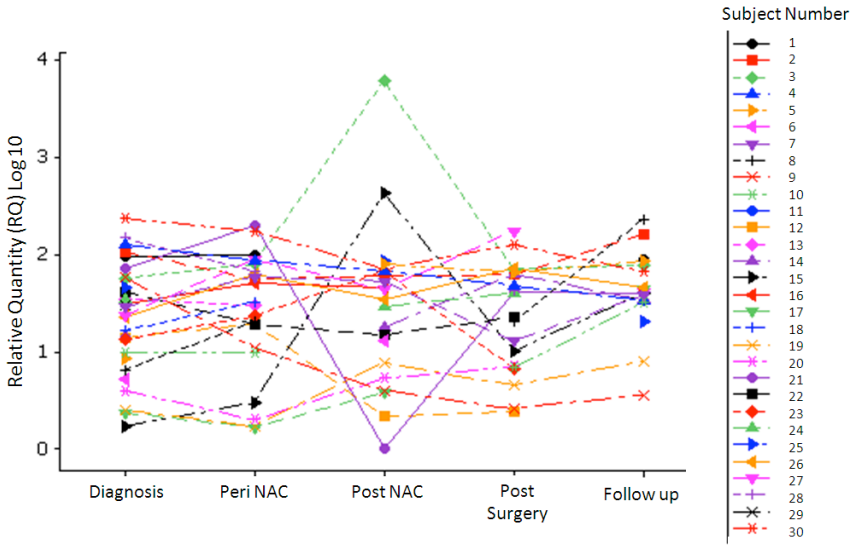
Timepoint	Nodal Status	HER2/*neu* status	ER status
Diagnosis	0.008*	0.223	0.076
Peri NAC	0.794	0.601	0.337
Post NAC	0.062	0.365	0.227
Post Surgery	0.851	0.208	0.775
Follow up	0.424	0.722	0.298

There was a significant difference between *miR-195* expression in the circulation of node positive and node negative women at diagnosis, in advance of commencing NAC (2-sample t-test, p=0.008).

7.4.4 MiR-497

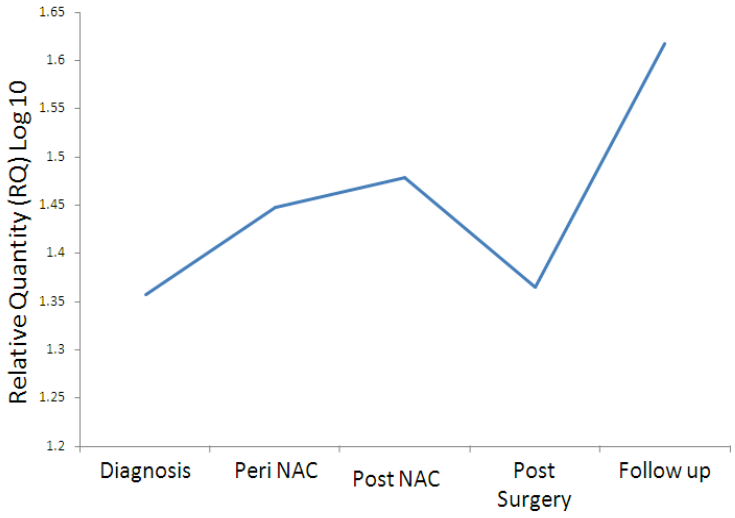
MiR-497 was detectable at all study timepoints in all patient blood samples (Figure 7.8). The mean *miR-497* expression profile was generated to examine the overall trend over the course of NAC. Figure 7.9 outlines that although not significant, *miR-497* increased in expression at annual follow up (p=0.601, ANOVA). The correlation between *miR-497* expression at specific timepoints was then assessed (Figure 7.10).

Figure 7.8 Individual patient *miR-497* profiles



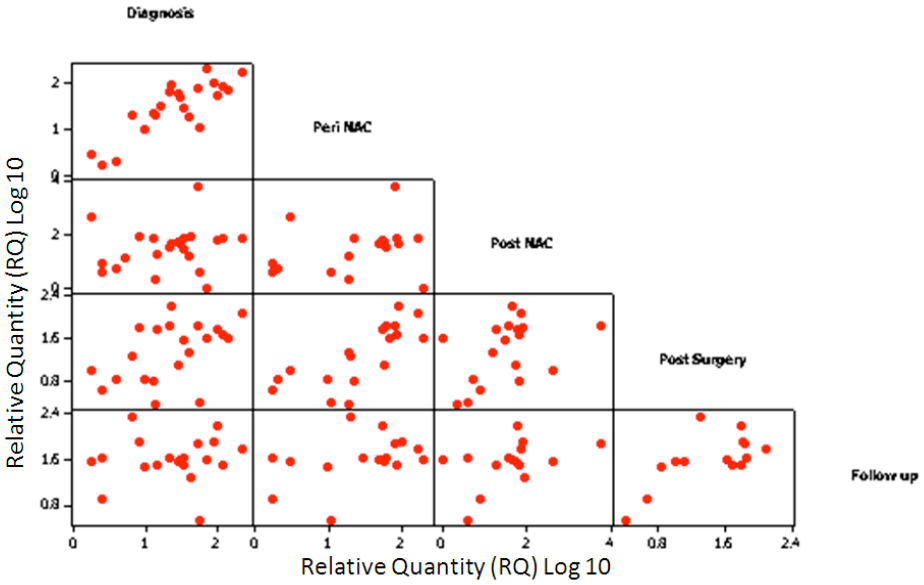
MiR-497 expression was detectable at each timepoint for each patient with the individual patient profile outlined above.

Figure 7.9 Mean *miR-497* expression



MiR-497 was underexpressed at diagnosis and again post-surgery, with expression increasing at the follow up period.

Figure 7.10 Correlation between *miR-497* expression at various timepoints



The matrix plot above represents the correlation between *miR-497* at each timepoint plotted against each other timepoint. Of note, there was a positive correlation (R=0.85) between *miR-497* at diagnosis and at the peri NAC timepoint; Patients with reduced levels of *miR-497* at diagnosis also had low *miR-497* peri-NAC.

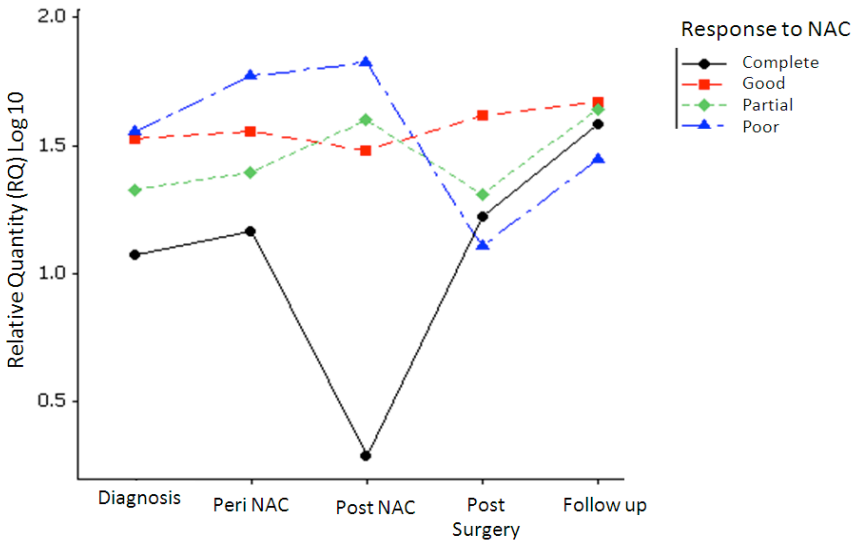
Table 7.9 Correlation between *miR-497* expression at various timepoints

Timepoint	Diagnosis		Peri NAC		Post NAC		Post Surgery		Follow up	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Diagnosis	NA	NA	0.859	0.001*	0.179	0.414	0.495	0.022*	0.109	0.648
Peri NAC			NA	NA	0.260	0.313	0.748	0.001*	0.420	0.106
Post NAC					NA	NA	0.425	0.079	0.448	0.082
Post Surgery							NA	NA	0.686	0.007*
Follow up									NA	NA

*P value <0.05

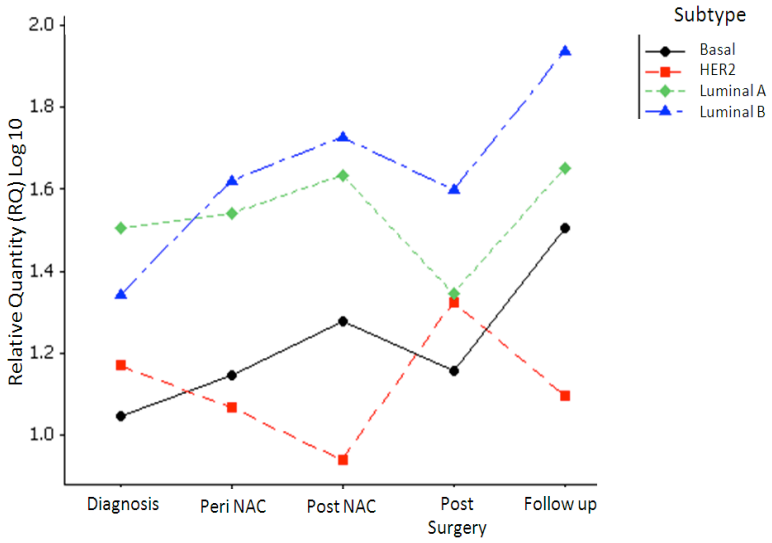
The relationship between *miR-497* expression and both response to NAC (Figure 7.11) and molecular subtype (Figure 7.12) was then determined. The statistical analyses are presented in Table 7. 10, highlighting that there was no significant difference between groups at any specific timepoint.

Figure 7.11 *MiR-497* expression and response to NAC



Although no obvious trend was observed, it is interesting to note that those with a complete response to NAC had a lower *miR-497* level at diagnosis than those with a poor response.

Figure 7.12 *MiR-497* expression and molecular subtype



Although not significant, Luminal A and Luminal B breast cancers had higher *miR-497* expression than those with Basal and HER2/*neu* subtypes.

Table 7.10 Difference in *miR-497* expression across timepoints for different molecular subtypes and response groups

Difference in *miR-497* expression (ANOVA)		
Timepoint	**Subtype**	**Response**
Diagnosis	0.338	0.670
Peri NAC	0.507	0.811
Post NAC	0.515	0.161
Post Surgery	0.786	0.710
Follow up	0.117	0.935

The relationship between *miR-497* expression and nodal status, HER2/*neu* status and ER status was assessed (Table 7.11). There was no significant difference in *miR-497* expression across positive and negative nodal, HER2/*neu* or ER status.

Table 7.11 Difference in *miR-497* expression with clinicopathological parameters

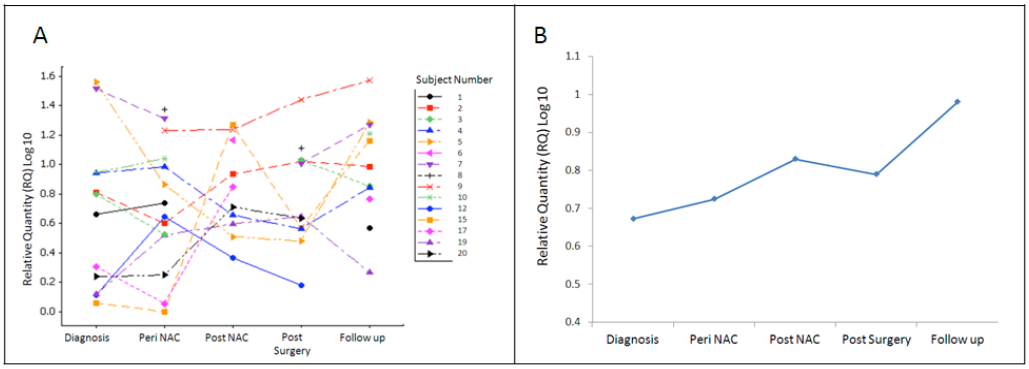
Timepoint	Nodal Status	HER2/*neu* status	ER status
Diagnosis	0.420	0.656	0.123
Peri NAC	0.849	0.659	0.232
Post NAC	0.912	0.551	0.186
Post Surgery	0.552	0.507	0.612
Follow up	0.533	0.950	0.161

There was no significant difference in *miR-497* expression across different clinicopathological parameters.

7.4.5 MiR-21

MiR-21 was profiled on 15 patients across each of five timepoints. No clear trend was noted when each patient was analysed in isolation, however when the mean *miR-21* level was determined it was possible to see that its expression increased from diagnosis to follow-up. The relationship between *miR-21* expression and clinicopathological variables is presented in Table 7.12 and Table 7.13. Of note, *miR-21* is significantly higher in those with ER positive disease at diagnosis (p=0.008), although significantly lower in this group post NAC (p=0.021).

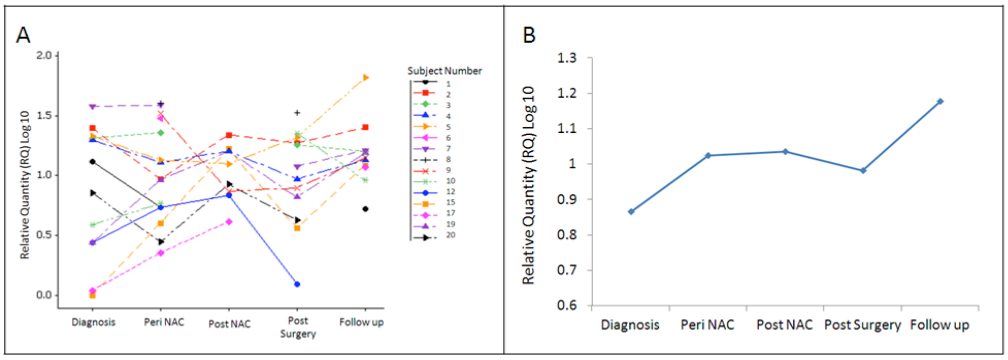
Figure 7.13 *MiR-21* expression (A) overall (B) mean



7.4.6 MiR-29a

The mean *miR-29a* expression showed a similar trend to *miR-21*, with levels rising from diagnosis to follow up. The relationship between *miR-29a* expression and clinicopathological variables is presented in Table 7.12 and Table 7.13.

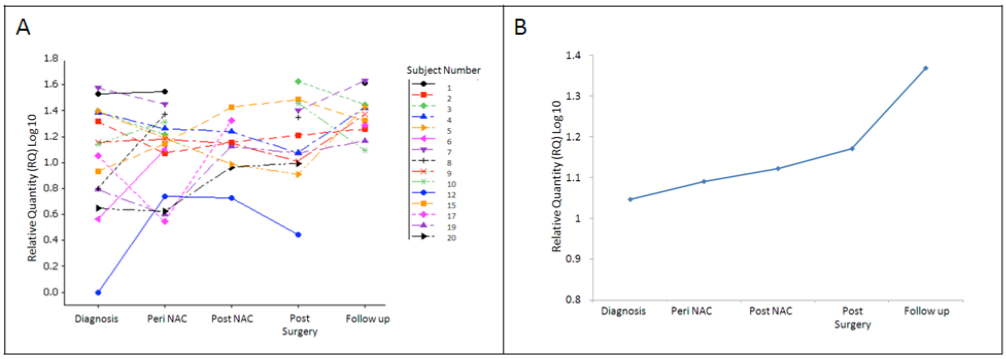
Figure 7.14 *MiR-29a* expression (A) overall and (B) mean



7.4.7 MiR-181a

Interestingly, mean *miR-181a* levels also rose gradually from diagnosis to follow up. The relationship between *miR-181a* expression and clinicopathological variables is presented in Table 7.12 and Table 7.13. *MiR-181a* was significantly higher at follow up in women with node negative disease at diagnosis ($p<0.001$).

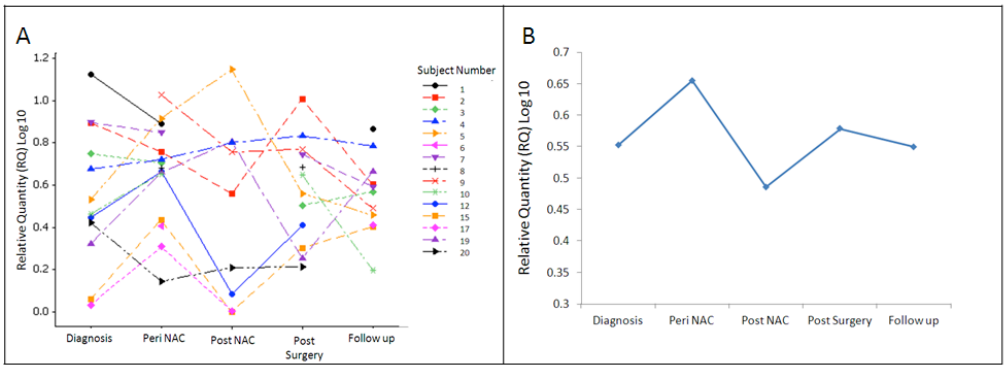
Figure 7.15 *MiR-181a* expression (A) overall and (B) mean



7.4.8 MiR-223

The relationship between *miR-223* expression and clinicopathological variables is presented in Table 7.12 and Table 7.13. *MiR-223* was significantly higher at diagnosis in women with ER positive disease compared to those with ER negative disease.

Figure 7.16 *MiR-223* expression (A) overall and (B) mean



7.4.9 MiR-652

The relationship between *miR-652* expression and clinicopathological variables is presented in Table 7.12 and Table 7.13. *MiR-652* is significantly higher post-surgery in those with ER positive disease than those with ER negative disease (p=0.04).

Figure 7.17 *MiR-652* expression (A) overall and (B) mean

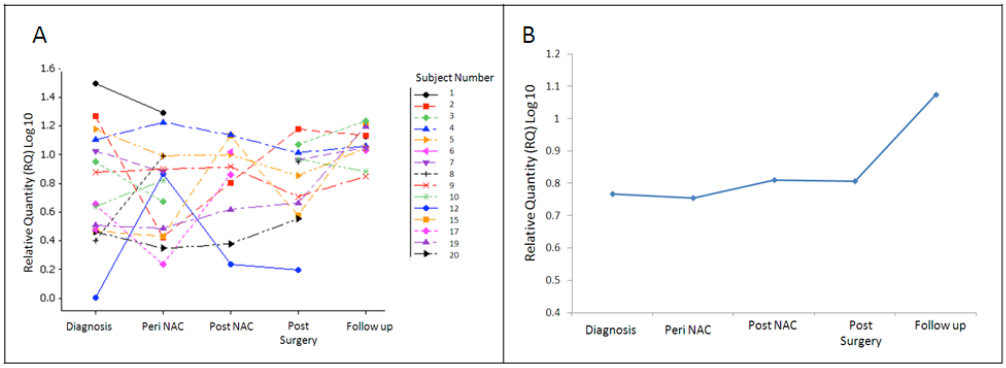


Table 7.12 MiRNA expression across subtype and response to treatment at each timepoint

miRNA	Timepoint	Subtype	Response
miR-21	Diagnosis	0.164	0.468
	Peri NAC	0.106	0.578
	Post NAC	0.184	0.809
	Post Surgery	0.292	0.673
	Follow up	0.751	0.894
miR-29a	Diagnosis	0.147	0.283
	Peri NAC	0.666	0.132
	Post NAC	0.275	0.168
	Post Surgery	0.383	0.790
	Follow up	0.456	0.863
miR-181a	Diagnosis	0.555	0.678
	Peri NAC	0.483	0.558
	Post NAC	0.700	0.657
	Post Surgery	0.942	0.694
	Follow up	0.957	0.181
miR-223	Diagnosis	0.110	0.473
	Peri NAC	0.044*	0.275
	Post NAC	0.223	0.539
	Post Surgery	0.209	0.914
	Follow up	0.716	0.137
miR-652	Diagnosis	0.582	0.917
	Peri NAC	0.096	0.800
	Post NAC	0.341	0.966
	Post Surgery	0.551	0.889
	Follow up	0.296	0.545

The expression of each of the five miRNAs was determined for each timepoint on a subgroup of 15 patients. There was a difference in *miR-223* expression between subtypes at the peri NAC timepoint. No miRNA had a significant difference at any timepoint with regards to response to treatment. *P value <0.05

Table 7.13 MiRNA expression across HER2/*neu* status, ER status and nodal status for each timepoint

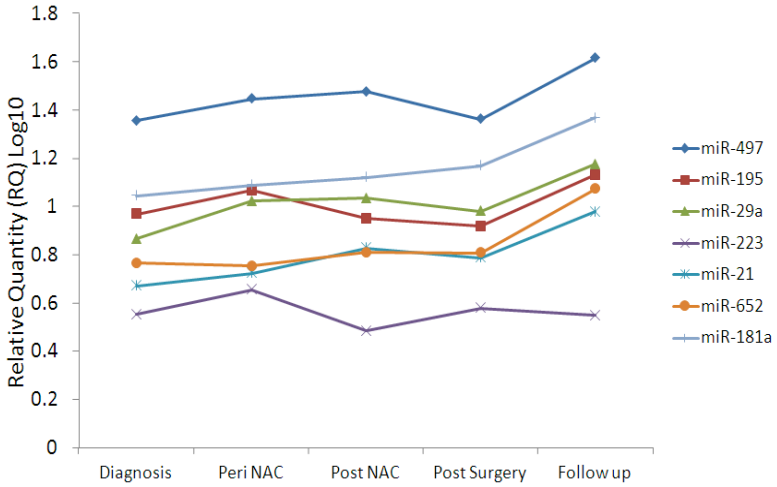
miRNA	Timepoint	HER2 status	ER status	Nodal Status
miR-21	Diagnosis	0.471	0.008*	0.669
	Peri NAC	0.334	0.215	0.913
	Post NAC	0.902	0.021*	NA
	Post Surgery	0.902	0.717	0.803
	Follow up	0.401	0.405	0.877
miR-29a	Diagnosis	0.952	0.096	0.144
	Peri NAC	0.508	0.469	0.752
	Post NAC	0.374	0.191	NA
	Post Surgery	0.205	0.062	0.693
	Follow up	0.405	0.397	0.538
miR-181a	Diagnosis	0.400	0.193	0.476
	Peri NAC	0.878	0.168	0.677
	Post NAC	0.510	0.258	NA
	Post Surgery	0.462	0.967	0.896
	Follow up	0.866	0.472	0.001*
miR-223	Diagnosis	0.472	0.029*	0.216
	Peri NAC	0.456	0.136	0.932
	Post NAC	0.280	0.131	NA
	Post Surgery	0.111	0.403	0.835
	Follow up	0.862	0.075	0.365
miR-652	Diagnosis	0.587	0.129	0.399
	Peri NAC	0.395	0.073	0.814
	Post NAC	0.124	0.631	NA
	Post Surgery	0.434	0.040*	0.946
	Follow up	0.111	0.637	0.713

NA; not available as not enough miRNA expression levels to perform statistical analyses
*P value <0.05

7.4.10 Overall trend of miRNA expression

MiRNA expression was not uniformly altered over the course of NAC. As evident in Figure 7.18 below, 4 miRNAs (*miR-21, miR-181a, miR-195 and miR-497*) display a pattern of post-surgery reduction in relative expression while 2 miRNAs (*miR-223* and *miR-181a*) show a moderate increase in expression. *MiR-652* showed relatively no alteration in mean expression between the post-neoadjuvant chemotherapy and post-surgical timepoints.

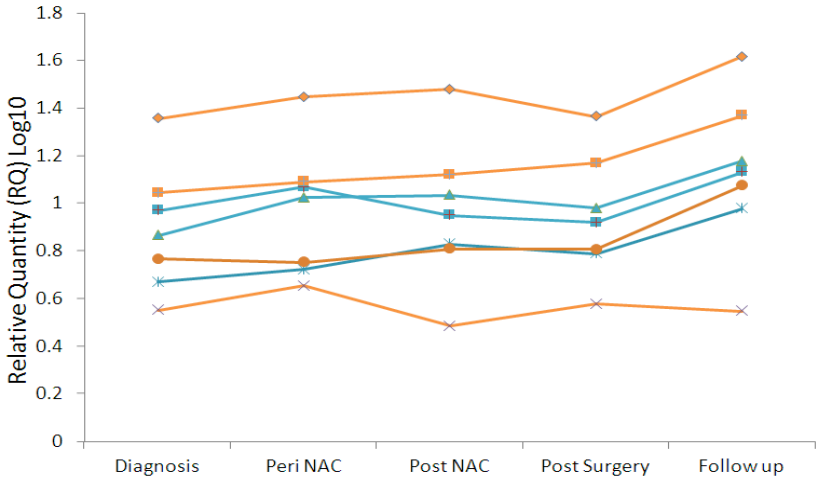
Figure 7.18 Mean expression for each miRNA



Mean miRNA expression of each of the 7 miRNAs is displayed above. A post surgical relative reduction in expression was observed for 4 miRNAs (*miR-21, miR-181a, miR-195, miR-497* and *miR-652*) while *miR-181a* and *miR-223* showed a relative increase in expression. *MiR-652* showed no alteration between the post neoadjuvant and post-surgical timepoints (post NAC 0.811, post surgery 0.808).

When the target miRNAs were separated into two groups, those with increased expression in the cancer group (oncomiRs) and those with reduced expression in the cancer group (tumour suppressor miRs), no obvious pattern emerged (Figure 7.19).

Figure 7.19 MiRNA expression patterns of oncomiRs and tumour suppressor miRNAs



Mean expression for miRNAs underexpressed in the breast cancer state (orange) and overexpressed in the breast cancer state (blue).

7.5 Discussion

Neoadjuvant chemotherapy (NAC) has become an increasingly popular treatment modality in the management of breast cancer. The goal of treatment is to downstage the primary tumour, thus permitting breast conserving surgery (BCS) over mastectomy and providing a more favourable cosmetic outcome (319). It is also used in the management of locally advanced inoperable breast cancers deemed unsuitable for surgical resection at the outset. In these cases the aim is to reduce tumour volume sufficiently to enable surgical resection (50). As mentioned, the safety and benefit of NAC has been demonstrated by numerous large studies, namely the NSABP-18, NSABP-27 and the European Organisation for Research and Treatment of Cancer trial 10902 (51, 320, 321). Chemotherapy drugs mainly exert their effects by inducing apoptosis, inhibiting mitosis or both. These agents cannot discriminate between neoplastic and healthy tissue, and thus toxic side effects (usually dose related) commonly occur such as gastrointestinal upset, bone marrow suppression (with subsequent neutropenia and anaemia) and cardiac or neurological toxicity.

However, not all individuals derive the same benefit from NAC. Response to treatment is mainly assessed with the use of serial imaging (mammography, ultrasonography or MRI). However this method frequently fails to accurately assess tumour burden and true response to treatment. Still, monitoring response to treatment is crucial to avoid unnecessary side effects from ineffective therapies. Only 2 predictive markers are validated and routinely assessed in the management of breast cancer; ER status and HER2/*neu* receptor status. These markers convey that a likely benefit would be achieved from treatment with hormonal therapies or Trastuzumab, respectively, but are of no benefit in NAC. Furthermore, both of these markers are tested on breast tissue. Cancer Antigen 15-3 (CA 15-3) is a serum biomarker but its relevance in clinical practice is limited with no useful application to treatment response in NAC. There is a need to develop sensitive, specific, minimally invasive molecular biomarkers for breast cancer that can be utilised to predict response and outcome, therefore facilitating the pre-therapeutic classification of patients into subgroups for appropriate treatment. Similarly, in the evolving era of individualised, targeted cancer therapy there is a demand for circulating biomarkers to monitor response to such treatment.

MiRNAs appear to be well suited to this task; they are easily detectable in the peripheral circulation facilitating relatively easy sampling in comparison to tumour biopsy. In addition, miRNAs may have the ability to reflect tumour biology. Moreover, the expression of several

specific miRNAs is altered in the cancer state, and could be further inhibited or induced over the course of neoadjuvant chemotherapy in line with clinicopathological parameters, thus providing a unique opportunity for a 'fluid biopsy' denoting the nature of the response to treatment. Ideally a suitable panel of miRNA biomarkers would show significant changes in expression level in good-responders whilst little or no change would be observed in miRNA expression in non-responders. Several studies to date have reported on the ability of miRNA expression profiles to predict chemo-sensitivity which may be due to the downstream effects of these specific miRNAs on their mRNA targets and protein expression. Altered protein expression may interfere with the ability of the neoplastic cell to respond to the chemotherapeutic agent, by altering pathways such as the DNA repair systems or apoptosis (327). Circulating *miR-200c* has been identified as a predictor of response to chemotherapy and prognosis in oesophageal cancer (328). In patients with metastatic colorectal cancer, elevated *miR-625-3p* is associated with a poor response to oxaliplatin chemotherapy regimens (329). However, these studies mainly use simply pre-treatment miRNA measurements.

This study is the first of its kind. At the time of writing there were 81 patients enrolled in the study. At the outset, this was a single centre trial (Galway University Hospital) but has since expanded to four other sites (St James's Hospital, Letterkenny General Hospital, Sligo General Hospital and Beaumont Hospital). The primary goal of the ICORG 10-11 study at large is to identify a panel of circulating miRNA markers which could help identify those breast cancer patients who are most likely to respond well to neoadjuvant chemotherapy, to serve as an overall prognostic factor and stratify patients into risk categories which would further guide their management. Albain *et al* have identified that a large percentage of patients with hormone receptor positive (luminal A and luminal B) breast cancer do not derive a benefit from adjuvant anthracycline based chemotherapy regimens (330, 331). But there is no clinical test to discriminate between these groups. Similarly, we aim to identify a panel of circulating miRNA markers which could monitor patient's response to chemotherapy and hormonal therapies. Ideally a suitable panel of markers would show significant changes in expression level in good-responders whilst little or no change would be observed in miRNA expression in non-responders.

The results to date are both interesting and encouraging; while simultaneously raising further questions about what alterations at the molecular level over the course of NAC is

contributing towards the altering miRNA expression profiles. The consistency in miRNA yield irrespective of timepoint and patient suggests that the miRNA profiles are not merely reflecting the total miRNA concentration or cellularity of the sample. It is reassuring to note that not all miRNAs evaluated in this study had the same pattern of alteration. It alludes to the potential that changes in specific miRNA expression may reflect underlying changes in tumour biology, as a response to treatment as well as being a direct effect of chemotherapy. It would be remarkable if molecular changes at the tumour level were detectable in the circulation as a result of selective secretion or passive shedding over the duration of breast cancer management.

While firm conclusions cannot be drawn from this pilot cohort some interesting observations have been noted.

- There was no significant difference in mean miRNA yield (ng/µl) between each timepoint. This is reassuring as it suggests that differences in miRNA expression are not simply reflective of differences in total RNA or miRNA quantity. Total miRNA quantity could be influenced by cellularity or composition of the blood specimen, for example, following chemotherapy.
- The mean expression of all miRNAs increases relatively at follow up. This is interesting, particularly for miRNAs such as *miR-195* which have previously been reported to be over-expressed in the breast cancer state with a reduction to the level of healthy controls post-operatively (93).
- *MiR-195* expression at diagnosis in the study group overall was similar to what would be expected based on previous studies (Chapter 5).
- *MiR-195* has relatively increased expression in Luminal A breast tumours both at diagnosis and throughout each of the study timepoints.
- *MiR-497* has relatively increased expression in hormone receptor positive breast tumours (Luminal A and Luminal B) throughout the study timepoints.

Although in its infancy, these preliminary findings are exciting. Biomarkers that can exploit the molecular understanding of breast cancer are needed to stratify patients into groups, and sub-groups so that treatment pathways and therapeutic agents reflect individual tumour biology and patient needs, thus providing individualised target breast cancer treatment.

Chapter 8
Discussion

8.1 Introduction

The global burden of breast cancer is continuing to rise; it is the principle cause of death in developed countries and the second leading cause of death in developing countries with the incidence projected to increase steadily up to 2050 (1, 3). It is imperative that scientists and clinicians work in partnership to unravel the molecular mechanisms responsible for tumourigenesis so that breakthroughs which impact on the morbidity and mortality of this disease can be made in the diagnosis, prognosis, and treatment of breast cancer.

MiRNAs are small single-strand endogenous non-coding RNAs of approximately 18 to 22 nucleotides in length. They were first discovered 20 years ago, in 1993, and have since become the focus of much scientific and translational research. MiRNAs are known to play functional roles in almost all physiological and pathological processes, including carcinogenesis (53). In addition to a functional role in initiation, promotion, progression and malignant transformation of tumour cells, miRNAs are released from these cells and the tumour microenvironment, either actively or passively, and are detectable in a plethora of body fluids including blood making them exceptional biomarker candidates. Aberrant miRNA expression has been described in a host of disease states.

MiRNAs are a promising area of translational research, particularly for diseases such as breast cancer, which is in need of a novel class of biological agents to facilitate diagnosis, guide and monitor response to treatment, assist in prognostication and potentially act as an innovative therapeutic strategy.

8.2 Summary and implication of results

It was the aim of this study to investigate the expression of miRNAs in breast cancer with a particular emphasis on the application of miRNAs as circulating oncologic biomarkers for breast cancer.

8.2.1 Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies

Real-time quantitative (RQ-PCR) is widely used to quantify miRNA expression due to its sensitivity, specificity, speed, simplicity and the small amounts of template RNA required. To differentiate true biological variation from experimentally induced artefacts, target miRNA expression levels are normalised to those of a control(s). Despite the abundance of studies on circulating miRNA profiles to discriminate between normal and disease states, there had yet to be conclusive reports of appropriate ECs. Few validated endogenous controls for miRNA research in breast cancer have been described. Early studies on systemic miRNAs in breast cancer normalized to *miR-16* with few exceptions employing the use of candidates such as *U6, RNU44, RNU48*, *miR-142-3p*, *miR-484*, *miR-191* and *miR-425* (87, 93-102). Additional studies, on breast and other cancers, have suggested alternative EC candidates such as *U6, RNU44, RNU48*, *miR-142-3p*, *miR-484*, *miR-191* and *miR-425* (87, 95-102). However, there is a lack of validated reports of suitable ECs for circulating miRNAs. In the present study a high throughput miRNA array platform was used to analyse the expression of 387 candidate miRNAs in 20 blood samples (n=10 cancer, n=10 control) to screen for stably expressed miRNAs. Candidates for further validation by RQ-PCR were identified by GME analysis of the array data in combination with GeNorm analysis of candidate miRNAs chosen from the literature. Subsequent validation by RQ-PCR in a larger cohort (n=40 cancer, n=20 controls) revealed *miR-16* and *miR-425* as the most suitable EC candidates. This study has important implications for breast cancer translational research. The surge of interest in identifying specific miRNAs as biomarkers for health and disease requires that an equal amount of attention is focused on the establishment of suitable ECs with which to normalize the data such that appropriate conclusions can be derived. This study therefore provides a normalisation approach for future RQ-PCR studies.

8.2.2 Identification and Validation of Oncologic miRNA Biomarkers for Luminal A Breast Cancer

Despite recent advances in our understanding of the molecular portraits and biology of breast cancer, hematogenous spread of malignant cells from the primary tumour to distant organs with subsequent proliferation into metastases remains the leading cause of death for breast cancer patients (6). There are no reliable circulating biomarkers for breast cancer. Mammography is the most widespread screening tool, with a definitive diagnosis requiring an invasive tissue biopsy. This prevalent disease is in need of a minimally invasive biomarker which may be used in combination with radiological imaging to facilitate early subtype specific tumour diagnosis. Blood presents an excellent medium for biomarker discovery; it circulates throughout the body, distributing and collecting molecules, thus acting as a mosaic of all the processes occurring. Upon commencing this study, there were several reports that tumour-specific miRNAs were detectable in the circulation (94, 119, 120). However, no attempt had been made to determine if miRNA profiles could not only reflect the primary solid organ, but the tumour subtype. This work aimed to progress current understanding of miRNAs in Luminal A breast cancer. Microarray analysis identified 76 differentially expressed miRNAs, providing insight into the molecular mechanisms underpinning this breast cancer subtype. ANN revealed 10 miRNAs for further analysis (*miR-19b, miR-29a, miR-93, miR-181a, miR-182, miR-223, miR-301a, miR-423-5p, miR-486-5* and *miR-652*). The biomarker potential of 4 miRNAs (*miR-29a, miR-181a*, *miR-223* and *miR-652*) was confirmed by RQ-PCR, with significantly reduced expression in blood of women with Luminal A breast tumours compared to healthy controls (p=0.001, 0.004, 0.009 and 0.004 respectively). A miRNA profile of three circulating miRNA biomarkers (*miR-29a, miR-181a* and *miR-652)* for breast cancer was identified which in combination provided a sensitivity (77%) and specificity (74%) profile exceeding those of several current clinical biomarkers. This has important implications in breast cancer diagnosis as a complementary test, for use in combination with mammography would prove extremely advantageous for breast cancer diagnosis particularly in an era where prompt diagnosis, expeditious commencement of appropriate adjuvant treatments and surgical resection have a role to play in ultimately improving patient outcomes. It is clear that further studies analysing the expression of these miRNAs in other breast cancer subtypes and solid cancers is warranted.

8.2.3 Differential Expression of miR-15 Family Members in Breast Cancer

The process by which miRNAs manipulate gene expression largely depends on the degree of base-pair complementarity between the miRNA and its target mRNA which is largely governed by the seed sequence (55). MiRNA families are groups of miRNAs which share a common seed sequence. MiRNA related research to date has focused on individual miRNAs with sparse reports of miRNA families in specific disease states (230-233). *MiR-195* is a member of the *miR-15* family and has been implicated in several types of cancers with overexpression in the circulation of women with breast cancer compared to healthy controls (93, 119, 234-237). The aim of this study was to contribute to current understanding of miRNA families by profiling the *miR-15* family in blood and tissue of women with breast cancer. *MiR-15a*, *miR-15b* and *miR-497* expression were lower in the circulation of breast cancer patients versus healthy controls (p=0.001, 0.018, and 0.03) whereas *miR-195* was upregulated in breast cancer patients (p=0.001). In tissues, *miR-15b*, *miR-195*, *miR-424* and *miR-497* were dysregulated (p=0.001, 0.001, 0.03 and 0.03 respectively). *MiR-195* (p=0.04, R=0.64) and *miR-497* (p=0.04, R=0.61) tissue expression correlated with that of the blood. The variation in *miR-15* family expression across members and between tissue and blood within the same member was interesting and suggests that although all miRNAs were detectable, some may be more biologically relevant than others. The combination of *miR-15a, miR-195* and *miR-497* provided both a sensitivity and specificity of 82% and 76.5% respectively for breast tumour detection. *MiR-15* miRNAs have multiple cancer-related mRNA targets including BCL2, CCND1 and FGF2. This study is the first to examine expression of a miRNA family in the circulation of women with breast cancer and suggests that this miRNA family are promising biomarkers and therapeutic targets in the future.

8.2.4 Analysis of miR-195 and miR-497 in the tumour microenvironment

MiR-195 and *miR-497* are members of the *miR-15* family and are clustered on chromosome 17 (17p13.1) and are altered in both the circulation and tissue of women with breast. The origin of circulating *miR-195* and *miR-497* is not of critical relevance in exploiting these miRNAs as biomarkers for breast cancer. However, this becomes more critical in elucidating the role these miRNAs are playing in carcinogenesis and also in determining whether manipulation of miRNA expression could be explored as a viable therapeutic avenue for breast cancer. The initial aim of this study was to determine where in the tumour

microenvironment *miR-195* and *miR-497* were located using *in situ* hybridisation (ISH). *MiR-195* was detected in both the stromal and tumour epithelial cells.

The subsequent objective was to assess *miR-195* and *miR-497* expression in different breast cancer cell lines, to determine if all cell lines displayed a similar expression pattern. The MDA-MB-231 cell line (basal subtype) displayed the lowest *miR-497* expression. While the hormone receptor positive and HER2/*neu* overexpressing (ZR-75-1, T47D and SKBR3) cell lines display higher and similar rates of *miR-497* expression. Finally, it was evaluated if this miRNA cluster was subject to epigenetic modulation by methylation *in vitro* by treating breast cancer cell lines with azacytidine. The treated cells (10mM azacytidine) displayed increased expression of both miRNAs suggesting that the expression of these molecules could be influenced by epigenetic modification, thus highlighting potential upstream mechanisms to alter expression which could be harnessed as therapeutic strategies.

8.2.5 Circulating miRNAs: Novel breast cancer biomarkers and their use for guiding and monitoring response to chemotherapy

Despite recent advances in the understanding of the molecular mechanisms underpinning breast cancer aetiology and development, management of this heterogeneous disease has altered little over the past decade. This is particularly true in relation to adjuvant chemotherapy which is currently offered to women with lymph node involvement (318). Early experience with this treatment has demonstrated that response to therapy is not uniform with some deriving more of a benefit than others. At the time of commencing this study, there was no clinical test that could meticulously predict responders from non-responders, or that could be exploited to monitor treatment response in real time. The earlier studies outlined in this project in combination with the large body of published literature indicate that miRNAs are promising biomarker candidates. This study assessed the potential application of miRNAs (*miR-21, miR-29a, miR-181a, miR-195, miR-223, miR-497* and *miR-652*) as predictors of response to neoadjuvant chemotherapy and as tools for monitoring therapeutic response by assessing fluctuations in circulating miRNA expression levels over the course of neoadjuvant chemotherapy, overall and with respect to clinical and pathological variables. The results are both interesting and encouraging; while simultaneously raising further questions about what alterations at the molecular level over the course of NAC are contributing towards the altering miRNA expression profiles. Although in its infancy, the preliminary findings of this study are exciting and could provide an example of translational research moving from bench to bedside.

8.3 Potential clinical application

8.3.1 MiRNAs as biomarkers

Moving scientific revelations from the laboratory to the clinic is the goal of all translational research. The findings of this study support pursuing the application of miRNAs as disease biomarkers, in the diagnostic realm, and also as biomarker tools to facilitate monitoring and predicting response to treatment. MiRNAs exhibit several characteristics which deem them to be ideal biomarker candidates, including their stability, disease specific expression profiles and lower complexity. Perhaps the most exciting characteristic of miRNAs as a biomarker for breast cancer is their ability to be detected in the systemic circulation. Lawrie et al were the first to report that miRNAs were detectable in the blood, with elevated *miR-21* in the serum of patients with large B-cell lymphoma (116).

The diagnosis of breast cancer currently involves the combination of clinical examination, radiological imaging and an invasive tissue biopsy, providing histological confirmation. Mammography is currently considered the gold standard for diagnosis and the concept of breast screening is now accepted internationally as it is associated with an improved outcome due to earlier diagnosis (332). However, mammography is not without its constraints, with both ionisation exposure, a high proportion of lobular carcinomas are mammographically occult, dense breasts make interpretation of the mammogram difficult, there are high false negative rates and a false positive rate of up to 10% (333).
Small curable early cancers can potentially be missed. When diagnosed promptly, women are more likely to have early stage disease, confined to the breast. These cases are amenable to breast conserving surgery. In cases where tumour cells have metastasised to the lymph nodes at the time of diagnosis, more extensive local surgery (mastectomy) and axillary surgery (an axillary lymph node clearance) is often required. This procedure carries the potential for severe complications, including lymphoedema, which impacts extensively on ones quality of life. This body of work presents two distinct circulating miRNA profiles (*miR-29a/miR-181a/miR-652* and *miR-15a/miR-195/miR-497*) for the detection of breast cancer, each with an acceptable sensitivity and specificity profile for use in clinical practice (77% and 74%, and 82% and 76.5%, respectively). These findings have the ability to augment the diagnostic capacity, and perhaps even facilitate a 'fluid biopsy' by permitting tumour biology to be revealed from blood.

The potential of miRNAs as diagnostic biomarkers has already entered the commercial setting. Rosetta Genomics [TM, 25], is a leading organisation in this field with a number of commercially available products. The Rosetta Cancer Origin Test™ includes a panel of 64 miRNAs which can discriminate between 42 different cancers in patients with cancers of unknown origin. Other miRNA diagnostic products include the Rosetta Lung Cancer Test™, Rosetta Kidney Cancer Test™ and Rosetta Mesothelioma Test™. Mira Dx™ [26] is another company which was founded by Dr Frank Slack and Dr Joanne Weidhaas. Their main product is PreOvar®, a genetic test for the KRAS-variant which is significantly enriched in women with Hereditary Breast and Ovarian Cancer (HBOC). GenSignia[27] is another recently founded company which is focusing on miRNAs as diagnostic biomarkers for lung cancer.

These small molecules may also have the capacity as biomarkers in the prediction and prognostic sense. This would be particularly useful in facilitating decisions surrounding the provision of neoadjuvant and adjuvant chemotherapy in breast cancer. Chemotherapy is associated with toxic side effects and a varied response rate. There is currently no clinical test (tissue- or circulation-based) that can meticulously predict responders from non-responders, or that can be exploited to monitor treatment response in real time. Subsequently, a number of women are administered toxic treatment without any promise of deriving any benefit. As revealed in Chapter 7, miRNAs may have the potential to discriminate between these groups, thus sparing a proportion of patients from the toxic effects of treatments from which they would derive no benefit.

Customisation of cancer treatment, including the avoidance of unnecessary treatments, is a key philosophy of personalized cancer care. In addition isolating women likely to respond from chemotherapy, it is necessary to segregate those who do no require chemotherapy in the first instance. This could be executed by a prognostic tool. There are currently two commercially available prognostic tools. Both Oncotype DX ®(the 21-gene available assay) and Mammaprint® (the Amsterdam based 70-gene assay) are commercially available gene signatures that were derived from molecular profiling techniques with prognostic capabilities (46-49). Despite providing a more individualised approach to the management of breast cancer, there are many inherent weaknesses in the use of these messenger RNA signatures alone. Recent evidence suggests that miRNA signatures provide complementary

[25] http://www.rosettagenomics.com/
[26] http://www.miradx.com/
[27] http://www.gensignia.com

and additional information to traditional molecular marker studies, and could help stratify patients into prognostic groups (112).

All of the above benefits would have a significant positive impact on over-burdened healthcare sectors worldwide. At the primary care setting, the ability to differentiate benign from malignant breast disease from a simple blood test would allow early stratification and immediate referral to an appropriate centre of excellence for breast cancer management. This could even be performed by a simple point-of-care diagnostic test, with the ability to detect a panel of miRNAs, perhaps including those mentioned above. Furthermore, the health economic implications of such a discovery extend far beyond our local unit. The ability to predict responders and non-responders, as highlighted above, spares those who are unlikely to derive any benefit from chemotherapy from the toxicity and severe side effect profile while simultaneously reducing the cost to the health service.

8.3.2 MiRNAs as disease modulators and therapeutic targets

As evident by the findings of this study, miRNAs are intricately involved in tumourigenesis and so, the recent ability to manipulate miRNAs has become an appealing anti-cancer treatment strategy. The unique miRNA signatures of breast cancer described herein may be exploited as biomarkers for breast cancer as described above, but this also unveils the potential therapeutic strategy of restoring the miRNA expression profile to normal as an anti-cancer treatment approach. MiRNAs may be exploited as potential therapeutic targets in cancer by several approaches. Firstly, depleted miRNAs may be replaced by either miRNA mimetics or viral-vector encoded miRNAs; secondly, over-expressed miRNAs can be inhibited by miRNA masking, miRNA sponges or anti-miRNA oligonucleotides (AMOs) and thirdly, patient response to current treatment modalities can be enhanced by altering miRNA expression (334).

8.3.2.1 Potential Treatment Strategies for Oncogenic MiRNAs

Over-expressed ‘oncogenic’ miRNAs need to be knocked-down or deactivated in order to restore their normal expression level and reverse the mRNA inhibition that results from these aberrantly expressed miRNAs. Several strategies of RNA interference exist, some of which were originally developed for mRNA silencing but have been tailored for miRNA

manipulation. The main approaches include anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking.

Anti-miRNA Oligonucleotides (AMOs)

Anti-miRNA oligonucleotides are synthetic oligonucleotides which represent a new application of the antisense concept which was originally applied to mRNA. AMOs for miRNA use are designed with perfect reverse base pair complementarity and competitively inhibit binding between the miRNA and its mRNA target by binding with the miRNA molecule. The most commonly used types of chemically modified AMO are 2'-O-methyl AMOs, 2'methoxyethyl AMOs and locked nucleic acids (LNAs) (335).

MiRNA Masking

A miRNA mask is typically a single stranded 2'-O-methyl-modified oligonucleotide which exhibits perfect complementarity not to the miRNA itself, but rather to the miRNA binding site on the 3' UTR of a protein coding mRNA gene (336). In this way, miRNA masking has the ability to provide gene-specific and miRNA-specific miRNA targeting, as it does not directly interact with the miRNA, but prevents binding of the miRNA. In addition, this approach avoids off-target effects and provides a better platform to study the effects of a specific gene on miRNA function.

MiRNA Sponges

MiRNA sponges provide an alternative to antimiR inhibition of miRNA function. First described in 2007, miRNA sponges can be expressed in cells as RNA molecules produced from transgenes and have the advantage of being able to bind multiple over-expressed miRNAs at the same time, a remarkable challenge to have overcome (337). This is particularly attractive in the cancer setting, where multiple miRNAs are simultaneously deregulated and function together, particularly those within the same miRNA family, such as the *miR-15* family. These competitive inhibitors display numerous and tandem binding sites complementary to the miRNAs of interest. When a sponge is present at high levels, it specifically deactivates the effects of the family of miRNAs sharing the common seed sequence. MiRNA sponges target only mature miRNAs and therefore do not exert any effect on other miRNA precursors within the same cluster.

Figure 8.1 Potential miRNA-based therapeutic strategies[28]

Oncogenic (over-expressed) miRNAs can be knocked-down or inactivated in an effort to restore 'normal' expression levels by anti-miRNA oligonucleotides (AMOs), miRNA sponges or miRNA masking. MiRNAs with reduced expression in the cancer state (tumour suppressor genes) can be replaced by miRNA mimicry. Other replacement techniques include induction of miRNA over-expression by viral vectors and reversal of epigenetic silencing using small molecules.

8.3.2.2 Potential treatment strategies for tumour suppressor miRNAs

In the cancer state, miRNAs with decreased expression are regarded as tumour suppressor genes. The fundamental aim of the therapeutic approach in this case is to restore miRNA expression to a normal level. This can be achieved by miRNA mimicry, the induction of miRNA overexpression by viral vectors, or by reversing the epigenetic silencing of specific miRNAs using small molecules. These strategies are desirable as therapeutic approaches for cancer, as in general miRNA levels are found to be decreased in the cancer state (75).

MiRNA Mimicry

MiRNA oligonucleotide mimics are double stranded RNA molecules that contain a 'guide strand' that is identical to the mature miRNA, and is designed to mimic its function. The 'passenger strand', the second RNA strand, is usually perfectly complementary to the guide strand. The duplex formation provides better loading onto the mRISC and therefore improves the biological activity (338). MiRNA oligonucleotide mimics require chemical alteration to reduce degradation by nuclease. In addition, modifications can be made to

[28] McDermott *et al* Pharmaceutical Research 2011

enhance target organ specific uptake, for example cholesterol can be added to increase hepatic uptake. In theory, as miRNA mimics have the same functionality as the endogenous miRNA, this technique should reduce off-target effects.

Viral Vector Delivery of MiRNAs

Increasing the level of tumour suppressor miRNAs can also be achieved by DNA plasmid delivery of the pri-miRNA, usually with the aid of a viral vector. This form of gene therapy typically exploits adenoviral or lentiviral vectors. The vector is based on the adeno-associated virus (AAV) and the specific miRNA is incorporated into this system to enable tissue delivery. AAV miRNA replacement approaches have been studied *in vivo* and obtained encouraging results (339). MiRNA therapeutics companies such as Mirna Therapeutics[29] and Asuragen[30] have also shown interest in this strategy.

8.3.2.3 MiRNA therapeutics in breast cancer

The major advances made in the realm of miRNA therapeutics have been in liver disorders and hepatocellular carcinoma (HCC). MRX34, a synthetic *miR-34* mimic developed by Mirna Therapeutics has become the first miRNA mimic to enter Phase 1 trials in patients with primary hepatocellular carcinoma or metastatic cancer with liver metastases[31]. However, some therapeutic applications of miRNAs in other cancers, including breast cancer have also been described (Table 8.1). In a therapeutic capacity, there have been two predominant objectives and approaches to manipulating miRNA expression in breast tumours thus far. One is the knockdown of candidate breast cancer-related 'oncomirs', to suppress tumour growth and inhibit or prevent distant metastases. Another approach is to modulate miRNA expression with the intent of augmenting or altering tumour responsiveness to adjuvant chemotherapeutic or hormonal agents.

[29] http://www.mirnarx.com/
[30] http://asuragen.com/
[31] http://www.nature.com/nbt/journal/v31/n7/full/nbt0713-577.html

Table 8.1 MiRNAs implicated as therapeutic targets in common diseases

Disease	**miRNA**	**Expression level in disease state**	**Stage of Investigation *(in vitro/in vivo)***
Hepatitis B virus	miR-122, miR-31	n/a	*In vivo (340)*
Hepatitis C virus	miR-122	n/a	*In vivo (341)*
	miR-199a	n/a	*In vitro (342)*
Hepatic fibrosis	miR-27a, miR-27b	Overexpressed	*In vitro (343)*
	miR-29a , miR-29b	Underexpressed	*In vivo (344)*
Hepatocellular Carcinoma	miR-122	Underexpressed	*In vitro (345, 346)*
Lung Cancer (NSCLC)	Let 7 family	Underexpressed	*In vivo (347, 348)*
	miR-21	Overexpressed	*In vivo (349)*
Pulmonary arterial hypertension	miR-204	Underexpressed	*In vivo (350)*
Breast Cancer: Inhibition of metastases	miR-10b	Overexpressed*	*In vivo (351)*
	miR-21	Overexpressed	*In vivo (352)*
	miR-1258	Underexpressed	*In vitro (353)*
Breast Cancer: Adjuvant Response	miR-21	Overexpressed	*In vivo (354)*
	miR-205	Underexpressed	*In vitro (355)*
	miR-128a	Overexpressed	*In vitro (356)*
	miR-125b	Overexpressed	*In vitro (357)*
	miR-155	Overexpressed	*In vitro (358)*
	miR-34a	Overexpressed	*In vitro (359)*
	miR-342	Underexpressed	*In vitro (360)*
Haematology: Leukaemia (B-CLL)	miR-15, miR-16	Underexpressed	*In vitro* (140)
AML	miR-29b	Underexpressed	*In vitro* (361)
Lymphoma	miR-17-92 cluster	Overexpressed	Tumour: *In vivo* (362) Radiotherapy: *In vitro(363)*
Prostate Cancer	miR-34a	Underexpressed	*In vivo* (364)
	miR-16	Underexpressed	*In vivo* (365)
	miR-143	Underexpressed	*In Vitro (366)*
Bladder Cancer	miR-203	Underexpressed	*In vivo* (367)
Cardiac Hypertrophy induced arrhythmia	miR-1, miR-133	Overexpressed	*In vivo* (368)
	miR-208	Overexpressed	*In vivo (369)*
	miR-100	Overexpressed	*In Vitro* (370)
	miR-29	Underexpressed	*In vivo (371)*
Glioblastoma	miR-21	Overexpressed	*In vitro* (372)

*conflicting results reported by different studies

'OncomiR' Knockdown

It is widely accepted that metastases are responsible for most breast cancer related deaths. However targeting or interrupting the metastatic process with therapeutics has been generally unsuccessful, perhaps as a result of our limited understanding of this pathological process (373). Recent endeavours to explore the role of miRNAs in the metastatic cascade

have identified potentially key pathways in this process, and have identified novel therapeutic targets. Accumulating data have proven that miRNAs exert their effects at multiple steps in the metastatic cascade by influencing cancer cell adherence, migration, invasion, motility, and angiogenesis (137). In their miRNA microarray analysis of paired tumour tissues and metastatic lymph nodes, Baffa *et al* identified a metastatic cancer miRNA signature inclusive of *miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c,* and *miR-205. MiR-10b* is implicated in many cancers, including breast cancer, and is thought to promote tumour invasion and metastasis by inhibiting translation of the *HOXD10* gene thereby resulting in increased expression of the pro-metastatic gene, *RHOC* (374). Weinberg's group have also recently reported exciting findings from their work on antagonizing *miR-10b* in metastatic breast cancer cell lines (MDA-MB-231 cells). Silencing *miR-10b* with antisense oligonucleotides was found to inhibit *Twist*-mediated cell migration and invasion. They observed similar anti-metastatic effects after systemic *miR-10b* antagonism in a murine model (345).

Numerous other miRNAs have been implicated in the metastatic pathway. Yan *et al* performed *in vitro* LNA silencing of *miR-21* in two breast cancer cell lines (MCF-7 and MDA-MB-231), which resulted in significantly reduced cell proliferation and migration. Their subsequent *in vivo* studies resulted in similar inhibition of breast tumour growth following *miR-21* knockdown with antimiRs (352). Zhang *et al* have been the first to report that *miR-1258* inhibits breast cancer brain metastases by negatively regulating the heparanase pathway (353). Again, these results strongly support the potential of miRNAs to be applied to the clinical setting for therapeutic gain.

Augmenting response to adjuvant therapy

Chemotherapeutic drugs, radiotherapy and endocrine agents (aromatase inhibitors and selective oestrogen receptor modulators, SERMs) are the adjuvant therapies used in the routine management of women with breast cancer at present. Despite their success in improving disease free and/or overall survival, a proportion of women derive no benefit from these treatments or develop resistance to these agents over time (375). The basal subtype of breast cancer (classically ER, PR and HER2/*neu* negative) presents a specific therapeutic challenge as there are no targeted therapies currently available. Preliminary studies suggest that miRNA modulation in tumour tissue can augment the response to systemic therapies. *MiR-21* is again one of the most studied miRNAs in this setting. Mei *et*

al combined taxol chemotherapy with *miR-21* inhibitor treatment, via a polyamidoamine (PAMAM) dendrimer vector, to evaluate the effects of combination therapy on suppression of breast cancer cells (354). They found that cells treated with this combination demonstrated significantly reduced cell viability and invasiveness compared with cells treated with taxol alone, reflecting an enhanced chemotherapeutic effect of taxol in the presence of decreased *miR-21* levels (354). There is also experimental evidence that manipulation of *miR-205* levels can improve breast tumours response to anticancer agents (355).

Manipulation of several other miRNAs has been shown to have the potential to augment breast tumours' responsiveness to existing therapies. Inhibition of endogenous *miR-128a*, which is highly expressed in letrozole-resistant breast tumours, overcomes resistance to the aromatase inhibitor letrozole by modulating TGFß signalling (356). *MiR-125b, miR-155,* and *miR-342* have also been implicated in regulating chemosensitivity, whilst knockdown of *miR-34a* is associated with increasing cancer cells sensitivity to radiation (357-360).

8.4 Future perspectives

Over the past two decades major scientific advances have been made, from miRNA discovery to their application as novel treatment tools. Despite the progress that has been made miRNA research is still in its infancy and there are still some challenges which must be addressed before we can effectively move the findings of this study to the clinical setting.

- Validation by RQ-PCR

  MiRNAs identified in this study could be further validated, preferably on a larger scale with multicentre involvement and perhaps blinding of patient groups. The miRNAs of particular interest would be those with potential diagnostic biomarker application, namely *miR-15a, miR-29a, miR-181a, miR*-195, miR-223, miR-*497* and *miR-652.*

- Correlation with miRNA expression at diagnosis and survival

  Blood samples used in this study were prospectively collected. Further data regarding loco-regional recurrence, distant metastases and survival will be available on this study cohort in due course. Correlation of miRNA level at diagnosis and outcome data would be extremely interesting and could provide information on whether a miRNA or profile of miRNAs could predict outcome.

- Origin and transport of circulating miRNAs

  This study focused on the application of miRNAs as biomarkers for breast cancer. Since commencing this study, it has become apparent that little is known of the origin of circulating miRNAs and the methods by which they are transported in the circulation. It could be that these mechanisms vary depending on the specific miRNA in question. This is an extremely exciting avenue for further research.

- Predicting and monitoring response to neoadjuvant chemotherapy

  At the time of writing, 81 patients had been enrolled in the ICORG 10-11 trial, with 30 patients having completed all 5 blood sampling timepoints at the time that experimental analysis was being performed. Based on the power calculation a total of 122 patients will be accrued to this trial. This is an extremely exciting study with

the potential to influence patient management by facilitating in predicting and monitoring response to neoadjuvant chemotherapy.

- Standardisation of miRNA analysis

  Despite the abundance of miRNA research, disparity lies in the underlying methods making direct comparisons practically impossible. Standardised work practices should also be enforced before these platforms are approved for clinical utility. Academic research and industrial laboratories employ different methods of identifying and validating the specified miRNA, or indeed its precursor. To ensure reproducible results across laboratories and continents it will also be necessary to share methods of miRNA analysis as well as detailed information on the type of specimen evaluated and the ideal storage conditions. If patients are to be treated on the basis of their individual tumour's miRNA profile, it is necessary to ensure an international standard of tumour miRNA expression is achieved.

- Functional Studies

  There is an extensive gap in the current understanding of the precise function and metabolic pathway involvement of several miRNAs, both in health and disease. In this study *miR-195* and *miR-497* expression in breast cancer cell lines was determined. Functional *in vitro* or *in vivo* animal studies using normal breast and breast cancer cell lines could also be performed to determine the effects of the specific miRNAs identified in this study in health and disease, on cellular functions and tumour growth, for example. The effects of inducing further epigenetic modifications, and discovering further mRNA targets could also be evaluated.

- Therapeutic potential

  As mentioned, miRNA based therapeutic strategies is an emerging field. The effect of replenishing the depleted miRNAs outlined in this work and knocking down overexpressed miRNAs could be examined, with a view to harnessing their therapeutic application.

- Target prediction

  It would not be safe to launch a miRNA treatment strategy without first characterising the role of each therapeutic miRNA in other tissues and biological

pathways. The complexity of the situation is augmented by the knowledge that miRNAs can have dual functionality, with the potential to act both as an oncogene and tumour suppressor gene depending on the tissue and cancer state in question, and in addition, a single miRNA can have multiple gene targets. Gene target prediction is currently conducted computationally in a sequence specific manner, with software programmes such as miRBase, TargetScan and Pictar capable of providing numerous potential targets (183, 376). Yet it seems that sequence complementarity is not all that governs an efficient interaction between a miRNA and its mRNA target, as not all predicted targets are functional. Validation of such predicted targets and *in vivo* functional analysis needs to be carried out in order to accurately predict the wide range of side effects and toxicities which may be expected from a miRNA treatment approach. It would be prudent to identify all existing miRNAs, and their functions, before miRNA therapeutics can safely be employed.

8.5 Conclusion

This project has contributed to the current understanding of the molecular biology of breast cancer, namely miRNAs and their role as oncologic biomarkers for this prevalent heterogeneous disease. Since their original description over 20 years ago, miRNAs have been thrust into the forefront of translational research, with implications in almost all biological processes, in both health and disease states. MiRNAs are exciting molecules with the potential to revolutionise breast cancer diagnosis and treatment in pursuit of individualised targeted cancer treatment for patients.

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Appendices

Appendix 1 Department of Surgery BioBank Forms

1.1 Breast Unit Patient Consent Form

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Patient Information

Introduction
We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.
Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement
If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure
We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.
(i) **Tissue Samples**
By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.
(ii) **Blood Samples**
By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.
(iii) **Clinical Information**
By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

Further Information
If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).
If you would like further information about research projects that may be conducted, please contact your Consultant.
Thank you in anticipation of your assistance. Please read and sign the Consent section.
I have read the attached information sheet on the above project, dated _____ ☐
Please Initial Box

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand that I may withdraw from the study at any time.

(Name of sponsor): .....

PARTICIPANT'S NAME: .....

CONTACT DETAILS: .....

PARTICIPANT'S SIGNATURE: .....

DATE: .....

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR GUARDIAN: .....

SIGNATURE: .....

RELATION TO PARTICIPANT: .....

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME OF RESEARCH NURSE OR INVESTIGATOR: .....

SIGNATURE: .....

DATE: .....

CONSULTANT: .....

Keep the original of this form in the investigators file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

1.2 Specimen Request Form

BioBank Specimen Form Nov 2010

Complete form and send with specimens to Surgery Research Lab, CSI (Bleep 835, Ext. 4202)

Surname:	First Name:	Referral Reason (Please Circle): Breast - Colorectal - Prostate - Skin - Lung – Other _____	
Board No:	RH No:	Consultant:	Cancer ☐ Noncancer ☐ Awaiting Dx ☐
DOB:	Sex:	Date:	Blood ☐ Tissue ☐

Blood Specimen

		No. of Tubes
Tube	Yellow 5ml SST	1
	Purple 4ml EDTA	1
	Purple 10ml EDTA	1

Time Point (Tick Box)
- Pre Neoadjuvant ☐
- Peri Neoadjuvant ☐
- Pre Tumour Resection ☐
- Post Surgery (1 day) ☐
- Review / Follow Up ☐

Tissue Specimen

Type (Tick Box)	Liquid Nitrogen	RNA Later	Fresh (Media)
Tumour	☐	☐	☐
Tumour Assoc. Normal (TAN)	☐	☐	☐
Benign	☐	☐	☐
Normal	☐	☐	☐
Lymph Node	☐	☐	☐
Other_____	☐	☐	☐

General Information

Liquid Nitrogen Empty tubes. Immerse in Liquid Nitrogen (LN2)

RNA later Tubes labelled 'RNA later'. Place in biohazard bag

Fresh (Breast only) Tubes containing pink media.Place in biohazard bag

Laboratory use only

Written Informed Consent (Tick Box)
Consent Form with specimen ☐
Consent Form in Patient Chart ☐

Shire Pt No.: _____
Date: _____
Signature:

Time Point (Tick Box)
Diagnostic Core Biopsy ☐
Tumour Resection ☐
Local Recurrence ☐

Breast Side: Right ☐ Left ☐

Surgical Procedure:

Form completed by (Sig/Date):

Complete this section for Cancer Genetics Research Blood only

Age at Diagnosis: _____

Family History (Please tick): Yes ☐ No ☐

Describe Family History (Include blood relatives, maternal/paternal and cancer type):

_____

_____

_____

_____

1.3 9700 microRNA cDNA archive plate record

9700 microRNA cDNA Archive Plate Record

Date:	Time	Temperature
Hold	30mins	16°C
Hold	30mins	42°C
Hold	5mins	85°C
Hold	∞	4°C

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

		clear	yellow	blue	pink
MWG/ABI Mix	1X	X	X	X	X
	1 x 15µL rxn				
dNTP Mix (100mM)	0.17µL				
10x RT buffer	1.65µL				
Nuclease Free Water	4.57µL				
RNase Inhibitor (20U/uL)	0.21µL				
Multiscribe (50U/uL)	1.1µL				
Stem Loop Primer (50nM)	3.1µL				
Premix	10µL				
Total/miRNA (1-10ng/0.1-1ng)	5.0µL				
Total Rxn Volume	15µL				

Sample Details

Saved at M:\Surgery Research\SOPs\Molecular\9700 microRNA cDNA Archive Plate Record.doc

1.4 7900HT 96 well microRNA PCR Taqman plate record

7900HT 96 well microRNA Taqman Plate Record

Date			
Rxn Volume	**10uL**	20 sec	95°C
Target(s)		1 sec	95°C
Control(s)		20 sec	60°C
Calibrator		Cycles	x40

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

MWG/ABI Mix	1X	X	ABI miRNA Kit	1X	X
Mastermix (Fast)	5.0uL		Mastermix (Fast)	5.0uL	
NFW	1.6uL		NFW	3.8uL	
Probe (0.2uM)	0.5uL		miRNA PDAR	0.5uL	
F Primer (1.5uM)	1.5uL				
R Primer (0.7uM)	0.7uL				
cDNA	0.7uL		cDNA	0.7uL	
Total	**10uL**		Total	**10uL**	
Premix	(9.3uL)		Premix	(9.3uL)	

Sample Details

Saved at M:\Surgery Research\SOPs\Molecular\7900HT 96 well microRNA Taqman Plate Record.doc

Appendix 2 ICORG 10-11 Case Report Forms

2.1 Patient registration form

ICORG CONFIDENTIAL Version 2 01-Jun-2011

Protocol Number: 10-11

Centre Number: ☐☐☐

Patient Registration Form

Subject Initials: ☐☐☐

Date of Birth (dd/mon/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Gender ☐☐ 01=Female 02=Male

INCLUSION CRITERIA

Questions regarding INCLUSION must all be answered **YES** for subject study entry.

	Yes	No	N/A
1. **The patient has been newly diagnosed with breast cancer and is destined to undergo neoadjuvant chemotherapy**	☐	☐	☐
2. **The patient has a recurrent breast cancer or disease progression and will receive up-front chemotherapy**	☐	☐	☐
3. **The patient is older than 18 years of age.**	☐	☐	
4. **The patient is able to give written informed consent**	☐	☐	

REGISTRATION INFORMATION

Date of Informed Consent (dd/mon/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Date of Registration (dd/mon/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

I confirm that the above patient meets all the eligibility criteria

Investigator's Signature: _____

Date (dd/mon/yyyy): ☐☐ ☐☐☐ ☐☐☐☐

Page **1** of **1**

2.2 Baseline assessment form

ICORG | Confidential | Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Baseline Assessment Form

Date of Visit (dd/mon/yyyy)

DIAGNOSIS

Date of Core Biopsy (dd/mon/yyyy)

1. Clinical Assessment

Tumour

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO

S - Score ☐ (1 – 5)

2. Radiological Assessment

Ultrasound

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO

If Yes: size _____cm of _____cm in total

Mammogram

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO

If Yes: size _____cm of _____cm in total

Additional Features _____

_____

MRI

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO

If Yes: size _____cm of _____cm in total

Additional Features _____

_____

R - Score ☐ (1 – 6)

ICORG | Confidential | Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Baseline Assessment Form

Date of Visit (dd/mon/yyyy)

3. Histological Assessment

	POSITIVE	NEGATIVE
ER status	☐	☐
PR status	☐	☐
HER2 status	☐	☐
Tumour Grade	☐ 1 - 3	
Histological Type	_____	

Axillary Lymph Node Status

Biopsy performed	☐ YES	☐ NO
Result	☐ POSITIVE	☐ NEGATIVE

ICORG Confidential Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Baseline Assessment Form

Date of Visit (dd/mon/yyyy)

CURRENT TREATMENT

Is the patient currently on any other treatment? ☐ YES ☐ NO

If YES for what _____

With what medication _____

Other concomitant Medication (within the last 3 days)

Drug_____Dose_____

Drug_____Dose_____

Drug_____Dose_____

Drug_____Dose_____

BRIEF MEDICAL HISTORY

Is there any relevant medical history ☐ **YES** ☐ **No**

If Yes please fill in details below

Condition	Date started	Past	Ongoing	Severity
		☐	☐	
		☐	☐	
		☐	☐	
		☐	☐	

2.3 Specimen collection form

ICORG	Confidential	Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			0

Specimen Collection Form

Date of Visit (dd/mon/yyyy)

One to accompany each specimen please

SAMPLE DETAILS

Blood sample collection (dd/mon/yyyy)	
Sample Collection Timepoint (Please tick box and fill in detail)	Registration (Diagnosis) ☐ Perineoadjuvant Cycle Number _____ ☐ Postneoadjuvant and Presurgery ☐ Post Surgery (2-4 weeks post-operatively) Surgical Procedure _____ ☐ _____ 12-18 months after surgery ☐
Date:	**Signature:**

RECEIPT OF SAMPLE (Biobank Lab use only)

Type of samples received:	Tissue: ☐	Whole Blood: ☐	
Date:		**Signature:**	

2.4 Follow up assessment form

ICORG | Confidential | Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Follow Up Assessment Form
(Post – Op Follow Up)

Date of Visit (dd/mon/yyyy)

Treatment

Surgery ☐ YES ☐ NO

If YES date of surgery (dd/mon/yyyy)

Surgical procedure _____

Lymph node procedure _____

Neoadjuvant CT

Regimen — Regimen Name _____

Start Date — Date (dd/mon/yyyy)

End Date

Total No of Cycles

Page **1** of **4**

ICORG | Confidential | Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Follow Up Assessment Form
(Post – Op Follow Up)

Date of Visit (dd/mon/yyyy)

Post Neoadjuvant Chemotherapy Assessment

1. Clinical Assessment

Tumour

Size of Tumour _____ cm

Nodes involved ☐ Yes ☐ No

2. Radiological Assessment

Ultrasound - optional

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO ☐ N/A

If Yes: size _____cm of _____cm total

Mammogram - optional

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO ☐ N/A

If Yes: size _____cm of _____cm total

Additional Features _____

_____

MRI

Size of Tumour _____ cm

Nodes involved ☐ YES ☐ NO ☐ N/A

If Yes: size _____cm of _____cm total

Additional Features _____

_____

Response to Neoadjuvant CT based on MRI
(please tick only one box)

☐ Poor ☐ Partial ☐ Good ☐ Complete ☐ N/A

ICORG Confidential Version 1 22-Jun-2011

Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Follow Up Assessment Form
(Post – Op Follow Up)

Date of Visit (dd/mon/yyyy)

3. Histological Assessment from Surgical Resection

Tumour Size	Invasive _____ cm Total _____ cm		% Decrease in cellularity (If known)
Associated DCIS	_____ cm		% (if known)
Nodes involved		of	Total nodes

Response to Neoadjuvant CT based on histology report *(please tick only one box)*

☐ Poor ☐ Partial ☐ Good ☐ Complete ☐ N/A

CURRENT TREATMENT

Is the patient currently on any other treatment? ☐ YES ☐ NO

If YES for what _____

With what medication _____

Any planned hormonal treatment? ☐ YES ☐ NO

If YES please specify

Drug_____Dose_____

Drug_____Dose_____

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Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Follow Up Assessment Form
(Post – Op Follow Up)

Date of Visit (dd/mon/yyyy)

Any planned Radiotherapy ☐ YES ☐ NO

If YES please specify

How many Gy

How many fractions

Other Concomitant Medication (within the last 3 days)

Drug_____Dose_____

Drug_____Dose_____

Drug_____Dose_____

Drug_____Dose_____

2.5 Off study form

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Protocol Number	Subject Initials	Centre Number	Subject Number
10-11			

Off Study Form

Date (dd/mon/yyyy)

THE OFF STUDY FORM MUST BE COMPLETED FOR ALL PATIENTS, WHETHER THEY HAVE COMPLETED THE STUDY AS PER PROTOCOL CRITERIA OR THEY HAVE PREMATURELY DISCONTINUED THE STUDY FOR ANY REASON.

Date of last Visit (dd/mon/yyyy)

PLEASE SPECIFY THE REASON WHY THE PATIENT IS NOW OFF THE STUDY (please tick one)

☐ **Completed the study as per protocol criteria**

☐ **Lost to follow up** **Date of last contact (dd/mon/yyyy)**

☐ **Withdrawal of patient consent** **Date (dd/mon/yyyy)**

☐ **Death** **Date (dd/mon/yyyy)**

☐ **Other** **Specify:** _____

☐ **Sponsor Decision**

Investigator's Signature: _____

Date (dd/mon/yyyy):

Appendix 3 Reagents and Equipment

Company	Reagents & Equipments	Catalogue / Part / Model Number
Agilent Technologies Inc. 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA.	Agilent 2100 Bioanalyzer RNA 6000 Nano Assay Kit Guide RNA 6000 Nano LabChip Series II Assay Small RNA Assay Small RNA Kit Guide	- G2941-90126 5065-4476 5067-1548 G2938-90093
American Type Culture Collection (ATCC), Manassas, Virginia	Immortalised cell lines: MCF-10-2A, MCF-7, ZR-75-1, T47D, MDA-MB-231, SKBR3	-
Becton Dickenson, New Jersey, USA	Vacutainer Serum Separator Tubes II	-
Biogazelle Biogazelle NV, Technologiepark 3, B-9052 Zwijnaarde, Belgium	qBase PLUS Software	-
Chemometec ChemoMetec A/S Gydevang 43 DK-3450 Allerød Denmark	Nucleocounter NC-100 Reagent A100 Reagent B	-
Eppendorf Ltd. Endurance House, Vision Park, Histon, Cambridge, CB24 9ZR, UK.	Eppendorf 5417C Micro Centrifuge	-
Exiqon Skelstedet 16 DK-2950 Vedbæk Denmark	*miR-195* miRCURY™ LNA Detection probe, 5`-FAM and 3`-FAM labelled *miR-497* miRCURY LNA Detection probe, 5`-FAM and 3`-FAM labeled	38083-04 38256-15
Genial Genetics PO Box 3751 Chester, CH1 9UF, UK	Shire Genetic Database	-
Genomic Health Inc Corporate Headquarters 301 Penobscot Drive Redwood City, CA 94063-4700	Onco*type* DX®	-
Greiner Bio-One Inc. 4238 Capital Drive, Monroe, NC 28110, USA.	Vacuette EDTA K3E blood bottles	-
Haraeus, Germany	Class II Laminar Air Flow hoods	-
Invitrogen Corp. 5791 Van Allen Way, PO Box 6482, Carlsbad, CA 92008, USA.	dNTPs SuperScript™ III	18427-088 18080-085
Kinematica AG Inc. Luzernerstrasse 147a, CH-6014 Lucerne, Switzerland.	Polytron Bench-top Homogeniser	PT1600E
Leica, Herley, Denmark	Leica DM 6000B microscope	-

Life Technologies Foster City, CA, USA	Applied Biosystems 7900HT Fast Real-Time PCR System ABI Prism 7900 Sequence Detection System Fast Optical 96-well Reaction Plate with barcode 0.1ml High Capacity cDNA Reverse Transcription Kit Megaplex™ PreAmp Primers Megaplex™ Reverse Transcription Human Pool A MicroAmp Optical Adhesive Film TaqMan® miRNA assays TaqMan® Low Density Array Card A and Card B V3.0 TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG TLDA Universal PCR Master Mix, No AmpErase® UNG Trizol SDS RQ Manager Software RNase Inhibitor RNase Zap	 4366932 4368814 4399233 4399966 4313663 4398965 4444913 4324018 15596018
Lonza B4800 Verviers, Belgium	Fetal Bovine Serum RPMI 1640 cell culture media McCoy 5A cell culture media Lebowitz cell culture media Phosphate buffered saline Trypsin/EDTA 0.25% 1% L-Glutamine 1% Penicillin/Streptomycin	DE14-801F BE12-702F BE12-688F BE12-700F BE17-516F 17-161E BE17-605E BE17-622E
Milestone Medical Technologies Inc. 6475 Technology Avenue, Suite F, Kalamazoo, MI 49009, USA.	MicroMed T/T Mega Histoprocessing Labstation	-
Minitab Ltd, Brandon Court, Unit E1-E2, Progress Way, Coventry CV3 2TE UNITED KINGDOM	Minitab Version 16.0	-
Molecular Research Centre Inc., 5645 Montgomery Road, Cincinnati, OH 45212, USA.	BAN Phase Separation Reagent 4-Bromoanisole	BN 191
MWG Biotech Anzingerstrasse 7a, 85560 Ebersberg, Germany.	MiRNA-specific Primers (*miR-16* and *miR-21*)	-
Nanodrop Technologies Inc.,3411 Silverside Road, Bancroft Building, Wilmington, DE 19810, USA.	Nanodrop 1000® Spectrophotometer	-

Olympus, Pennsylvania, U.S.A	Olympus DP72 CCD	-
Qiagen Ltd., QIAGEN House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.	Buffer RDD, DNase I Buffer RPE RNA*later*® RNeasy® Mini Kit	79254 79306 76106 74204
Roche Mannheim, Germany	4-nitroblue tetrazolium (NBT) and 5-Brom-4-chloro-3'-indolylphosphate (BCIP)	-
Sarstedt AG & Co. Sarstedtstraße, Postfach 1220, 51582 Nümbrecht, Germany.	Sarstedt tubes Tissue culture flasks	-
Sigma-Aldrich Corp., St Louis MO, USA	Isopropanol	190764
Slee Technik Lise-Meitner-Strasse 1, 55129 Mainz, Germany.	Slee Cut 4055 Microtome	-
Syntec Scientific Ltd. Unit 2, The Business Centre, Northwest Business Park, Ballycoolin, Dublin 15, Ireland.	Cell Conditioning Solution Tris/Borate EDTA Buffer pH 8.0 EZPrep Solution Hematoxylin	950-124 950-102 760-2021 760-123 760-4205
Tecan, Mannedorf, Switzerland	Tecan Freedom Evo	-
Thermo Electron Corporation, 81 Wyman Street Waltham, MA 02454	HEPA Class 100 incubator	-
Vector Laboratories, Beringname, CA,USA	Nuclear-fast red counterstain	-
Visiopharm, Horsholm, Denmark	Visiopharm integrated microscope	-
VWR International Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland	Superfrost® Plus Slides Eukitt mounting medium	361387P 631-0108

Appendix 4 Publications arising from this work

1. Peer Reviewed Papers

Identification and validation of oncologic miRNA biomarkers for luminal A-like breast cancer

AM McDermott, N Miller, D Wall, LM Martyn, G Ball, KJ Sweeney, MJ Kerin
PLoS One
2014 Jan 31;9(1):e87032, PMID: 24498016

Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies

AM McDermott, MJ Kerin, N Miller
PLoS One
2013 Dec 31;8(12):e83718, PMID: 24391813

The therapeutic potential of microRNAs: Disease modifiers and drug targets

AM McDermott, HM Heneghan, N Miller, MJ Kerin
Pharmaceutical Research
2011, Vol 28, 3016-3029, PMID: 21818713

2. Book Chapter

Chapter Title: MiRNAs as potential therapeutic targets in cancer

AM McDermott, HM Heneghan, N Miller, MJ Kerin
In: *MicroRNAs in Cancer,* pages 333-363
Editors: C Lopez-Camarillo and LA Marchat
CRC Press, Florida 2013,
ISBN 978-1-57808-778-5

OPEN ACCESS Freely available online

PLOS ONE

Identification and Validation of Oncologic miRNA Biomarkers for Luminal A-like Breast Cancer

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Abstract

Introduction: Breast cancer is a common disease with distinct tumor subtypes phenotypically characterized by ER and HER2/*neu* receptor status. MiRNAs play regulatory roles in tumor initiation and progression, and altered miRNA expression has been demonstrated in a variety of cancer states presenting the potential for exploitation as cancer biomarkers. Blood provides an excellent medium for biomarker discovery. This study investigated systemic miRNAs differentially expressed in Luminal A-like (ER+PR+HER2/*neu*-) breast cancer and their effectiveness as oncologic biomarkers in the clinical setting.

Methods: Blood samples were prospectively collected from patients with Luminal A-like breast cancer (n = 54) and controls (n = 56). RNA was extracted, reverse transcribed and subjected to microarray analysis (n = 10 Luminal A-like; n = 10 Control). Differentially expressed miRNAs were identified by artificial neural network (ANN) data-mining algorithms. Expression of specific miRNAs was validated by RQ-PCR (n = 44 Luminal A; n = 46 Control) and potential relationships between circulating miRNA levels and clinicopathological features of breast cancer were investigated.

Results: Microarray analysis identified 76 differentially expressed miRNAs. ANN revealed 10 miRNAs for further analysis (*miR-19b, miR-29a, miR-93, miR-181a, miR-182, miR-223, miR-301a, miR-423-5p, miR-486-5* and *miR-652*). The biomarker potential of 4 miRNAs (*miR-29a, miR-181a*, *miR-223* and *miR-652*) was confirmed by RQ-PCR, with significantly reduced expression in blood of women with Luminal A-like breast tumors compared to healthy controls (p = 0.001, 0.004, 0.009 and 0.004 respectively). Binary logistic regression confirmed that combination of 3 of these miRNAs (*miR-29a, miR-181a* and *miR-652*) could reliably differentiate between cancers and controls with an AUC of 0.80.

Conclusion: This study provides insight into the underlying molecular portrait of Luminal A-like breast cancer subtype. From an initial 76 miRNAs, 4 were validated with altered expression in the blood of women with Luminal A-like breast cancer. The expression profiles of these 3 miRNAs, in combination with mammography, has potential to facilitate accurate subtype-specific breast tumor detection.

Citation: McDermott AM, Miller N, Wall D, Martyn LM, Ball G, et al. (2014) Identification and Validation of Oncologic miRNA Biomarkers for Luminal A-like Breast Cancer. PLoS ONE 9(1): e87032. doi:10.1371/journal.pone.0087032

Editor: Alessandro Weisz, University of Salerno, Italy

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Breast cancer is a prevalent disease, accounting for significant morbidity and mortality with a worldwide incidence of over 1,300,000 women [1]. It is the commonest female malignancy in almost all European countries and in North America and leading cause of female cancer mortality. Breast cancer is a heterogeneous disease, with distinct tumor phenotypes reflecting a spectrum of underlying molecular alterations and initiating events [2]. Analysis of gene expression patterns governing these events has resulted in the classification of breast tumors into subtypes broadly determined by expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2/*neu*). Targeted therapies including hormonal therapy for ER positive tumors and trastuzumab to inhibit HER2/*neu* signaling have become the major components of adjuvant breast cancer management. Consequently, when diagnosed and treated early, breast cancer is highly curable. Despite these advances, hematogenous spread of malignant cells from the primary tumor to distant organs with subsequent proliferation into metastases remains the leading cause of death for breast cancer patients [3]. Further insight into the molecular mechanisms underlying tumorigenic transformation is clearly warranted for the identification of additional molecular predictors and disease biomarkers in the clinical management of breast cancer.

Much current cancer research is focused on the identification of circulating cancer-specific biomarkers for application to disease diagnostics, as well as predicting and monitoring response to disease and tumor recurrence. There are no reliable circulating biomarkers for breast cancer. Mammography is the most widespread screening tool, with a definitive diagnosis requiring

an invasive tissue biopsy. This prevalent disease is in need of a minimally invasive biomarker which may be used in combination with radiological imaging to facilitate early subtype specific tumor diagnosis. Blood presents an excellent medium for biomarker discovery; it is minimally invasive and simple to obtain during routine clinical examination. Moreover, blood circulates throughout the body delivering nutrients and carrying proteins (including miRNAs), hormones and cells while eliminating waste substances, thereby reflecting the summation of physiological and pathological processes occurring in an individual at any one time.

Mi(cro)RNAs have shown much potential as cancer-specific biomarkers. MiRNAs regulate gene expression at the post-transcriptional level and are intimately linked with the cancer state; Firstly, miRNA expression has a causal effect on tumourigenesis, acting as oncogenes and tumor suppressor genes and secondly, altered miRNA expression occurs as a result of the carcinogenic process. In breast cancer, altered tissue miRNA expression patterns have been shown to correlate with molecular subtype and hormonal receptor status [4,5]. MiRNAs were originally studied in tissue, but several studies have demonstrated that tumor-specific miRNAs are detectable in the circulation [6–8]. These studies allude to the promising role of circulating miRNAs as biomarkers for detection of disease. Furthermore, speculation that circulating miRNA profiles could reflect not only the tumor tissue-type, but also the intrinsic molecular subtype thus acting as a fluid biopsy would be particularly valuable in breast cancer where management, even immediately following diagnosis, is governed by hormonal and HER2/*neu* receptor status, largely conveying molecular subtype.

Luminal A is the most common subtype, including over 70% of breast cancers. Confirmation of Luminal A subtype is performed using mRNA expression analysis however phenotypically Luminal A-like tumors are characterized as hormone receptor positive and HER2/*neu* negative. These tumors are frequently screen detected, node negative and therefore associated with a good prognosis. Recent advances such as the development of the Oncotype DX® test strive to prevent overtreatment of this common subtype by identifying women at high risk of recurrence for adjuvant chemotherapy.

The aim of this study was to utilize microarray profiling to identify circulating miRNAs that are differentially expressed in women with Luminal A-like breast cancer (ER positive, PR positive, HER2/*neu* negative) in comparison to healthy controls, to validate candidate miRNA expression using RQ-PCR, investigate their expression level in association with common clinicopathological parameters, and to study their effectiveness as circulating diagnostic biomarkers in the clinical setting.

Methods

Study Cohort and Sample Collection

Blood samples were prospectively collected from 110 women; this included 54 consecutive patients with a new diagnosis of Luminal A-like breast cancer and 56 healthy control participants. All patients had histologically confirmed Luminal A-like breast cancer; Hormone receptor positive and HER2/*neu* negative. Definitive confirmation of Luminal A subgroup would have required mRNA expression profiling which was not routinely performed or available at our institution. Healthy control blood samples were collected from women residing in the same catchment area as the cancer cases. These women were interviewed by a clinician in advance of sample collection to ensure that there was no personal history of malignancy or current inflammatory or infectious condition. Venous non-fasting whole blood samples were collected in BD vacutainers® containing 18 mg dipotassium EDTA anticoagulant (BD-Plymouth, PL6 7BP, UK). Microarray profiling was performed on RNA derived from blood on 10 of the above patients and 10 controls, the clinicopathological details of which are presented in Table 1. The remaining 44 cases and 46 controls were used to independently validate microarray findings. Clinicopathological details of the validation group are shown in Table 2. Tissue specimens both tumor (n = 11) and tumor-associated normal (TAN, n = 10) were prospectively collected from patients with Luminal A-like breast cancer at the time of surgical resection. Tissue samples were collected in RNAlater® RNA stabilization reagent (Qiagen, UK) prior to cryopreservation at −80°C. Clinicopathological details of this cohort are included in Table 3.

Ethics Statement

Ethical approval was granted by the Clinical Research Ethics Committee, Galway University Hospital. Written informed consent was obtained from all study participants.

RNA Extraction

Total RNA was extracted from 1 ml of blood using TRI Reagent BD (Molecular Research Centre, Inc) as previously described [9]. RNA concentration and integrity were examined by NanoDrop spectrophotometry (NanoDrop ND-1000 Technologies Inc., DE, USA) and Agilent Bioanalyzer RNA 6000 NanoChip Kit Series II (Agilent Technologies, Germany) analysis, respectively.

MiRNA Microarray Profiling

Expression profiling of circulating miRNAs was performed for 20 samples as described above using TaqMan human miRNA arrays and assays in accordance with the manufacturer's instructions (Taqman Low Density Array Human microRNA, Applied Biosystems, Foster City, CA, USA). In brief, total RNA was reverse transcribed using Megaplex primer pool A (Applied Biosystems) which contained sequence-specific primers for 381 specific miRNAs plus 3 controls (pool A). An additional panel of 384 miRNAs (381 miRNAs and 3 controls, pool B) was performed on a subset of 4 cancers and 4 controls. Real-time quantitative PCR was performed for 667 miRNAs, using A and B microfluidic cards, each containing primers and probes for 381 specific miRNAs plus 3 controls and thermal-cycled on an Applied Biosystems 7900 HT instrument. MiRNA expression data are available from the National Center for Biotechnology Gene Expression Omnibus (GEO) at accession number GSE46355.

Microarray Data Analysis

Within this study normalized miRNA array data were analyzed within a nonlinear ANN based data mining algorithm to identify those with altered expression in Luminal A-like breast cancer. This method comprised a feed-forward back-propagation algorithm utilizing a three layer architecture, a sigmoid transfer function, 2 hidden nodes and early stopping on unseen data full details are described by Lancashire *et al* [10]). Monte Carlo Cross validation was applied to the modeling approach to determine the performance of the miRNA probes on a randomly selected blind subset. This approach addressed issues with false discovery by preventing over fitting, driving the solution to one that has good predictability for a blind population.

The performance of single miRNA probes was determined by developing ANN models using the algorithm described above (each using a single probe intensity from the data), to classify

Table 1. Clinicopathological patient data for blood samples analyzed by microarray.

Cases	Age (yrs)	Inv. T size (mm)	Whole T size (mm)	Histological Subtype	Nodal Status	Grade	UICC Stage	ER	PR	HER2/neu	Controls*	Age (yrs)
1	70	22	22	Inv. Muc.	–	2	1	+	+	–	1	81
2	52	15	15	Inv. Ductal	–	2	2	+	+	–	2	61
3	60	13	20	Inv. Ductal	–	2	2	+	+	–	3	82
4	50	22	22	Inv. Ductal	–	2	1	+	+	–	4	76
5	46	38	38	Inv. Ductal	+	1	2	+	+	–	5	74
6	59	120	120	Inv. Ductal	+	2	3	+	+	–	6	87
7	56	53	53	Inv. Lobular	+	2	2	+	+	–	7	94
8	55	61	61	Inv. Ductal	+	2	3	+	+	–	8	94
9	44	45	45	Inv. Ductal	–	2	2	+	+	–	9	96
10	75	50	60	Inv. Ductal	+	2	3	+	+	–	10	72

Yrs, Year; mm,Millimeters; Inv. T size, Invasive Tumor size; UICC, Stage of breast tumor according to the International Union Against Cancer staging criteria; ER, Estrogen receptor; PR, Progesterone receptor: HER2/*neu*, Human epidermal growth factor receptor; –, negative; +, positive; NA not applicable. *All control subjects had no personal or family history of breast or ovarian cancer and were clinically well at the time of sampling.
Luminal A-like is phenotypically defined as ER positive, PR positive, HER2/neu negative.
doi:10.1371/journal.pone.0087032.t001

between Luminal A-like breast cancer and healthy controls (Figure 1). This process was repeated for all of the probes on the array and their classification performance on blind data determined. In this way a rank order of miRNAs was determined. From this rank order the key miRNAs were taken forward for validation.

Validation by RQ-PCR

Quantification of individual miRNAs in both blood and tissue samples was determined by RQ-PCR using TaqMan miRNA assays (Applied Biosystems). Ten of the most differentially expressed miRNAs from the microarray screen were selected for validation. Following RNA isolation, 100 ng of total RNA was reverse transcribed using stem-loop primers and MultiScribe reverse transcriptase. PCR reactions were performed in triplicate in final volumes of 10 µl on 96 well plates. Each plate included an inter assay control (IAC) to account for run-to-run variation. Plates were run on a 7900 HT instrument (Applied Biosystems) using standard thermal-cycling conditions.

Raw fluorescence (cycle threshold, C_T) data were subsequently calculated. High C_T values indicated low miRNA expression and vice versa. The threshold standard deviation for intra- and inter-assay replicates was 0.28. PCR amplification efficiencies (E) were calculated for each miRNA and Taqman miRNA assay using the formula $E = (10-1/\text{slope}-1)\times 100$, using the slope of the semi-log regression plot of Ct versus log input of cDNA (10-fold dilution series of five points). A threshold of 10% above or below 100% was adopted. C_T values were scaled to lowest expressing sample and normalized to *miR-16*, which has been shown to be stably expressed in breast cancer and is the most widely used endogenous control miRNA for breast cancer thus far [9,11]. MiRNA expression was calculated by the comparative cycle threshold (ΔC_T) method, using qbase$^{PLUS®}$ software (Biogazelle, NV, Belgium).

Statistical Analysis

Statistical analysis was performed using Minitab version 16.0 (Minitab Ltd, Coventry UK). The Kolmogorov-Smirnov test for normality was conducted. Data were log transformed ($\log_{10}$) for analysis when non-normal distribution was identified. Significance and associations of circulating miRNA levels were determined using the Mann-Whitney U test, t-test, ANOVA, Spearman's Rho or Pearson correlation, as appropriate. Results with p-value less than 0.05 were deemed to be significant. Binary logistic regression analysis was used and receiver operating characteristic (ROC) curves were generated to evaluate the ability of chosen miRNAs to distinguish between cancer cases and controls. This was performed both individually and for combinations of miRNAs.

Results

Identification of Dysregulated miRNAs in Luminal A-like Breast Cancer

The ANN data mining algorithm identified 76 miRNAs with detectable and altered expression in blood of patients with Luminal A-like breast cancer compared to healthy controls (Table S1).

Validation of Microarray

To further evaluate the expression patterns of individual miRNAs derived from the microarray dataset, real-time quantitative PCR was performed. A subset of three candidate miRNAs was chosen for sample to sample expression analysis and in most cases revealed good correlation between the microarray profiling data and RQ-PCR validation (Figure S1). Expression of ten of the most deregulated miRNAs was confirmed in an independent cohort of blood from patients with luminal A-like breast cancer ($n = 44$) and healthy controls ($n = 46$). MiRNA expression levels were also measured in tumor tissue derived from patients with Luminal A-like breast tissue. The miRNAs selected for validation and results obtained are outlined in Table 4. Two miRNAs (*miR-181a* and *miR-652*) were found to be over-expressed in the microarray and were down-regulated in the circulation of women with Luminal A-like breast tumors in the validation group ($p = 0.004$, and 0.009, respectively, Figure 2). Both *miR-181a* and *miR-652* miRNAs were also under-expressed in Luminal A-like tumor tissue compared to TAN ($p = 0.019$ and $p<0.001$, respectively Figure 2). *MiR-29a* and *miR-223* were under-expressed in the circulation of those with Luminal A-like breast cancer compared to healthy controls, in both the array and the validation cohorts ($p<0.001$ and $p = 0.004$, Figure 2).

Table 2. Clinicopathological Patient Data for Blood in Independent Validation Cohort.

Luminal A Breast Tumors		**Number (%) 44**
Mean age, years (range)		59.86 (±13.45)
Median Whole. T size (mm)		32.82 (±26.30)
	Missing data	11
Median Inv. T size (mm)		27.41 (±20.74)
	Missing data	22
Histological Subtype		
	Invasive ductal	35
	Invasive lobular	1
	Other	7
	Missing	1
Nodal status		
	Positive	22
	Negative	19
	Missing	3
Grade		
	1	7
	2	32
	3	4
	Missing	1
UICC stage		
	Stage 1	15
	Stage 2	14
	Stage 3	8
	Stage 4	2
	Missing	3
Estrogen Receptor		
	Positive	44 (100%)
	Negative	0
Progesterone Receptor		
	Positive	44 (100%)
	Negative	0
HER2/*neu* Receptor		
	Positive	0
	Negative	44 (100%)
Subtype		
	Luminal A-like	44 (100%)
Controls		**46**
Mean Age, years (range)		44.21 (±20.61)

doi:10.1371/journal.pone.0087032.t002

Association/Relationship between miRNA Expression and Clinicopathological Parameters

MiRNA (*miR-29a*, *miR-181a* and *miR-652*) expression data was compared with clinicopathological variables, namely grade, nodal status, tumor size and stage of disease. *MiR-29a*, *miR-181a* and *miR-652* were significantly down-regulated in the blood of patients compared to controls, irrespective of tumor grade, nodal status or stage of disease (Table 5). Altered expression in both early and late stage disease is an important biomarker characteristic. Interestingly, *miR-181a* was significantly down-regulated in the blood of patients with node positive disease compared to healthy controls ($p = 0.006$) but not node negative disease ($p = 0.09$). There was no difference in *miR-181a* expression between node positive or node negative disease. There was a negative correlation between *miR-181a* expression and invasive tumor size (Pearson correlation coefficient $r = -429$, $p = 0.059$).

Table 3. Clinicopathological Patient Data for Breast Tumors.

Luminal A Breast Tumors		**Number (%) 11**
Mean age, years (range)		54.27 (±9.26)
Median Whole. T size (mm)		34.1 (±42.5)
Histological Subtype		
	Invasive ductal	11
Grade		
	1	1
	2	1
	3	8
	Missing	1
UICC stage		
	Stage 1	3
	Stage 2	8
Estrogen Receptor		
	Positive	11 (100%)
	Negative	0
Progesterone Receptor		
	Positive	11(100%)
	Negative	0
HER2/*neu* Receptor		
	Positive	0
	Negative	11 (100%)
Subtype		
	Luminal A-like	11 (100%)

doi:10.1371/journal.pone.0087032.t003

Biomarker Potential of miRNAs

The evident dysregulation of *miR-29*, *miR-181a* and *miR-652* in the blood of women with Luminal A-like breast cancer, irrespective of tumor stage or grade, revealed a potential role for these miRNAs as circulating biomarkers for Luminal A-like breast cancer detection. We compared the area under the curve (AUC) produced from receiver operator characteristic (ROC) curve generation using binary logistic regression analysis for each individual miRNA and miRNA combination profiles. The best AUC cut-off of 0.80 was generated from a combination of *miR-29a*, *miR-181a* and *miR-652*, providing a sensitivity and specificity of 77% and 74%, respectively (Figure 3). The addition of *miR-223* did not improve the sensitivity or specificity profile achieved.

Discussion

Mammography is currently the gold standard screening tool for breast cancer diagnosis; however accurate diagnosis and intrinsic subtype confirmation requires histological evaluation from tissue obtained at breast biopsy, an invasive procedure. The identification of novel reliable minimally invasive breast cancer biomarkers would represent a significant development in the clinical management of this complex disease. The concept of a panel or

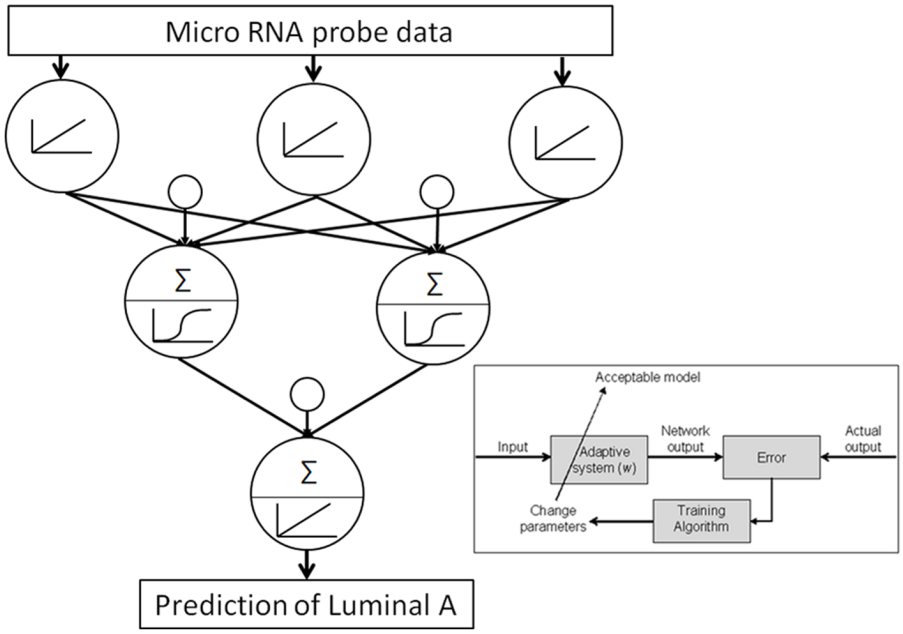

Figure 1. ANN architecture and algorithm as applied to data mining for miRNA markers of Luminal A-like breast cancer.
doi:10.1371/journal.pone.0087032.g001

profile of miRNAs for diagnostic purposes is a realistic approach, as to date no single miRNA has been reported with the qualities (sensitivity, specificity and reproducibility) for use in isolation. The 3 miRNAs identified in this study yielded a sensitivity and specificity of 77% and 74% respectively, and could be evaluated from blood collected during a simple blood test. Although not perfect, this sensitivity and specificity profile exceeds that of several currently used clinical biomarkers [12–15] and could be improved with the combination of mammography. There is no routinely used circulating biomarker for breast cancer detection. Carcinoma Antigen 15-3 (CA 15-3) and Carcinoembryonic Antigen (CEA) are circulating biomarkers. However their clinical application in breast cancer management is, if any, confined to detecting and monitoring disease recurrence and progression. These markers are

Table 4. Candidate miRNAs for validation by RQ-PCR.

miRNA	Chromosomal Location	Sequence	Expression	p-value (Circulation, RQ-PCR) *	p-value (Tissue, RQ-PCR)*
miR-19b	Chromosome 13: 92001446–92005532 (+)	UGUGCAAAUCCAUGCAAAACUGA	Unchanged	0.775	NA
miR-29a	Chromosome 7: 130561506–130561569 [−]	UAGCACCAUCUGAAAUCGGUUA	**Down**	**0.001**	**0.330**
miR-93	Chromosome 7: 99691391–99691470 [−]	CAAAGUGCUGUUCGUGCAGGUAG	Unchanged	0.399	NA
miR-181a	Chromosome 1: 198828173–198828282 [−]	AACAUUCAACGCUGUCGGUGAGU	**Down**	**0.004**	**0.019**
miR-182	Chromosome 7: 129410223–129410332 [−]	UUUGGCAAUGGUAGAACUCACACU	Unchanged	0.355	NA
miR-223	Chromosome X: 65238712–65238821 [+]	UGUCAGUUUGUCAAAUACCCCA	**Down**	**0.004**	**NA**
miR-301a	Chromosome 17: 57228497–57228582 [−]	CAGUGCAAUAGUAUUGUCAAAGC	Unchanged	0.179	NA
miR-423-5p	Chromosome 17: 28444097–28444190 [+]	UGAGGGGCAGAGAGCGAGACUUU	Unchanged	0.519	NA
miR-486-5p	Chromosome 8: 41517959–41518026 [−]	UCCUGUACUGAGCUGCCCCGAG	Unchanged	0.333	NA
miR-652	Chromosome X: 109298557–109298654 [+]	AAUGGCGCCACUAGGGUUGUG	**Down**	**0.009**	**0.001**

Table Legend: *p-value determined by t-test.
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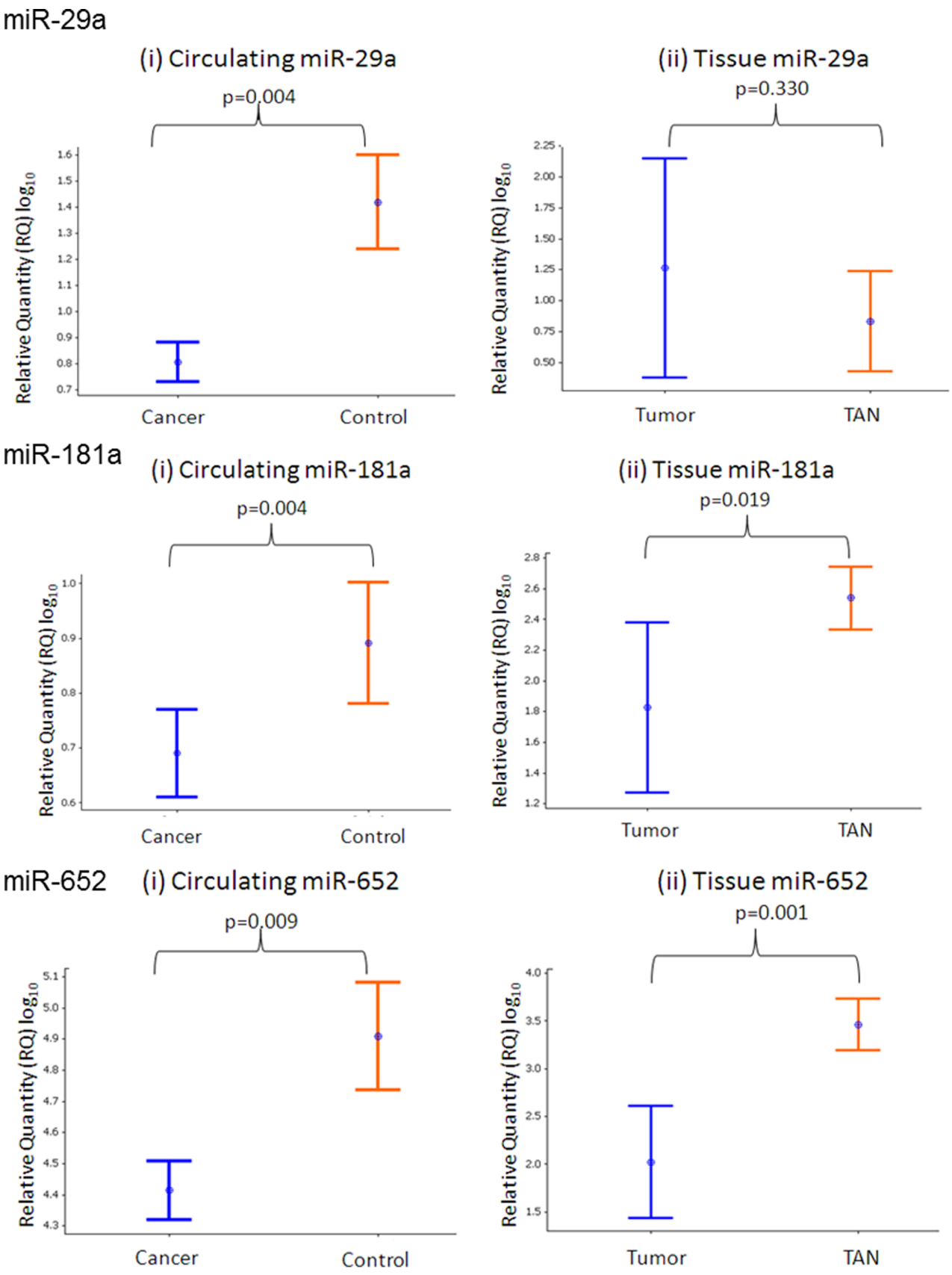

Figure 2. Expression levels of (A) *miR-29a* (i) in the circulation of patients with Luminal A-like breast cancer versus healthy controls and (ii) *miR-29a* levels in tumour and associated normal (TAN) tissue; (B) *miR-181a* expression (i) in the circulation of cases and

controls and (ii) and in tumour and TAN tissue; (C) *miR-652* expression in the circulation of cases and controls (i) and in tumour and TAN tissue (ii).
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merely elevated in 10% of stage 1 disease and 20% of stage 2 disease, precluding any usefulness in the diagnostic arena.

Early miRNA-related research mainly focused on tissue, with several reports of aberrant miRNA expression in breast cancer correlating with clinico-pathological variables such as stage and hormone receptor status [5,16–20]. Furthermore, individual miRNAs have been associated with metastatic potential of breast tumors [21]. The rush to identify non-invasive diagnostic biomarkers for breast cancer has resulted in a surge of interest in circulating miRNAs. Several studies to date have evaluated miRNA expression in blood of women with breast cancer [11,22]. Not all reports in the literature are directly comparable, as although circulating miRNAs are analyzed in each case, three alternative blood components have been used, namely whole blood, serum and plasma. We chose to analyze whole blood in this study as stability of miRNAs in EDTA-whole blood and the potential to profile miRNAs from this medium have been demonstrated [11,23,6]. In addition, given that circulating miRNA research is still in its infancy, it was chosen to utilize methods that could potentially be exploited in larger multi-centric trials by collecting whole blood stored in a refrigerator until transport rather than plasma or serum that requires prompt centrifugation, alloquotting and freezing.

It has been suggested that circulating miRNAs may reflect the presence of breast tumors but not the specific profiles of miRNAs within the breast tumors [24,25]. In the current study, we identified four miRNAs (*miR-29a*, *miR-181*, *miR-223* and *miR-652*) with dysregulated expression in the circulation of women with Luminal A-like breast cancer. *MiR-181a* and *miR-652* were down-regulated in Luminal A-like breast tumor tissue, while *miR-29a* was not. These findings support the hypothesis that circulating miRNA expression profiles may not act as a direct window on tumor activity and brings into question the mechanism by which they enter the blood stream, in addition to their functional role, if any, in the peripheral circulation. These processes remain poorly understood. MiRNAs can enter the peripheral circulation following selective secretion from tumor cells or circulating micro-vesicles [26]. Other cells in the tumor microenvironment can also secrete miRNAs. Meanwhile another school of thought suggests that miRNAs may be detectable in the circulation as a consequence of passive leakage from apoptotic and necrotic cells [27]. In reality it is likely that both of these theories are true, with accumulating evidence to support both plausible proposals.

Once in the circulation, miRNA transport is not uniform. Some miRNAs are encapsulated in microvesicles, apoptotic bodies, exosomes or high-density lipoprotein (HDL) particles while others are in combination with proteins of the Argonaute (AGO) family [28–30]. The protection conveyed by microparticles or in combination with AGO proteins explains the stability of miRNAs in nuclease rich and protease rich environments, such as the circulation, when compared to mRNA [31]. The majority of circulating miRNAs, as much as 90–95%, are transported in combination with the AGO protein family [30,31]. The functional role of miRNAs in circulation has yet to be fully elucidated; are these tiny particles merely secreted as by-products of physiological and pathological processes or are they circulating messengers, with important intercellular and inter-organ cell to cell messaging capabilities? Some recent studies allude to the potential for exosomally-packaged miRNA to act as cell to cell signaling molecules, during viral infection, the immune response and most significantly cancer progression [32–34]. However, despite these reports, it is likely that the majority of circulating extracellular miRNAs, particularly the AGO-transported form, have no functional role. Nonetheless, regardless of their source, their presence, relative stability and ease of detection can be exploited for biomarker means.

In this study ANN identified four specific miRNAs as being significantly altered in the circulation of women with Luminal A-like breast cancer. ANN data-mining algorithms have been shown to provide a robust solution to issues encountered within miRNA array data [5]. They have been shown to cope with non-linearity, and complexity; whilst offering the ability to identify biomarkers of high biological relevance and good predictive sensitivity and specificity [10]. *MiR-181a* has previously been reported as being

Table 5. miRNA expression and clinicopathological parameters.

Clinicopathological Parameter	miRNA	P-value*
Stage	*miR-29a*	0.737
	miR-181a	0.058
	miR-652	0.511
Grade	*miR-29a*	0.193
	miR-181a	0.924
	miR-652	0.998
Nodal Status	*miR-29a*	0.845
	miR-181a	0.257
	miR-652	0.845

This table demonstrates that although *miR-29a*, *miR-181a* and *miR-652* are under-expressed in women with Luminal A-like breast tumors, there is no significant difference in miRNA expression levels in the blood of women with breast cancer, regardless of stage of disease (1 to 5), grade of disease (1 to 3) or nodal status (positive or negative). This is an important biomarker trait, as it reflects miRNA expression alteration in early, as well as late stage disease.
*p-value determined by one-way ANOVA.
doi:10.1371/journal.pone.0087032.t005

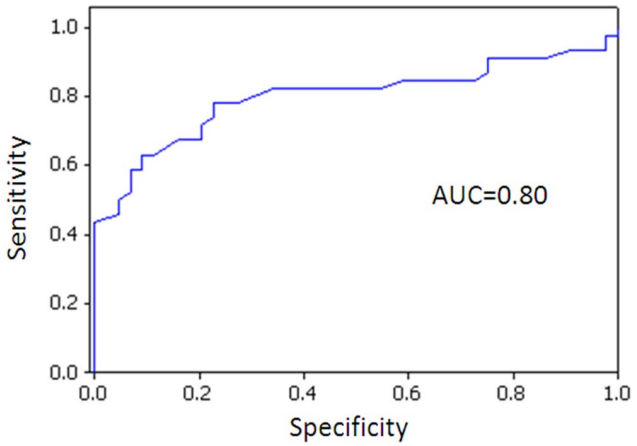

Figure 3. Receiver Operator Characteristic (ROC) Curve for 3 miRNA combination (*miR-29a, miR-181a* and *miR-652*).
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significantly under-expressed in the serum of women with breast cancer compared to healthy controls [35]. It has also been shown to be downregulated in tumor tissue of lung, oral, hepatocellular, and ovarian cancers [36–39]. In addition, *miR-181a* was identified as a potential prognostic factor for colorectal and gastric cancer [40,41]. A recent study, using NGS-SOLiD sequencing followed by validation with RQ-PCR reported *miR-29a* as being over-expressed in the serum of women with breast cancer [42]. This miRNA has been implicated in other cancers, predominantly colorectal where it may have a role in prognostication [43,44]. *MiR-223* has been reported in serum of patients with nasopharyngeal carcinoma and gastric cancer [45,46]. *In vitro* analysis revealed that *miR-223* was detected within exosomes and increased invasiveness of co-cultured cell lines (SKBR2 and MDA-MD-321) [47]. In the present study, validation of *miR-223* expression was examined in fewer samples than were available for *miR181a*, *miR29a* and *miR-652* validation (29 cancers, 40 controls), however we found it to be significantly lower in the circulation of cancer patients, $p = 0.004$). There are no previous reports, to our knowledge, of a role for *miR-652* as a diagnostic biomarker for breast cancer.

Despite the rapidly evolving field of circulating miRNAs as oncologic biomarkers, there are still a number of challenges which much be overcome before miRNA profiling can be routinely incorporated into the diagnostic arena. Real time is the most common technique employed for miRNA quantification. Despite significant technological advances in PCR instrumentation, and levels of detection, there remains little consensus on assay design through to data analysis. In particular, there is a lack of concordance on protocols for data normalization.

Although these results are extremely promising, and substantiate the potential application of miRNAs as biomarkers for breast cancer, we recognize that this study has limitations. The sample size is relatively small; larger validation analyses, involving blinded samples are needed to confirm the clinical utility of the 3 miRNA panel for luminal A-like breast cancer detection. Such studies should ideally include blood samples from all breast tumor subtypes, namely Luminal B, HER2/*neu* over-expressing and basal subgroups, as well as from patients with benign breast disease. Future studies to evaluate the mechanism of action of these miRNAs, if any, in breast tumors and determine the exact processes by which *miR-29a*, *miR-181a*, *miR-223* and *miR-652* are shed into the circulation are also warranted.

The potential value of the miRNAs outlined in this study is not restricted to diagnostic biomarkers for breast cancer. The realm of miRNA-related therapeutic strategies is gaining increased momentum, particularly in hepatitis and hepatocellular carcinoma. MiRNAs with depleted expression levels may be restored to 'normal' levels by viral vector encoded miRNAs or miRNA mimetics. It seems plausible if these miRNAs have a functional role in the tumor microenvironment, tumourigenesis could potentially be halted or reversed by restoring their expression levels.

Conclusions

In conclusion, this study presents 76 miRNAs with differential expression in the circulation of women with Luminal A-like breast cancer compared to those who do not have breast cancer. A miRNA profile of three circulating tumor-associated miRNA biomarkers (*miR-29a*, *miR-181a* and *miR-652)* for breast cancer are identified which in combination provide a sensitivity and specificity profile which exceeds that of several current clinical biomarkers. A complementary test, for use in combination with mammography would prove extremely advantageous particularly in an era where swift diagnosis, expeditious commencement of appropriate adjuvant treatments and surgical resection have a role to play in ultimately improving patient outcomes. Further large prospective studies are required, to include all breast cancer subtypes and to elucidate the potential of miRNAs in the systemic circulation as subtype-specific diagnostic or therapeutic breast cancer markers.

Supporting Information

Figure S1 Correlation between microarray and RQ-PCR data. Correlation (Pearson's) of miRNA expression levels between microarray (dark) and RQ-PCR (light) detected expression levels (A) *miR-29a* (B) *miR-181a* (C) *miR-182*.
(PNG)

Table S1 MiRNAs with altered expression in Luminal A breast cancer.
(DOC)

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Author Contributions

Conceived and designed the experiments: AMMD NM KJS MJK. Performed the experiments: AMD LM. Analyzed the data: AMMD DMW GB. Contributed reagents/materials/analysis tools: NM MJK. Wrote the paper: AMMD NM.

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Identification and Validation of miRNAs as Endogenous Controls for RQ-PCR in Blood Specimens for Breast Cancer Studies

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Abstract

Introduction: A prerequisite to accurate interpretation of RQ-PCR data is robust data normalization. A commonly used method is to compare the cycle threshold (C_T) of target miRNAs with those of a stably expressed endogenous (EC) miRNA(s) from the same sample. Despite the large number of studies reporting miRNA expression patterns, comparatively few appropriate ECs have been reported thus far. The purpose of this study was to identify stably expressed miRNAs with which to normalize RQ-PCR data derived from human blood specimens.

Methods: MiRNA profiling of approximately 380 miRNAs was performed on RNA derived from blood specimens from 10 women with breast cancer and 10 matched controls. Analysis of mean expression values across the dataset (GME) identified stably expressed candidates. Additional candidates were selected from the literature and analyzed by the geNorm algorithm. Further validation of three candidate ECs by RQ-PCR was performed in a larger cohort (n = 40 cancer, n = 20 control) was performed, including analysis by geNorm and NormFinder algorithms.

Results: Microarray screening identified 10 candidate ECs with expression patterns closest to the global mean. Geometric averaging of candidate ECs from the literature using geNorm identified *miR-425* as the most stably expressed miRNA. *MiR-425* and *miR-16* were the best combination, achieving the lowest V-value of 0.185. Further validation by RQ-PCR confirmed that *miR-16* and *miR-425* were the most stably expressed ECs overall. Their combined use to normalize expression data enabled the detection of altered target miRNA expression that reliably differentiated between cancers and controls in human blood specimens.

Conclusion: This study identified that the combined use of 2 miRNAs, (*miR-16* and *miR-425*) to normalize RQ-PCR data generated more reliable results than using either miRNA alone, or use of *U6*. Further investigation into suitable ECs for use in miRNA RQ-PCR studies is warranted.

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Introduction

Accumulating evidence has shown that miRNAs play pivotal roles in regulatory functions pertaining to cell growth, development and differentiation and are associated with a wide variety of human diseases. Despite their discovery over a decade ago, it is only recently that the extent of the complexity of these regulatory molecules is beginning to be understood. Expression analysis studies have revealed differential miRNA expression in tumors compared to normal tissues. MiRNAs have been found to be dysregulated in a wide variety of human cancers. Accordingly, miRNAs have elicited much interest as biomarkers for cancer diagnosis and disease monitoring and are rapidly emerging as novel targets for disease intervention.

Real-time quantitative (RQ-PCR) is widely used to quantify miRNA expression due to its sensitivity, specificity, speed, simplicity and the small amounts of template RNA required. To differentiate true biological variation from experimentally induced artifacts, target miRNA expression levels are normalised to those of a control(s). To accurately quantify miRNA expression by RQ-PCR, samples are assayed during the exponential phase of the PCR reaction during which time the amount of target miRNA is presumed to double with each cycle, without influence from limiting reagents. Comparison of cycle threshold (C_T), the cycle number at which fluorescence signals are detected above background, to C_T values to an endogenously expressed control RNA is used to determine miRNA expression levels by relative quantification (EC). The accuracy of this method is heavily reliant on the choice of endogenous control. Other methods of normalization such as normalization to the global mean, use of spike-in controls, among others have also been described [1]. Regardless of the approach, the normalization technique and specific control RNA(s) used directly influence the results produced from RQ-PCR and thus validity. The selection of a suitable EC(s),

with which to normalize RQ-PCR data, is an important first step in the accurate and reliable determination of miRNA expression levels.

Ideally a reliable EC(s) should remain stably expressed regardless of disease status or other clinical variables. A set of robust ECs that are steadily uniformly expressed across all body tissues, fluids and disease pathways has yet to be described, and is unlikely to exist. Several miRNA expression analysis studies based on tissue have reported the use of small RNAs (such as *U6, RNU44* or *RNU48*) or *miR-16* to normalize expression data [2–6]. However, use of these reference genes cannot simply be applied to miRNA analysis in blood or other body fluids as miRNA expression patterns are known to be disease-specific and perhaps specimen-type specific [7]. Despite the abundance of studies on circulating miRNA profiles to discriminate between normal and disease states, there have yet to be conclusive reports of appropriate ECs. This remains a significant hurdle that must be addressed to substantiate biomarker discovery and validate single miRNA expression analysis using RQ-PCR.

Breast cancer is a prevalent disease with increasing incidence worldwide. This growing social and economic burden has stimulated the search for novel biomarkers to aid in diagnostics, prognostication and disease monitoring of adjuvant treatment. Few validated endogenous controls for miRNA research in breast cancer have been described. Initial miRNA studies on breast tissues by Mattie *et al* normalized miRNA expression to *miR-16* and *let-7*, which were later shown to be stably expressed across malignant, benign and normal breast tissue by Davoren *et al* [2,8]. Early studies on systemic miRNAs in breast cancer normalized to *miR-16* [9,10]. Additional studies, on breast and other cancers, have suggested alternative EC candidates such as *U6, RNU44, RNU48*, *miR-142-3p*, *miR-484*, *miR-191* and *miR-425* [3,11-18]. However, there is a lack of validated reports of suitable ECs for circulating miRNAs.

The aims of this study were to evaluate a panel of candidate ECs (using microarray profiling) from which to validate the most stably expressed EC(s) to normalize RQ-PCR data derived from blood specimens in breast cancer. In addition we wished to determine the effect of different normalization strategies on target miRNA expression.

Table 1. Clinico-pathological data for blood samples derived from breast cancer cases and controls for microarray and RQ-PCR analysis.

Tumors	Array	RQ-PCR
	Number (%)	Number (%)
	10	**40**
Mean age, years (range)	56.7	56.17
Median whole. T size (mm)	45.6 (±31.27)	30.55 (±19.47)
Missing data	-	2 (5)
Nodal status		
Positive	5 (50)	20 (50)
Negative	5 (50)	20 (50)
Grade		
1	1 (10)	7 (17.5)
2	9 (90)	25 (62.5)
3	-	8 (20)
UICC stage		
Stage 1	2 (20)	15 (37.5)
Stage 2	5 (50)	12 (30)
Stage 3	3 (30)	12 (30)
Missing	-	1 (2.5)
Intrinsic Subtype		
Luminal A	10 (100)	30 (75)
Luminal B	-	2 (5)
HER2/*neu*	-	5 (12.5)
Basal	-	3 (7.5)
Controls	**10**	**20**
Mean Age, years (range)	81.7	49.65

Mm, millimeter; UICC, breast tumor staging according to the International Union Against Cancer criteria; HER2/*neu*, human epidermal growth factor receptor. Control subjects had no personal or family history of breast or ovarian cancer and were clinically well at the time of sampling.
doi:10.1371/journal.pone.0083718.t001

Materials and Methods

Study cohort and sample collection

Blood samples were prospectively collected from 80 women including 50 consecutive patients with a new diagnosis of breast cancer and 30 healthy control participants. All patients had histologically confirmed invasive breast cancer. Samples of venous non-fasting blood were collected in BD vacutainers containing 18 mg dipotassium EDTA (K2E) anticoagulant (BD-Plymouth, PL6 7BP, UK) following written informed consent. Microarray profiling was performed on RNA derived from blood on 10 of the above patients and 10 of the controls (Table 1). The remaining 40 cases and 20 controls were used to validate candidate ECs and target miRNA expression (Table 1). Ethical approval was granted by the Clinical Research Ethics Committee, Galway University Hospital, Ireland.

RNA extraction and analysis

Total RNA was extracted from 1 mL of blood using TRI Reagent BD (Molecular Research Centre, Inc). RNA concentration and integrity were examined by NanoDrop spectrophotometry (NanoDrop ND-1000 Technologies Inc., DE, USA) and Agilent Bioanalyzer RNA 6000 Nano Chip Kit Series II (Agilent Technologies, Germany) analysis, respectively.

Microarray profiling

Expression profiling of circulating miRNAs was performed on RNA extracted from 20 blood specimens using TaqMan human miRNA microarrays as instructed by the manufacturer (TaqMan Low Density Array Human microRNA Card A, Life Technologies, Foster City, CA, USA). Megaplex primer pools were used to reverse transcribe RNA samples (700 ng) which were then PCR amplified in 2 μL volumes on 384 well pre-configured microfluidic cards. Each card contained TaqMan probes for 377 miRNAs plus 3 pre-defined ECs (*U6* in quadruplicate, *RNU44* and *RNU48*) and a negative control (*ath-miR-159a*).

Candidate EC selection

In addition to the candidate ECs identified by microarray profiling, the expression of 7 additional candidates, as chosen from a review of published studies, was investigated in the array dataset (*miR-16, miR-425, miR-484, miR-142-3p U6, RNU44* and *RNU48*, Table 2). Three of these (*miR-16, U6*, and *miR-425*) were further

Table 2. Candidate endogenous controls.

MiRNA Name	Molecule type	Accession Number*	Chromosomal Location	Blood Component	Reference
miR-16	miRNA	MI0000070*	13q14.2	Whole blood, Serum, Plasma	[9,14,25,30-34,49]
U6 (RNU6B)	snoRNA	26826**	10p13	Plasma	[11,23]
RNU48	snoRNA	26801**	6p21.33	Serum	[50]
miR-425	miRNA	MI0001448*	3p21.31	Whole blood	[17]
RNU44	snoRNA	26806**	1q25.1	Whole blood	[51]
miR-484	miRNA	MI0002468*	16p13.11	Serum	[15]
miR-142-3p	miRNA	MI0000458*	17q22	Plasma	[14]

*mirBase database accession number
**NCBI Gene ID
doi:10.1371/journal.pone.0083718.t002

validated in a larger cohort of blood from breast cancer patients and controls.

Data analysis

Microarray data was analyzed in two ways. Firstly, to identify the most stably expressed miRNA(s) from the panel of 377 miRNAs across the 20 blood samples, global mean expression normalization was applied [19]. MiRNAs were then ranked commencing with those with expression profiles closest to that of the mean. The second approach used the GeNorm algorithm to assess the stability of the 7 candidate ECs chosen from the literature. The most stably expressed ECs were assessed and ranked both individually (M variable) and in combination (V variable) [20].

RQ-PCR validation

Total RNA (100 ng) was reverse transcribed using miR-specific stem-loop RT primers (Life Technologies) and components of the High Capacity cDNA Reverse Transcription kit (Invitrogen Life Technologies) according to the manufacturer's protocols. Expression levels of individual miRNAs were detected by subsequent RQ-PCR using TaqMan MicroRNA assays (Invitrogen Life Technologies) and a 7900HT instrument (Life Technologies) using standard thermal cycling conditions in accordance with manufacturer recommendations. PCR reactions were performed in triplicate in final volumes of 10 µl on 96 well plates, including inter-assay controls (IAC) to account for variations between runs.

Amplification efficiency

PCR amplification efficiencies (E) were generated using the formula $E = (10\text{-}1/\text{slope} - 1)\times 100$, using the slope of the semi-log regression plot of C_T versus log input of cDNA (10-fold dilution series of five points). A threshold of 10% above or below 100% was adopted.

PCR data analysis

Cycle threshold (C_T), or quantification cycle (Cq) is the cycle number during a PCR reaction at which the fluorescence generated is sufficient to pass the threshold, ten times the standard deviation of the baseline fluorescence emission. C_T values inversely correlate with the logarithm of the initial expression such that candidates with high expression had low C_T, and vice versa. The threshold standard deviation for intra- and inter-assay replicates was 0.28.

Candidate EC stability analysis

Stability of candidate ECs was assessed using geNorm and NormFinder algorithms. GeNorm is based on the assumption that none of the candidate ECs is co-regulated. It was used to rank ECs according to stability values (M), which represented the variation in expression of candidate ECs in comparison to each other. This was done by selecting optimal pairs of ECs by calculating and comparing M values for all candidates and stepwise exclusion of the least stable EC [20]. NormFinder is a Microsoft Excel add-in that can accommodate both inter- and intra-group variation [21], in this case cancer and control, by accounting for variability and bias between groups. It was used to estimate the most stable EC in isolation, and the most stable 2-EC combination. The lower the stability value, the more stable the expression of the EC candidate.

Comparative quantification of target miRNAs relative to EC

Target miRNA (*miR-93*, *miR-181a* and *miR-652*) expression levels were estimated using qBase software (Biogazelle, Belgium) to calculate amplification efficiency-corrected relative quantities following normalization to each candidate EC. The comparative cycle threshold ($\Delta\Delta C_T$) method, using the formula $\Delta\Delta C_T = (C_T$ target, test sample - C_T EC, test sample) – (C_T target, calibrator sample - C_T EC, calibrator sample) was applied. To test the effect of alternative EC on target miRNA detection, the expression of miRNA targets (*miR-181a, miR-652* and *miR-93*) with previously documented expression in the circulation of breast cancer patients were measured using the ECs.

Statistical analysis

Statistical analysis was performed using Minitab version 16.0 (Minitab Ltd, Coventry UK). The Kolmogorov-Smirnov test for normality was conducted and parametric tests were used where appropriate. A log transformation ($\log_{10}$) of the data was performed when necessary. Significance of circulating miRNA levels was determined using t-tests or Mann-Whitney U test, as appropriate. Results with p values <0.05 were considered significant.

Results

Identification of candidate ECs (using microarray)

To identify the most stably expressed miRNAs from the microarray dataset global mean expression normalization was applied. This involved the use of the mean expression value of all expressed miRNAs in a given sample (in this case, 20 samples) as a

normalization factor for miRNA RQ-PCR. MiRNAs with expression profiles closest to the mean were *miR-103, miR-185, miR-532-3p, miR-194, miR-126, miR-155, let-7e, miR-345, miR-425 and miR-15b* as illustrated in Table 3. The first 9 miRNAs on this list were excluded from further EC analysis on the basis of their documented roles in breast cancer (Table 3). *MiR-425* was the only miRNA in this group not previously implicated in breast cancer and hence was chosen for further validation by RQ-PCR.

There was no significant difference in expression of the candidate miRNAs and snoRNAs between the cancer and control group across the microarray dataset (Figure 1). Expression stability of the means of snoRNAS (*U6, RNU44* and *RNU48*) and miRNAs (*miR-16*, *miR-425*, *miR-142-3p*, and *miR-484*) were assessed using GeNorm (Figure 2). *MiR-425* was found to be the most stably expressed, with a geNorm M-value 0.907. RNU48 was the least stably expressed candidate. Combination of *miR-425* and *miR-16* resulted in the lowest V- value of 0.185 (Figure 3).

The stability of 3 of the above miRNAs (*miR-425*, *miR-16* and *U6*) was further investigated by RQ-PCR in a larger cohort: *miR-425* was selected as it was identified by GME analysis and had the lowest geNorm M value. *MiR-16* had the next lowest geNorm M value and has been used to normalize PCR data in several cancer studies both in tissue and blood [8,9,14,22]. *U6* was selected, not on the basis of its apparent stability (it did not feature in GME analysis, and ranked comparatively low by geNorm), rather due to its wide use in the literature [11,23].

Relative quantities of candidate EC genes

RQ-PCR was performed to validate the expression patterns of 3 candidate ECs in 60 blood samples, comprised of 40 samples from women with cancer and 20 from healthy controls (2). All candidate ECs were expressed in abundance, with mean C_T values less than 25. *MiR-16* showed the highest expression, with mean C_T of 15.5 (range 13.5–18.7), followed by *miR-425*, mean C_T 20.7 (range 17.4–24.2) and then *U6*, mean C_T 21.0 (range 19.0–22.8), see Table 4.

The C_T values of each candidate EC were assessed for differential expression between cancer and control blood samples (Figure 4). *U6* was significantly more abundantly expressed in the control group ($p = 0.009$). In this manner it was identified that there was no difference in expression of *miR-16* or *miR-425* between the cancer group and the controls, as would be expected for good candidate ECs.

Relative expression values of candidate ECs were log transformed and expressed as means with matching symmetrical confidence intervals (CI). Confidence intervals between -1 and +1 represented fold changes of ≤ 2, while those between -1.58 and $+1.58$ equated to fold changes of ≤ 3. A fold change cut off of 3 was applied as previously established [24]. Confidence intervals with an upper border >1.58 signaled over-expression of a candidate EC in the cancer group. Confidence intervals with lower borders <1.58 indicated under-expression of the candidate EC in the control group (Figure 5). Equivalence testing was then performed to confirm that all three candidate ECs were equivalently expressed between the cancer group and the control group.

There was a significant difference in variance between ECs (Bartlett's test, $p<0.001$) indicating their differing stabilities, with *miR-16* showing the greatest variation (Figure 6).

Analysis of reference gene expression stability geNorm and NormFinder

The stability and variability of the candidate ECs was further assessed using NormFinder and geNorm as summarized in Table 5. Lower stability values indicate greater stability. GeNorm provided two values: a gene stability (geNorm M) value and a geNorm V value. The geNorm M value ranked candidate ECs according to their stability, from the least stable (highest M value) to the most stable candidate (lowest M value). These values were generated on the basis of the average pairwise variation between all tested genes accompanied by stepwise exclusion of the least stable gene. GeNorm V values determined the optimum EC pairing for normalization, by defining the pairwise variation between two sequential normalization factors. GeNorm identified *miR-16* as the single most stably expressed miRNA, with a GeNorm M value of 1.191. NormFinder identified *miR-16* and *U6* as the best combination, with a stability value of 0.102. The single best EC as calculated by this algorithm was *miR-425*, followed by

Table 3. GME analysis to determine 10 most stably expressed miRNAs from the microarray dataset.

Rank	miRNA	Previous Reports	Standard Deviation from GME	Reference
1	miR-103	Upregulated in serum of breast cancer patients	0.121	[52,53]
2	miR-185	Suppress tumor growth and progression in human ovarian cancers, pediatric renal tumors and breast cancer cell lines	0.128	[54]
3	miR-532	Associated with triple negative(ER-ve/PR-ve/HER2/neu-ve) breast cancer in tumor tissue	0.132	[55]
4	miR-194	Upregulated following Traztuzumab therapy in breast cancer cells; overexpression of miR-194 results in cell migration and invasion inhibition in breast cancer cell lines	0.139	[56]
5	miR-126	Under-expressed in breast cancer, with restoration associated with metastases suppression in breast cancer cell lines and breast tumors	0.150	[57-59]
6	miR-155	Over-expressed in breast tissue and circulation of women with breast cancer	0.155	[60,61]
7	let-7c	Under-expressed in breast cancer with a tumor suppressor role	0.158	[9,62,63]
8	miR-345	Targets MRP in multidrug resistant breast cancer cells compared to normal cells	0.158	[64]
9	miR-425	No reports of altered expression or functional role in breast cancer	0.160	-
10	miR-15b	Altered expression in circulation and tumor of those with breast cancer	0.162	McDermott *et al.*, unpublished data

The top 10 most stably expressed miRNAs following normalization of the microarray data using global mean expression (GME). Nine of these miRNAs have been implicated in breast cancer. *MiR-425* is the only candidate miRNA with no reported association with breast or any other cancer type.
doi:10.1371/journal.pone.0083718.t003

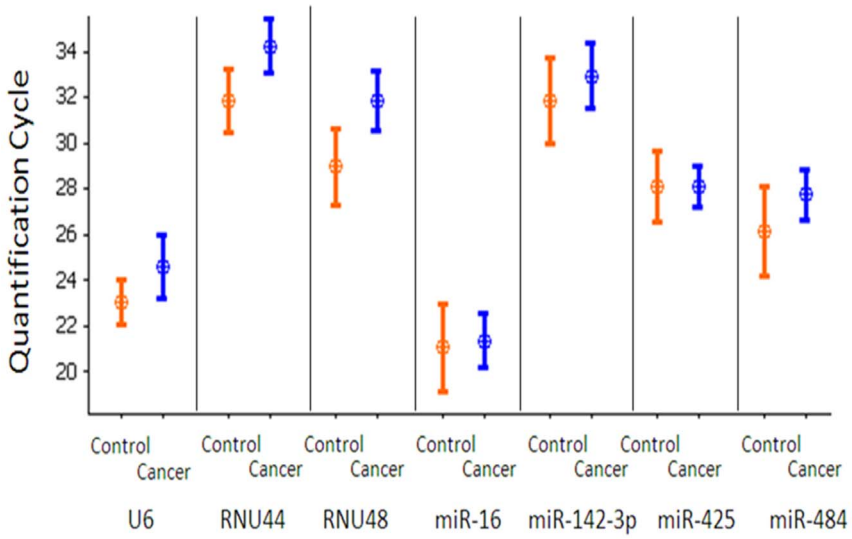


Figure 1. Quantity of each candidate miRNA on microarray analysis. The quantity of each miRNA or snoRNA (quantification cycle/cycle threshold) was determined by microarray for the cancer group and the control group. There was no significant difference (t-test) in candidate EC expression between the cancer group and the control group.
doi:10.1371/journal.pone.0083718.g001

miR-16 and *U6*. Consistent with the geNorm analysis on the microarray data *miR-16* and *miR-425* were identified as being the most appropriate ECs.

Effect of candidate EC selection for normalization on relative expression of target miRNAs

To test their efficacy on target miRNA quantification, each of the candidate ECs was used to determine relative quantities of

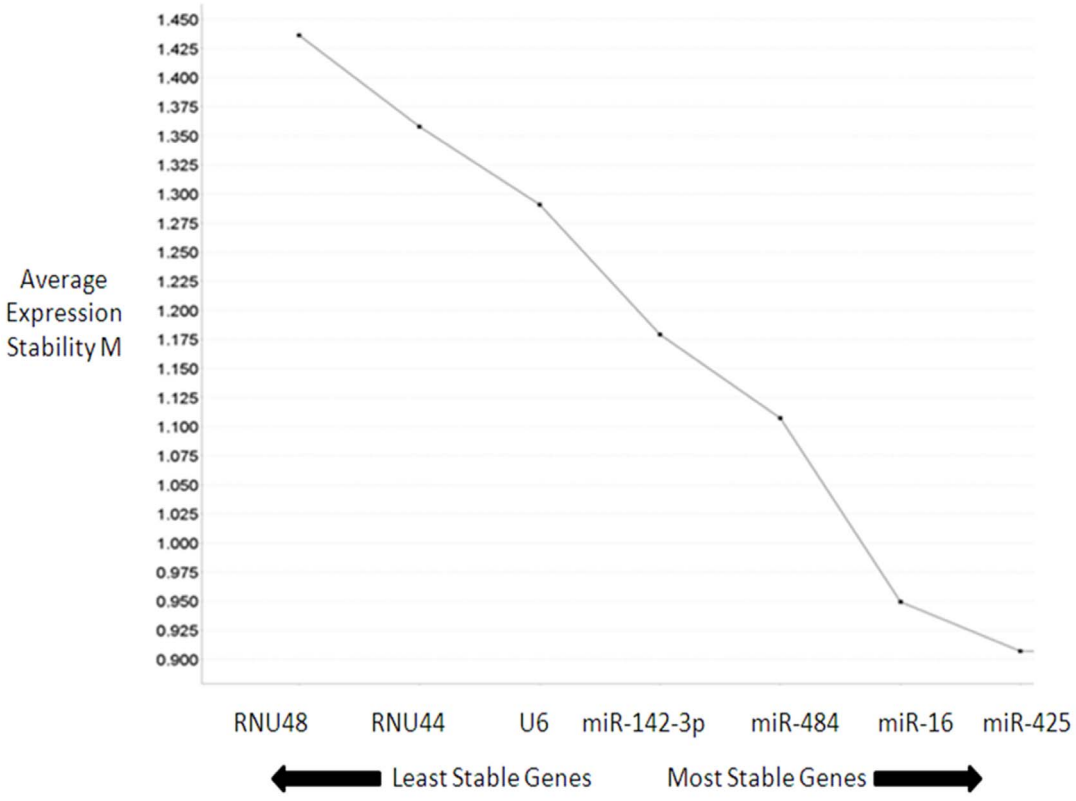


Figure 2. GeNorm analysis of average expression stability of candidate ECs. Ranking of candidate ECs according to average expression stability. The least stable candidate ECs with the highest stability measure (M) are on the left side of the graph, with the most stable ECs with the lowest M value on the right. *RNU48* and *RNU44* are the least stable ECs while *miR-425* and *miR-16* are the most stable candidate ECs.
doi:10.1371/journal.pone.0083718.g002

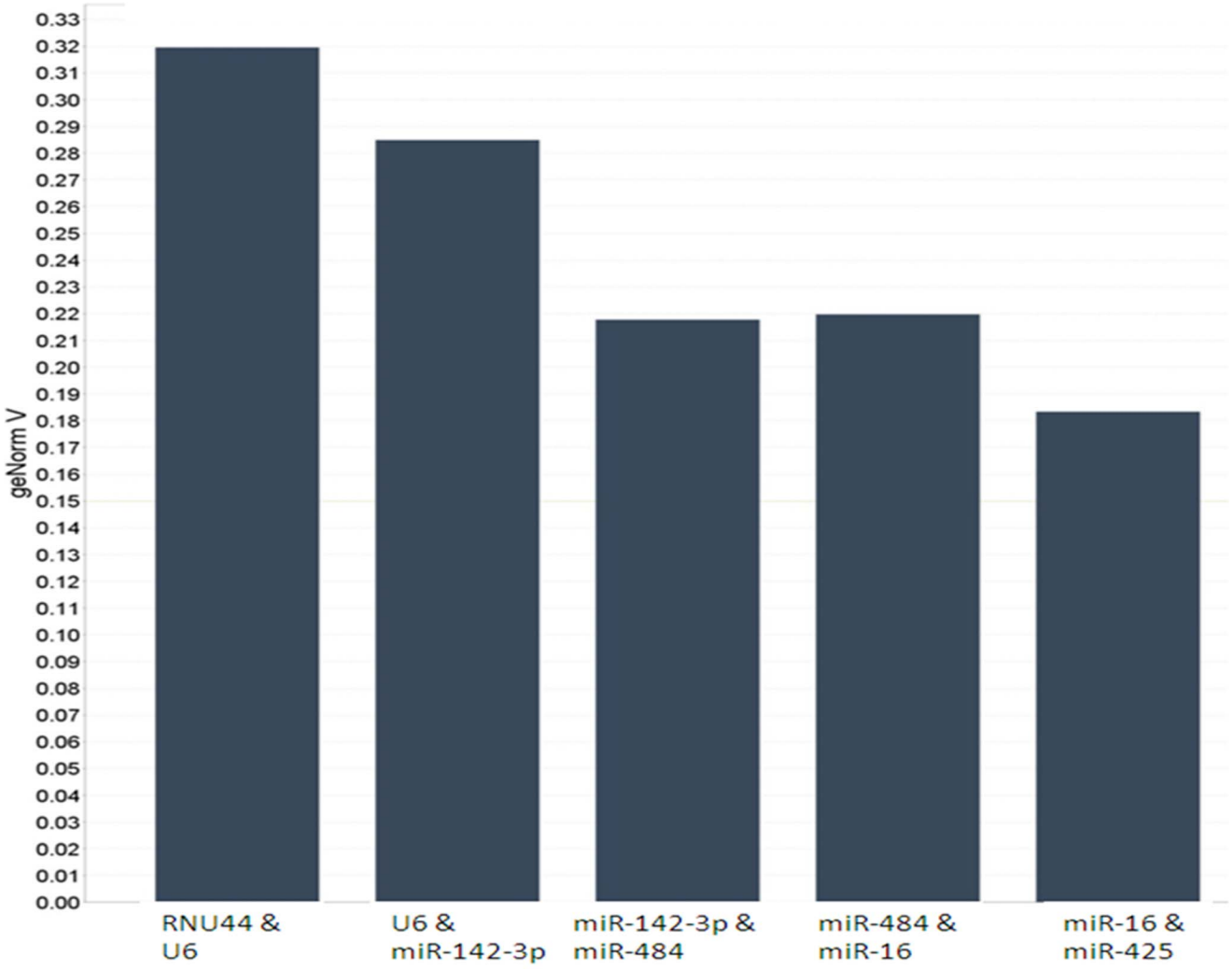


Figure 3. Determination of the best combination of ECs for normalization. Determination of optimum number of candidate ECs for normalization. The GeNorm programme establishes the optimum combination of candidate ECs for normalization, producing the lowest V variable. This factor is calculated using the variable 'V' as the pairwise variation (Vn/Vn+1) between two sequential normalization factors (NFs) (NF_n and NF_{n+1}). The combination of candidate ECs is deemed optimal when the V variable achieves the lowest value. The optimal combination was achieved by combining *miR-16* and *miR-425*.
doi:10.1371/journal.pone.0083718.g003

Table 4. Cycle Threshold (C_T) values for ECs in validation cohort.

miRNA	Mean C_T ± St Dev	C_T Range	C_T Min	C_T Max
U6	21.042±0.848	3.843	19.065	22.898
Cancer	21.23±0.854		19.568	22.898
Control	20.661±0.712		19.065	21.804
miR-16	15.460±1.342	5.2	13.565	18.765
Cancer	15.398±1.346		13.565	18.765
Control	15.584±1.361		13.585	17.812
miR-425	20.740±1.415	6.746	17.459	24.206
Cancer	20.772±1.416		18.100	24.206
Control	20.676±1.447		17.459	23.395

doi:10.1371/journal.pone.0083718.t004

known breast cancer-associated miRNAs (Figure 7). *MiR-93*, which was previously shown not to be dysregulated in breast cancer (McDermott *et al.*, unpublished data) was overexpressed in the cancer group when *U6* ($p = 0.017$) was used as an EC but was unaltered with other candidate ECs. *MiR-181a*, which was previously shown to be under-expressed in breast cancer [25](McDermott *et al.*, unpublished data) was under-expressed when *miR-16* ($p = 0.011$) used as EC. Of the four target miRNAs, the choice of EC did not influence the relative quantity of circulating *miR-652* between cancers and controls ($p<0.001$) suggesting a highly significant differential expression of *miR-652* in breast cancer (McDermott *et al.*, unpublished data). Relative quantities of target miRNAs in cancer and control groups are shown in Figure 7.

These results highlight the importance of selecting appropriate and validated ECs. Despite the large sample size, true biological differences in miRNA expression were not detected when using less stable ECs for normalization.

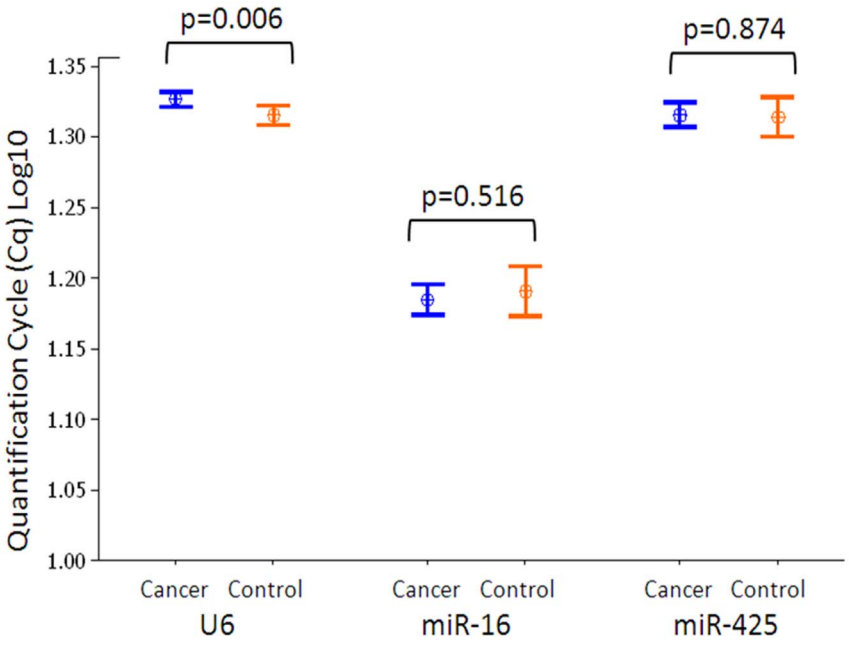


Figure 4. Relative quantity candidate ECs. Relative quantity of candidate EC miRNAs in blood of breast cancer patients (blue, n = 40) and healthy controls (n = 20) expressed as quantification cycle (Cq) values. Interval plots display the mean and 95% confidence interval. There was no significant difference ($p>0.05$, t-test) for *miR-16* and *miR-425*. However, *U6* was more abundant in the control group ($p = 0.006$).
doi:10.1371/journal.pone.0083718.g004

Discussion

Altered miRNA expression is associated with most pathological disease processes, including carcinogenesis. Their ease of detection in biological fluids, including blood, makes them ideal candidates for exploitation as minimally invasive biomarkers. RQ-PCR is the most common technique for miRNA expression analysis. However, the high sensitivity of this approach means that accurate interpretation of RQ-PCR results depends heavily on the use of suitable, stably expressed ECs for data normalization in an effort

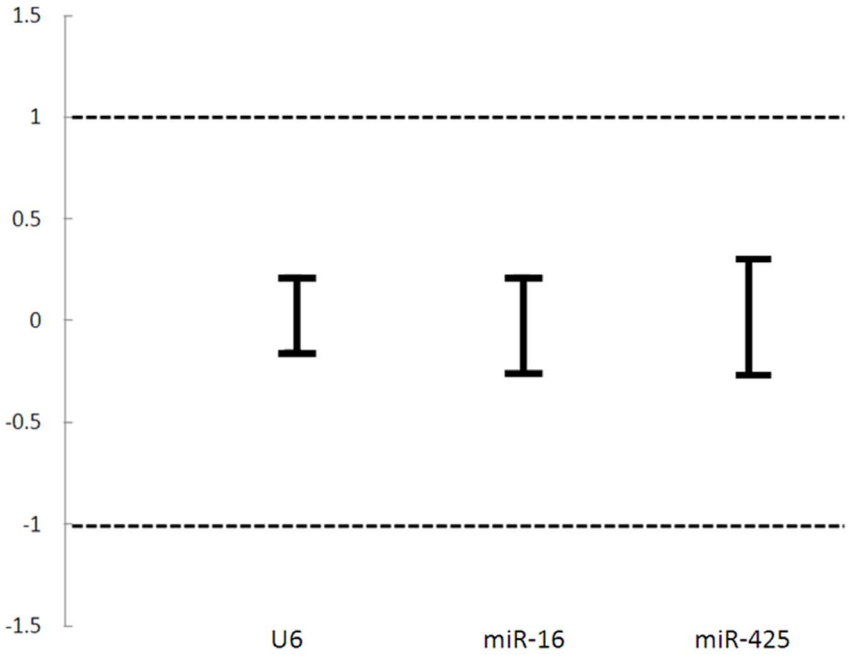


Figure 5. Equivalence test for candidate ECs. Each line represents the difference in logarithmic (log base2) expression between the cancer and control groups. The upper and lower bars of individual candidate ECs represents the upper and lower limits of symmetrical confidence intervals, respectively. Confidence intervals between −1 and +1 corresponded to fold changes of ≤2. No candidate EC displayed a fold change greater than 2. All three candidate ECs were equivalently expressed.
doi:10.1371/journal.pone.0083718.g005

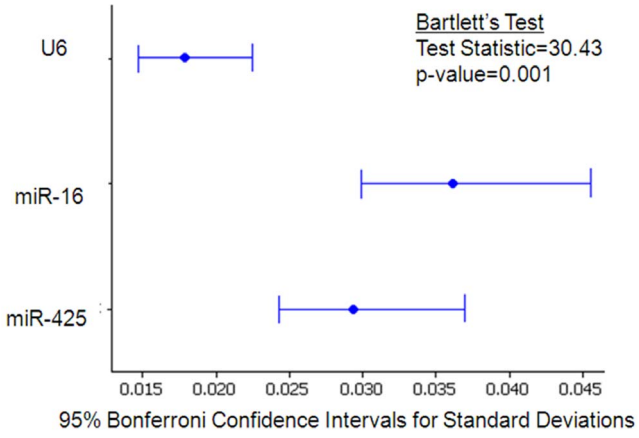


Figure 6. Variation associated with each candidate EC. Bonferroni confidence intervals for standard deviations. There was a significant difference in variance ($p<0.001$, Bartletts's test) associated with each candidate EC, indicating differing stabilities.*MiR-16* showed greater variance than *miR-425* and *U6*.
doi:10.1371/journal.pone.0083718.g006

to minimize non-biological variation between samples. Reference genes for mRNA studies have been well established but validated ECs for miRNA research are scarce. In addition, ECs for use in tissue miRNA research may not be directly translated to other tissues or body fluids. Scrupulous miRNA data normalization may be more important than other functional RNA classes [26].

The first systematic assessment of candidate ECs for miRNA RQ-PCR was conducted by Davoren *et al* [8]. This study examined the expression stability of five miRNAs (*let-7a, miR-10b, miR-16, miR-21* and *miR-26b*) and 3 small nucleolar RNAs (*RNU19, RNU48* and *Z30*) was determined in normal, benign and malignant breast tissue. The best normalization strategy for miRNA analysis in breast tissue was found to be a combination of *miR-16* and *let-7a*. There have been subsequent isolated reports of suitable ECs for specific disease states and specimen types, but the focus of this issue in the literature is disproportionate to the number of accounts of altered miRNA expression in specific disease states [15,27–29]. Chang *et al* conducted a similar systematic approach to identify suitable ECs for application to colorectal cancer tissue [28]. MiRNA profiling was performed on a small cohort of paired colorectal tumor tissues and normal tissue. Global mean expression analysis was performed to identify stably expressed candidate ECs. Six candidate miRNAs (*let-7a*, *miR-16*, *miR-26a*, *miR-345*, *miR-425* and *miR-454*) and 2 small nucleolar RNAs (*RNU48* and *Z30*) were chosen for for further validation by RQ-PCR in a larger cohort of colorectal tissues. *MiR-16* and *miR-345* were identified as the best combination of reference miRNAs by both geNorm and NormFinder, with *miR-16* and *miR-345* being the single best normlizers identified by NormFinder and geNorm, respectively. Genovesi *et al* identified ECs for use in medulloblastoma studies involving TLDA cards, and recommended the combination of *miR-301a* and *miR-339-5p* for normalization of card A data, with a combination of *miR-425** and *RNU24* being used for Card B data analysis [27]. Few studies have examined suitable ECs for use in circulating miRNA studies. Hu *et al* identified and validated candidate miRNAs as ECs for serum miRNA expression studies in breast cancer [15]. In this cohort, a combination of *miR-191* and *miR-484* provided the best normalization approach for target miRNA expression. Song *et al* focused on gastric cancer, examining 6 miRNAs (*let-7a, miR-16, miR-93, miR-103, miR-192, and miR-451*) and one small nucleolar RNA, *RNU6B* for suitability as candidate ECs [29]. This study advocated the use of *miR-16* and *miR-93*, the most stably expressed candidate ECs, for normalization of miRNA expression in serum for gastric cancer.

Table 5. GeNorm and NormFinder expression stability analysis.

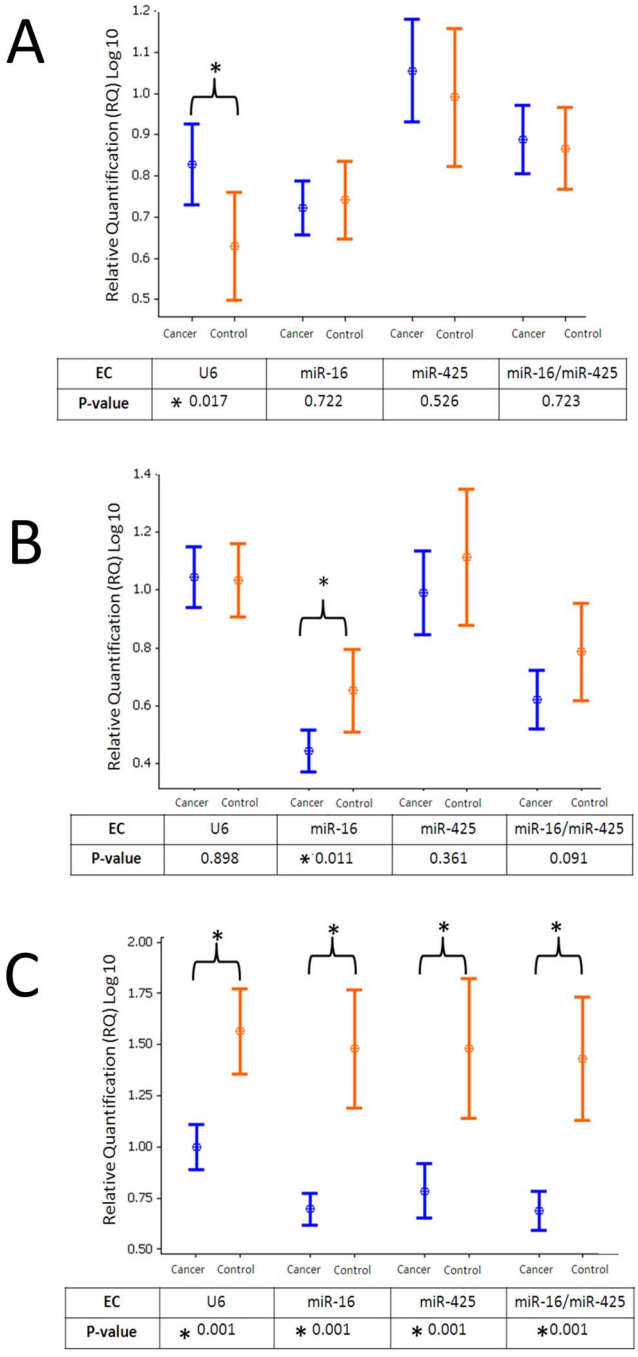
Rank	geNorm		NormFinder	
	Gene	Stability	Gene	Stability (M)
1	miR-16	1.191	miR-425	0.038
2	U6	1.232	miR-16	0.064
3	miR-425	1.251	U6	0.067

Ranking of candidate reference genes based on expression stability values calculated by NormFinder and geNorm
doi:10.1371/journal.pone.0083718.t005

The present study identified that the combined use 2 miRNAs, (*miR-16* and *miR-425*) to normalize RQ-PCR data generated more reliable results than using either miRNA alone, or use of *U6*, which has been used by several authors to date. In the absence of a comprehensive analysis of reliable ECs for RQ-PCR data from blood samples, a microarray screen was performed at the outset. We profiled 20 blood samples (10 from women with breast cancer and 10 from healthy control women) for the expression of in excess of 380 miRNAs (including *U6* rRNA). The dataset was analyzed using global mean expression (GME) to identify miRNAs with expression patterns closed to the mean expression of the entire dataset. We selected *miR-425* from the GME analysis, and both *miR-16* and *U6* from the literature for further analysis by RQ-PCR in a validation cohort ($n = 60$). Our initial validation step using raw C_T values of these 3 candidate ECs displayed that *U6* was more abundant in the control group, while there was no difference in *miR-16* or *miR-425* expression between the cancer or control group. Equivalent expression of candidate ECs between the cancer and control group was then confirmed using a fold change cut off of ≤ 3, corresponding to confidence intervals between -1.58 and $+1.58$. We then used both GeNorm and NormFinder algorithms which identified *miR-16* and *miR-425*, respectively, as the most stably expressed candidate ECs, with NormFinder suggesting their combination as the best combination.

As evident from the results presented in this study, use of an inappropriate EC for normalization can significantly alter the apparent expression of target miRNAs. Combination of *miR-16* and *miR-425* as EC detected significant dysregulation of *miR-652* ($p = 0.001$). *MiR-181a* has previously been shown to be under-expressed in breast cancer, but was shown in this study to be significantly under-expressed when *miR-16* alone was used as an EC. Normalization with the combination of *miR-16* and *miR-425* increased the p-value to 0.091. *MiR-93* has been shown to be stably expressed in blood of women with breast cancer compared to healthy controls. However, when *U6* was applied as the normalizer *miR-93* appeared to be upregulated in the cancer group. A combination of miRNAs for normalization augments the reliability of the data produced, and has been advocated by other studies [21,28].

MiR-16 appears to be the most widely used EC for blood-related miRNA studies with application to breast, ovarian, pancreatic, gastric, prostate and renal cell cancer, melanoma and the hematological malignancies [9,14,25,30–34]. Recent studies have reported on the origin of circulating *miR-16*, indicating red blood cell hemolysis as a major source of this miRNA in blood [35,36]. This may be more a concern in studies



EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	* 0.017	0.722	0.526	0.723

EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	0.898	* 0.011	0.361	0.091

EC	U6	miR-16	miR-425	miR-16/miR-425
P-value	* 0.001	* 0.001	* 0.001	*0.001

Figure 7. Effect of candidate EC selection on relative expression of target miRNAs. This figure demonstrates the impact of candidate EC selection on the accuracy of target miRNA expression. The relative expression of three target miRNAs (*miR-93, miR-181a* and *miR-652*) is presented following normalization using each of four distinct EC strategies: *U6* alone, *miR-16* alone, *miR-425* alone and finally *miR-16* and *miR-425* in combination. Interval plots depict mean and 95% confidence intervals for relative *miRNA* expression (Log_{10}) in the blood of women with breast cancer (blue) and healthy controls (orange) normalized to different candidate ECs with p-values indicated in the table below. A. *MiR-93* expression. *MiR-93* appears to be elevated in the circulation of women with breast cancer when *U6* was used to normalise RQ data. However, when the other EC candidates are used for normalization there is no difference in *miR-93* expression between the cancer and control group. B. *MiR-181a* expression. *MiR-181a* is underexpressed with *mIR-16* was used to normalise RQ data (p = 0.011). There is no difference *in miR-181a* expression when other EC candidates are used for normalization. C. *MiR-652*. *MiR-652* is under-expressed in the circulation of women with breast cancer, regardless of the choice of candidate EC (*U6, miR-425* or *miR-16*) indicating that it was highly differentially expressed in blood of those with breast cancer.
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where cell-free blood fragments (serum/plasma) are the source of miRNAs. We utilized whole blood in a disease where it has been previously shown that patient red blood cell and hemoglobin levels are within the normal range in the majority of cases, particularly those with early stage disease [9]. Therefore, as blood samples from both cancer and control patients are treated identically one would not anticipate this to have a direct effect on *miR-16* expression, as evidenced by our results where they was no difference in *miR-16* expression between the cancer and control groups. This issue may be more of a concern, when RNA extraction protocols not utilizing chaotrophic agents such as Trizol are used, implying that individual sample treatment and storage in advance of RNA extraction would directly influence results. There are few reports of *miR-425* in the literature. This is reassuring as it denotes that *miR-425* may be a miRNA with little functional value in disease processes, an attractive trait of an EC. *U6* was selected for further validation by RQ-PCR as although not the most stably expressed EC based on GME or GeNorm analysis of the microarray data, it is commonly used for miRNA studies [11,12,37,38]. *U6* (*RNU6B*) is a small nucleolar RNA (snoRNA) that forms part of the *U6* small nuclear ribonucleoprotein, a component of the spliceosome responsible for splicing of pre-mRNA. The use of *U6* and other snoRNAs in miRNA related research is contentious [39]. These larger molecules are likely to be less reliable than miRNA ECs as their expression is less stable than miRNA with studies showing more frequent degradation in serum samples [40,41]. This makes it difficult to draw conclusions pertaining to miRNA expression when snoRNAs are utilized as ECs. In this study we showed that *U6* was aberrantly expressed in the cancer group compared to the control group (p = 0.009).

This study focuses on RQ-PCR data normalization using candidate ECs which is the most prevalent method. Two alternative normalization strategies for circulating miRNA expression have been proposed to date; Global Mean Expression (GME) and exogenous (spiked-in) miRNAs. GME was recently introduced by Metsdagh *et al* for use in high-throughput miRNA profiling. GME uses the average expression of all the miRNAs detected in a sample as the normalizer presuming that the mean miRNA expression of all miRNAs is constant when the same starting amount of total RNA is used, regardless of the sample type. This technique reduces technical variation and preserves biological variation and is very suited to large genome wide miRNA profiling [19]. It is better suited to large expression profiling studies, with several such studies reporting its use [42–44]. This technique is largely unsuited to biomarker studies as bias may be introduced in such studies when several of the target miRNAs being analyzed show variation in expression (over-expression or under-expression) in one study group compared to another. Spiked-in non-human exogenous miRNAs, such as *cel-miR-39*, *cel-miR-54* and *cel-miR-238*, have also been used for normalization [45–47]. This method presumes that by adding a known quantity of spiked in miRNA to an equal volume of serum/plasma/whole blood, a stable quantity of reference gene is obtained. However, this technique leaves room for technical and human error.

Accurate normalization strategies are crucial for miRNA related research, as detecting even small changes in miRNA expression can have major biological implications, as a single miRNA can target multiple mRNAs, even in the same pathway thus augmenting its effect [48]. In truth, a single universal EC for use

in all specimen types across all diseases, malignant or otherwise, is unlikely to exist. Suitable ECs need to be validated for use in specific disease states and specimen types. The surge of interest in identifying specific miRNAs as biomarkers for health and disease requires that an equal amount of attention is focused on the establishment of suitable ECs with which to normalize the data such that appropriate conclusions can be derived.

Conclusion

This study is of relevance in translational miRNA research for circulating miRNAs in breast cancer. It identifies a combination of two miRNAs, *miR-16* and *miR-425*, with application for use as ECs for normalization. Further investigation into suitable ECs for use in miRNA RQ-PCR studies is warranted.

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Author Contributions

Conceived and designed the experiments: AMD MJK NM. Performed the experiments: AMD. Analyzed the data: AMD. Contributed reagents/materials/analysis tools: MJK NM. Wrote the paper: AMD NM.

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EXPERT REVIEW

The Therapeutic Potential of MicroRNAs: Disease Modulators and Drug Targets

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ABSTRACT MiRNAs are a class of small, naturally occurring RNA molecules that play critical roles in modulating numerous biological pathways by regulating gene expression. The knowledge that miRNA expression is dysregulated in many pathological disease processes, including cancer, has led to a rapidly expanding body of literature as we try to unveil their mechanism of action. Their putative role as oncogenes or tumour suppressor genes presents a wonderful opportunity to provide targeted cancer treatment strategies. Additionally, their documented function in a host of benign diseases broadens the potential market for miRNA-based therapeutics. The present review outlines the underlying rationales for considering mi(cro)RNAs as therapeutic agents or targets. We highlight the potential of manipulating miRNAs for the treatment of many common diseases, particularly cancers. Finally, we summarize the challenges that need to be overcome to fully harness the potential of miRNA-based therapies so they become the next generation of pharmaceutical products.

KEY WORDS miRNA manipulation · miRNA therapeutics · oncomirs · tumor supressors

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ABBREVIATIONS

AGO	argonaute
amiRNA	artificial microRNA
AML	acute myeloid leukemia
AMO	anti-miRNA oligonucleotide
ECM	extracellular matrix
ER	estrogen receptor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HO-1	heme oxygenase-1
HSC	hepatic stellate cells
LNA	locked nucleic acids
MCL	mantle cell lymphoma
miRAGE	miRNA serial analysis of gene expression
miRISC	miRNA-associated RNA-induced silencing complex
miRNA	microRNA
mRNA	messenger RNA
NSCLC	non-small-cell lung cancer
PAMAM	polyamidoamine
PR	progesterone receptor
RAKE	RNA-primed array-based Klenow enzyme
SAGE	serial analysis of gene expression
SERM	selective estrogen receptor modulator
TGFß	transforming growth factor beta
UTR	untranslated region

INTRODUCTION

Mi(cro)RNAs are a class of small non-coding RNA fragments that have captured the attention of the scientific world since their discovery almost two decades ago. They have since been demonstrated to play critical roles in

almost all aspects of the cell cycle, and their expression is known to be dysregulated in various pathological conditions, including carcinogenesis (1). The functional roles of miRNAs in health and disease have been partly elucidated over the last 5 years; this process has unravelled their remarkable potential as disease biomarkers and therapeutic targets (2).

The association of aberrant miRNA expression with almost every cancer and common disease, along with functional analyses of specific miRNAs, has exposed the remarkable potential of manipulating miRNA expression as a therapeutic strategy for these conditions (Table I). The therapeutic application of miRNAs involves various strategies: first, through antisense-mediated inhibition of over-expressed miRNAs; second, through replacement of under-expressed miRNAs with either miRNA mimetics or viral vector-encoded miRNAs; and third, by modulating miRNA expression to augment a patient's response to existing treatment modalities (2).

MIRNA BIOGENESIS

The biogenesis of human miRNA originates in the nucleus, where there is transcription of a large primary (pri-)

Table I MiRNAs Implicated as Therapeutic Targets in Common Diseases

Disease	miRNA	Expression level in disease state	Stage of Investigation *(in vitro/in vivo)*
Hepatitis B virus	miR-122, miR-31	n/a	*In vivo* (21)
Hepatitis C virus	miR-122	n/a	*In vivo* (24,26)
	miR-199a	n/a	*In vitro* (29)
Hepatic fibrosis	miR-27a, miR-27b	Over-expressed	*In vitro* (30)
	miR-29a , miR-29b	Under-expressed	*In vivo* (31)
Hepatocellular carcinoma	miR-122	Under-expressed	*In vitro* (34–36)
Lung cancer (NSCLC)	Let 7 family	Under-expressed	*In vivo* (42–44)
	miR-21	Over-expressed	*In vivo* (47)
Pulmonary arterial hypertension	miR-204	Under-expressed	*In vivo* (48)
Breast cancer:			
Inhibition of metastases	miR-10b	Over-expressed[a]	*In vivo* (54)
	miR-21	Over-expressed	*In vivo* (55)
	miR-1258	Under-expressed	*In vitro* (56)
Breast Cancer:			
Response to adjuvant therapy	miR-21	Over-expressed	*In vivo* (58)
	miR-205	Under-expressed	*In vitro* (59)
	miR-128a	Over-expressed	*In vitro* (60)
	miR-125b	Over-expressed	*In vitro* (61)
	miR-155	Over-expressed	*In vitro* (62)
	miR-34a	Over-expressed	*In vitro* (63)
	miR-342	Under-expressed	*In vitro* (64)
Haematology:			
Leukaemia (B-CLL)	miR-15, miR-16	Under-expressed	*In vitro* (66)
AML	miR-29b	Under-expressed	*In vitro* (69)
Lymphoma	miR-17-92	Over-expressed	Tumour: *In vivo* (71)
	cluster		Radiotherapy: *In vitro* (72)
Prostate cancer	miR-34a	Under-expressed	*In vivo* (73)
	miR-16	Under-expressed	*In vivo* (74)
	miR-143	Under-expressed	*In vitro* (75)
Bladder cancer	miR-203	Under-expressed	*In vivo* (76)
Cardiac hypertrophy induced arrhythmia	miR-1, miR-133	Over-expressed	*In vivo* (77)
	miR-208	Over-expressed	*In vivo* (78)
	miR-100	Over-expressed	*In vitro* (79)
	miR-29	Under-expressed	*In vivo* (80)
Glioblastoma	miR-21	Over-expressed	*In vitro* (105)

[a] conflicting results reported by different studies

miRNA by RNA polymerase II or III. Seventy percent of human miRNAs are transcribed from introns and/or exons, suggesting that regulation of this process is under gene promoter control. The remaining 30% of pri-miRNAs are located in intergenic regions, and so have independent promoters (3,4). Pri-miRNAs are several hundred or thousand nucleotides in length and contain at least one miRNA stem loop. This single unit may contain up to six precursor (pre-) miRNAs, which are produced by the cleaving action of the RNase III enzyme Drosha, combined with the microprocessor complex subunit DGCR8. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a hairpin structure that is critical for their transport to the cell cytoplasm by the energy-dependent Exportin-5 (5). Once in the cytoplasm this hairpin can then be cropped by the RNAase III enzyme Dicer, to produce a double-stranded structure, miRNA:miRNA*, consisting of the miRNA and its complement. This multi-step process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC interacts with its target mRNA and exhibits its cellular effects (6) (Fig. 1).

MIRNA FUNCTION

MiRNAs have been implicated in almost every part of the cell cycle. They exhibit their function by sequence-specific modulation of gene expression at a post-transcriptional level. It is estimated that miRNAs govern over 30% of protein coding genes in this way (7). An understanding of their mechanism of action is crucial for their application in a therapeutic setting. The seed-sequence, the short region of importance in miRNA target recognition, extends from bases 2 to 8 on the 5′ tail of the mature miRNA strand (4). Each miRNA has two possible mechanisms of action, determined by the degree of complementarity between the miRNA seed sequence and its mRNA target, which is governed by Watson and Crick base pairing. First, if the target mRNA and miRISC have perfect base pairing

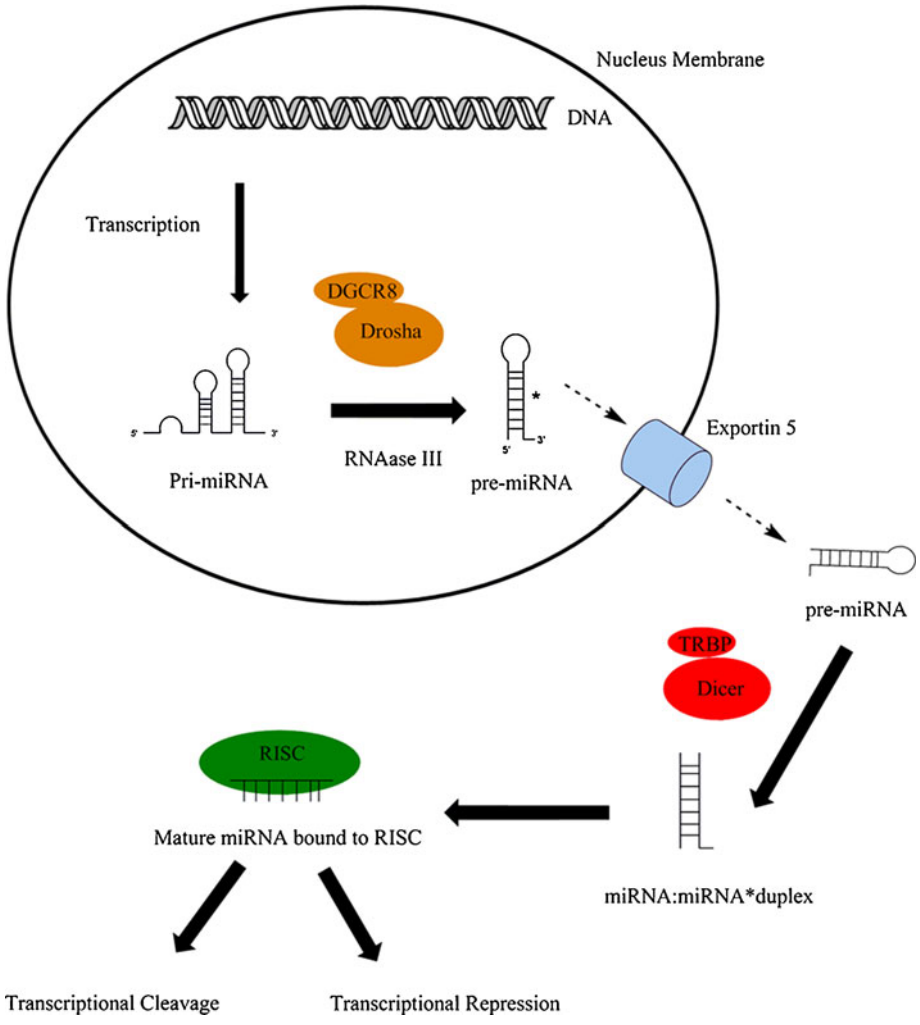

Fig. 1 MiRNA biogenesis and processing. Simplified representation of the steps involved in miRNA biogenesis and processing in human cells. This multi-step process begins in the nucleus of the cell, where there is transcription of a large primary (pri-) miRNA by RNA polymerase II. This large pri-miRNA is then cleaved by the RNase III enzyme Drosha and coupled with the microprocessor complex subunit DGCR8 to produce pre-miRNA. Pre-miRNAs range from 70 to 90 nucleotides in length and contain a stem loop structure for their transport to the cell cytoplasm by Exportin-5 (5). Once in the cytoplasm, this hairpin structure is cropped off by the RNase III enzyme, Dicer, producing the double-stranded miRNA:miRNA* duplex. This process culminates in the mature miRNA strand being incorporated into a miRNA-associated RNA-induced silencing complex (miRISC). It is in this formation that miRISC targets complementary mRNA sequences and exerts its cellular effects, via transcriptional cleavage or transcriptional repression.

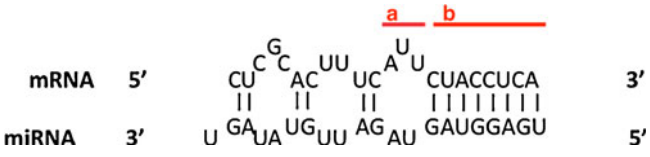

Fig. 2 Typical pattern of base pairing, with imperfect complementarity, between a miRNA and its target mRNA. Typically, the miRNA binds to a specific site or sites within the 3′UTR region of the mRNA sequence. According to thermodynamic analysis, some degree of complex formation occurs along the entire miRNA-mRNA duplexed region. Base pairing is particularly weak in the central region due to mismatched 'bulges' in the miRNA sequence (**a**), and particularly strong at the 5′ end (seed region) of the miRNA (**b**). Base pairing between *let-7* miRNA and *hbl-1* mRNA in *C. elegans* is shown as an example (Lin *et al.,* 2003).

homology, the mRNA is cleaved and degraded through activation of the RNA-mediated interference pathway. Second, and more commonly, miRNAs modulate their gene targets by repression of protein translation. MiRNAs exhibit this effect by imperfectly binding to partially complementary sequences located often in the 3′ untranslated region (UTR) of target mRNAs, although miRNAs can also bind to the coding region and 5′UTR of target genes (8). The proposed mechanism by which imperfect pairing between a miRNA and its target results in translation inhibition or repression is that efficiency of translation is reduced consequent to various mismatched 'bulges' in the central region, or to a lesser extent the 3′end, of the miRNA (Fig. 2). These bulges appear to affect the strength with which the miRNA binds to its mRNA target and can affect the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA (9). Thus far, over 1,000 human miRNAs have been identified (10), each with the capacity to influence several mRNA targets through imperfect base pair homology (Fig. 3).

MIRNA PROFILING AND IDENTIFICATION OF DISEASE-SPECIFIC MIRNAS

MiRNA expression profiling of a variety of human tissues, both healthy and pathological, has given remarkable insight into the developmental stages of many diseases. It has been shown that distinct patterns of miRNA expression are observed in individual tissues and in different disease states. These tissue- and disease-specific expression patterns reflect mechanisms of cellular transformation and further support the idea that miRNA expression patterns encode the developmental history of human disease. In contrast to mRNA expression profiles, it is even possible to successfully classify poorly differentiated tumors using miRNA expression patterns (11,12). A number of different techniques are available for miRNA expression profiling. Oligonucleotide microarray-based miRNA analysis was first described in 2004 and has since become the most commonly used method for detecting cancer-specific miRNA expression profiles involving large numbers of samples (13). Bead-based flow cytometric technology is a highly specific high-throughput method of miRNA expression profiling, devel-

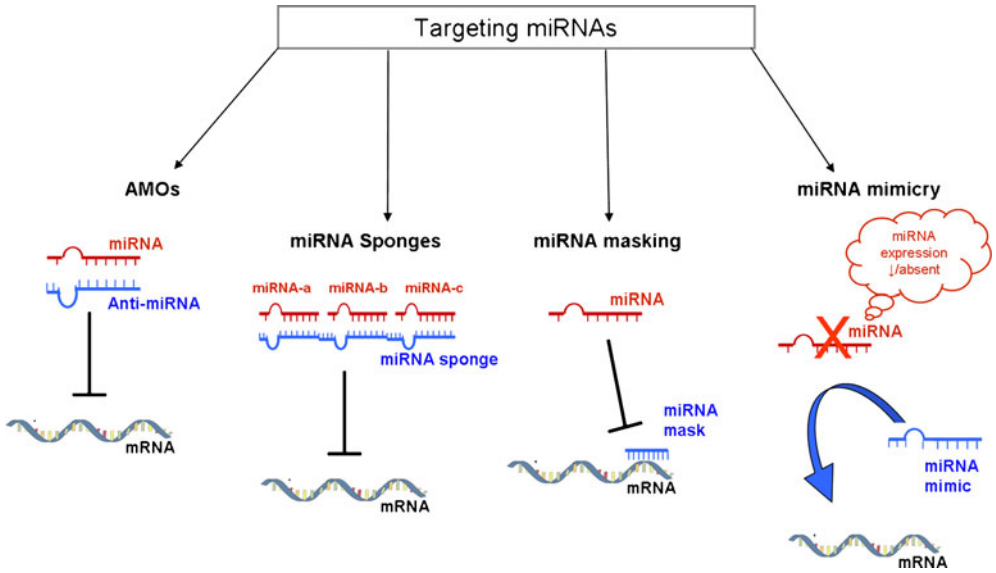

Fig. 3 Strategies of miRNA manipulation and potential miRNA therapeutic strategies. The effects of oncogenic miRNAs can be down-regulated by anti-miRNA AMOs (anti-miRNA oligonucleotide), miRNA sponges, and miRNA-masking. AMOs can bind to complementary miRNAs and induce either duplex formation or miRNA degradation. MiRNA sponges exhibit multiple miRNA binding sites, resulting in the ability to simultaneously sequester multiple miRNAs. MiRNA masks are complementary to the 3′UTR of the target miRNA, resulting in competitive inhibition of the downstream target effects. The downstream effects of tumour suppressor miRNAs can be restored by introducing synthetic miRNAs (miRNA mimicry).

oped by Lu *et al.* (12). While microarray-based miRNA profiling experiments are technically more challenging to perform, bead-based flow cytometry provides a higher specificity. Other technologies in this realm include tag-based sequencing methods such as miRNA serial analysis of gene expression (miRAGE) (14) and the high-throughput RNA-primed array-based Klenow enzyme (RAKE) assay, which is an enzymatic on-chip-labeling technique (15). However, laborious and costly cloning and sequencing steps have limited the use of SAGE, and widespread use of the RAKE assay has been hindered primarily by the fact that a large amount of starting RNA is required (16). More recently, the introduction of platforms that permit large-scale parallel analysis of genome-wide sequences have advanced miRNA identification and analysis even further. Deep sequencing technology is one such platform which enables the simultaneous sequencing of millions of different RNA molecules in a single sample. Deep sequencing overcomes many of the limitations of microarray-based profiling. The latter is susceptible to cross-hybridization and measures only the relative abundance of miRNAs that have already been identified. In contrast, deep sequencing is not dependent on any prior sequence information. Instead, it provides unbiased information about all RNA species in a given sample, thus allowing for discovery of novel and disease-specific miRNAs or other types of small RNAs that have eluded previous cloning and standard sequencing efforts. In conjunction with the evolution of next-generation sequencing technologies, advanced bioinformatic tools have had to evolve simultaneously in order to analyze the massive amounts of data generated (17–20). As these highly sophisticated techniques continue to develop, the extent and significance of miRNA regulation of gene expression will become even more evident. The future of miRNA expression profiling may lie in techniques which can be applied to profile miRNA expression *in vivo*, and not just in archived specimens. Molecular imaging of miRNAs presents a non-invasive method of monitoring miRNA biogenesis and function based on reporter and fluorescent beacon imaging approaches. Molecular imaging is superior to traditional miRNA expression profiling methods, as it can be applied to living cells and provides further insight into potential disease altering miRNAs for consideration in therapeutic modalities (21). This exciting development could be invaluable in the clinical setting, allowing individual response to treatment to be evaluated at a cellular level.

MIRNAS AND THERAPEUTICS

The rapidly expanding body of knowledge on miRNA expression and function is ideal for exploiting as the next generation of disease therapeutics. The fact that these tiny RNA fragments are implicitly involved in many pathological states and that they mediate potent and specific gene silencing makes them attractive therapeutic targets. To date, the greatest efforts in this setting have been in exploring the potential application of miRNA therapeutics for various cancers. In the cancer state, miRNAs have been demonstrated to play a dual role, that of an oncogene or a tumor suppressor. Gain or loss of function of individual miRNAs has been reported in almost every solid and hematological cancer, with pathological roles in tumor cell proliferation, progression of tumors and the metastatic process (1,22). Early *in vitro* work involving miRNA manipulation in cancer cell lines demonstrated the remarkable therapeutic potential of this strategy. A number of different molecular and pharmacological strategies may be employed to help realize this potential.

MiRNAs with oncogenic capacity can be deactivated or silenced by several RNA interference-type strategies, namely miRNA-specific knockdown by anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking. AMOs are synthetic antisense oligonucleotides that competitively inhibit the interaction between miRNAs and their mRNA targets. The most widely employed types of AMOs are 2′-O-methyl AMOs, 2′-O-methoxyethyl AMOs and locked nucleic acids (LNAs) (23). Locked nucleic acids (LNA) are modified oligonucleotides with many advantages over traditional AMOs, including the fact that they do not require a vector and have superior thermal stability and lower toxicity (24). These latter molecules are being utilized in the majority of current *in vivo* studies in this field. As a potential therapeutic approach, however, AMOs have several inherent weaknesses, such as their transient duration of action and inability to target more than one miRNA at a time.

Given that miRNAs have been observed to function not in isolation but often in clusters in pathological processes, knockdown of multiple over-expressed miRNAs presents a therapeutic challenge. The unique concept of 'miRNA sponges' holds great appeal in this context. These competitive miRNA inhibitors are transcripts expressed from strong promoters that display numerous and tandem binding sites for the miRNAs of interest. Sponges, which may be located in non-protein coding RNA or in the 3′-UTR of a reporter gene, are frequently under the control of potent promoters, such as CMV, to ensure large quantities of the transcript are produced (25). Ebert *et al.* demonstrated the efficacy of these miRNA inhibitors *in vitro* by transiently transfecting cultured cells with vectors encoding miRNA sponges. This resulted in a reduction in the level of miRNA targets to at least that attainable with AMOs (12,26). A single sponge bearing a heptameric seed sequence can target families of over-expressed miRNAs which share this seed. In doing so, the sponge can effectively manipulate abnormal expression levels, thereby

preventing their binding with endogenous mRNA targets (26). Drosphilia miR-SP is a dynamic technology that allows transgenic miRNA silencing, with precise *in vivo* spatial resolution (27). This advanced miRNA-sponge technology aims to overcome the lack of tissue specificity associated with traditional miRNA-sponges, while providing insight into interactions between miRNAs and other genes. Transgenic miRNA sponges (miR-SPs) are synthesised by locating modified miRNA complementary oligonucleotides downstream of repetitive upstream activation sequences (UAS).

MiRNA masking is an alternative miRNA knockdown strategy to the AMO approach, with the advantage of targeting miRNAs in a gene-specific manner (28). A miR-mask is synthesized as a single-stranded 2′-O-methyl-modified oligoribonucleotide, which has perfect complementarity to an endogenous miRNA binding site in the 3′ UTR of a protein coding mRNA gene. Unlike an AMO, which binds to the target miRNA directly, a miR-mask binds with high affinity to the target miRNA's binding site in the 3'UTR of its mRNA target. This specific mechanism avoids off-target effects. The miR-mask technology has already been validated *in vivo*, thereby highlighting its potential clinical utility. Using a zebrafish model, Choi *et al.* successfully inhibited the repressive action of *miR-430* on transforming growth factor beta (TGFß) using a miR-mask, which was complementary to the *miR-430* binding site in its target mRNAs *squint* (*sqt*) and *lft2* (29).

With regard to tumor suppressor miRNAs or those with decreased expression in benign disease states, the fundamental principle in miRNA-based treatment strategies is to restore their expression level to normal. This can be achieved through miRNA mimicry or viral vector-encoded miRNA replacement. MiRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs (30), or the precursor pre-miRNA molecules. The viability of this approach has been demonstrated in numerous *in vitro* and *in vivo* settings, the details of which will be discussed later in this review. Gene therapy in the form of viral vectors is another approach for the therapeutic replacement of miRNAs. Adenoviral and lentiviral vectors encoding miRNAs have been investigated as miRNA delivery vehicles in this context, with encouraging results (31,32). In fact, adenoviral vector-encoded miRNA replacement strategies have already been studied *in vivo* (33) and have attracted interest from miRNA therapeutics companies such as Mirna Therapeutics and Asuragen. These studies reported transduction efficiency and minimal toxicity. However, Grimm *et al.* highlighted the potential for serious toxicity to occur with this miRNA replacement strategy. Systemic administration of short RNAs was achieved in adult mice using a delivery vector based on duplex-DNA-containing adeno-associated virus type 8 (AAV8), resulting in down-regulation of critical liver-derived miRNAs, resulting in morbidity and even fatalities (34). The authors postulated that mortality in this instance was consequent to oversaturation of endogenous miRNA pathways. Their experience is important to consider in bringing this strategy from bench to bedside. We will now discuss the rationale and evidence for miRNA therapeutic applications in many common diseases.

LIVER DISEASES

The seminal advances with respect to miRNA therapeutics have been in the field of liver disorders: hepatitis, hepatic fibrosis, and hepatocellular carcinoma (HCC). HCC is one of the most common cancers worldwide and among the leading causes of cancer-related deaths (35). It usually arises in the setting of pre-existing chronic liver disease, which is caused by viral hepatitis (B or C) in 80% of cases worldwide (36). The role of miRNAs in viral hepatic diseases is particularly complex. In addition to miRNA-mediated RNA-silencing pathways influencing viral-host cell interactions (37), viruses not only exploit the hosts cellular miRNAs, but also encode their own miRNAs (38).

Viral Hepatitis B

There is compelling evidence to suggest that miRNAs participate in the development of and host reponse to hepatitis B viral infection (39). Using computational analysis, Jin *et al.* identified that HBV putatively encodes only one candidate pre-miRNA and that viral miRNA only targeted viral mRNA, not host cellular transcripts. The authors proposed that HBV had evolved to use viral miRNAs as a means to regulate its own gene expression to its benefit (40). This hypothesis was confirmed *in vitro* when vector-based artificial miRNA (amiRNA) successfully inhibited HBV replication and expression (41). Ely *et al.* confirmed the *in vivo* viability of this potential therapeutic approach to HBV by employing RNA polymerase II promoter cassettes that transcribes anti-HBV primary miRNA shuttles, specifically pri-mi-122 and pri-mi-31, with a resulting decreases in HBV expression (42).

Viral Hepatitis C

Relative to HBV, there is less evidence to support the involvement of viral miRNAs in the replication of hepatitis C virus (HCV). However, HCV replication appears to be subject to the regulatory miRNAs of the human host cell (39). *MiR-122* was the first liver-specific cellular miRNA identified and constitutes over 70% of miRNAs in the liver. It is known to have two potential binding sites for HCV and enhances the replication of HCV by targeting the viral 5′

non-coding region. Within hepatic tissue, *miR-122* is only detected in the HuH-7 human hepatoma cell line, which is interesting, as HCV can only replicate in these cells. When *miR-122* is inactivated *in vitro* by transfection with 2′-O-methylated RNA oligonucleotide with exact complementarity to *miR-122*, HCV replication in these cells decreases by over 80% (43). This confirms that *miR-122* plays an important direct role in HCV translation by targeting the 5′ untranslated region and enhancing the association of ribosomes at an early stage (44). Krutzfeld *et al.* provided the first report of successful miRNA antagonism *in vivo* when *antagomir-122* was conjugated with cholesterol and delivered intravenously, resulting in *miR-122* knockdown for 23 days (45). However, this method of antagmiR delivery, employing synthetic 2′-O-methyl anti-miRNA oligonucleotides (AMOs), raises concerns regarding their stability and toxicity. Locked nucleic acid (LNA)-modified oligonucleotides, as mentioned, present significant advantages for sequence-specific antagonism of miRNAs; they display advanced thermal stability when combined with their target RNA and have a low toxicity profile in mammals (24,46). The use of LNAs *in vivo* as a mechanism of delivering miRNAs for therapeutic purposes was demonstrated by Elmen *et al.* In a murine model, this group delivered unconjugated LNA-antimiR oligonucleotide complementary to the 5′end of *miR-122,* and observed specific dose-dependent *miR-122* silencing without hepatotoxicity (47). It has also been documented that *miR-122* is an indirect facilitator of HCV replication; Heme Oxygenase-1 (HO-1) is capable of inhibiting HCV replication, and *miR-122* down-regulates this pathway. The combination of *miR-122* down-regulation, with up-regulation of HO-1, is a potential new strategy for antiviral therapies directed towards HCV (48).

MiR-199a is another liver-specific miRNA that has been associated with HCV replication. *In vitro* studies have demonstrated that over-expression of *miR-199a* results in inhibition of HCV replication, independent of the interferon pathway, while inactivation of *miR-199a* induces accelerated viral replication (49).

Hepatic Fibrosis

Liver fibrosis is a largely irreversible condition that occurs in association with most chronic liver diseases. Hepatic stellate cells (HSCs) become activated in response to repeated injury and exposure to inflammatory mediators. They subsequently lose their lipid droplets and migrate to the injured area, where they secrete large amounts of extracellular matrix (ECM), resulting in fibrosis (50). This process can result from chronic hepatitis, and ultimately leads to liver cirrhosis and potentially hepatocellular carcinoma. Many miRNAs have been implicated in the pathogenesis of hepatic fibrosis. *MiR-27a* and *miR-27b* have recently been studied in rat HSCs *in vitro*. They are normally over-expressed in the inflammatory state, and down-regulation of both miRNAs resulted in the HSCs returning to a more quiescent state, with decreased proliferation and restored lipid droplets (51). *MiR-29a* and *miR-29b* are also of interest in the setting of hepatic fibrosis. A recent microarray conducted on murine livers identified the *miR-29* family as being significantly down-regulated in fibrotic liver tissue (52). In this elegant study by Roderburg *et al.*, *miR-29* was shown to play a regulatory role in pathways involving the genes TGF-β and NF-κβ. The authors also found that over-expression of *miR-29b* resulted in down-regulation of collagen expression in murine HSCs (52). These data illustrate the future potential for *miR-29b* as an antifibrotic agent.

Hepatocellular Carcinoma

MiR-122 is one of the most extensively investigated miRNAs; it is now known that its function extends far beyond virus replication and infection of the liver. Computational tools and *in vitro* expression data suggest that *miR-122* also has a role in the cellular stress response (53) and hepatocellular carcinogenesis (54). Converse to the major positive role of *miR-122* in HCV replication, it has a negative role in hepatic tumorigenesis and in fact is a tumor suppressor in the liver. MiRNA expression profiling has revealed that *miR-122* is down-regulated by at least 50% in human HCC tissue compared to normal or non-cirrhotic liver.

Transfection of HCC cell lines with *miR-122* has been shown to induce cellular apoptosis and reduce cell viability (55,56). This presents a novel chemotherapeutic strategy in HCC, a disease with a typically poor prognosis for which there are limited treatment options. An increase in *miR-122* expression in malignant cells could result in targeted cell death. Young *et al.* devised a mechanism to test this theory by developing small molecule modifiers of *miR-122* function (57). These miRNA modifiers (1–3) act at the transcriptional level to either up- or down-regulate *miR-122* expression. More specifically, the authors observed that small molecule *miR-122* inhibitor 2 inhibited HCV replication, while small molecule *miR-122* inhibitor 3 induced an increased expression of the pro-apoptotic *miR-122* in the HCC cell line HepG2, leading to *caspase* activation and reduced cell viability. This study highlights the remarkable potential of miRNA manipulation as a plausible novel therapeutic strategy.

LUNG DISEASES

Lung cancer is the leading cause of cancer death worldwide, with non-small-cell lung cancer (NSCLC) accounting

for 80–85% of lung cancer cases. To date, over 40 miRNAs are known to be dysregulated in NSCLC. Various miRNA signatures, derived from lung tumor tissue or even plasma/serum samples, have been proposed as biomarkers of this disease with utility in diagnosis and prediction of overall survival (58,59). With regard to miRNA therapeutic strategies for lung cancer, much of the work to date has focused on harnessing the tumor suppressor properties of the *let-7* family for this purpose. *Let-7* is stably expressed in normal adult lung; however, expression profiling of NSCLCs has revealed that various members of the *let-7* family are decreased in tumor tissue compared to normal lung. *Let-7a*, among other miRNAs, has been shown to have prognostic value in that low levels correlate with poor survival (60). Functional work has identified and defined the regulatory roles of the *Let-7* family in several oncogenic pathways, including the RAS pathway, where it represses activity of the *KRAS* oncogene, mutations of which are commonly implicated in adenocarcinoma of the lung (61,62).

Esquela-Kerscher and Slack *et al.* pioneered many of the early *in vitro* and *in vivo* investigations into the role of let-7 miRNAs in NSCLC. They identified that the tumor suppressor effect of *let-7* was transient and that replacement of let-7 through gain-of-function techniques could reduce cell proliferation in various human lung cancer cell lines. In a murine model of human lung cancer, this group demonstrated how loss of *let-7* induced lung tumor formation and growth, through loss of its regulatory effect on the oncogenes *RAS* and *HMGA2* amongst others. Restoration of *let-7* expression in lung cancers, using intranasal delivery techniques, restrained the growth of tumors by repressing multiple cell cycle and proliferation pathways together with *ras* and *MYC* suppression (63,64). This work paved the way for further investigations into the therapeutic feasibility of miRNAs in the clinical treatment of lung cancer.

Although promising, the application of *let-7* as a therapeutic agent for cancer is premature as yet, given that details of the immunogenic and cytotoxic effect of *let-7* administration remain to be determined. Its ubiquitous expression and involvement in multiple cellular pathways imply that manipulation of its levels is likely to have diverse off-target effects. The development of safe, effective, and tissue-specific delivery methods for *let-7* requires further effort before this strategy advances as a cancer therapy.

Kumar *et al.* have demonstrated similar tumor suppressor effects of the *let-7* miRNAs on lung cancers *in vivo* (65). Using a lentiviral system, they first transfected murine *KRAS*-expressing lung adenocarcinoma cells (LKR 13) with a *let-7 g* miRNA duplex; this resulted in decreased cell proliferation and induction of cell death. In tumor xenografts, the authors observed significant reduction of both murine and human non-small-cell lung tumors when *let-7 g* was over-expressed using these lentiviral vectors. Furthermore, they found that *let-7 g*-mediated tumor suppression was more pronounced in lung cancer cell lines harboring oncogenic K-Ras mutations than in lines with other mutations. The potential of *let-7* in the treatment of lung cancer extends beyond its direct effects on the tumor. Preliminary *in vitro* data suggest that there is potential to use miRNA modulation to enhance standard treatments for cancer, such as radiotherapy for lung tumors. Weidhaas *et al.* provide evidence that over-expressing members of the *let-7* family in lung cancer cells and in a *C. elegans* model of radiation-induced cell death results in increased sensitivity to radiation therapy, whereas decreasing *let-7* levels induces a state of radioresistance (66). These effects were mediated through altered *RAS* signalling.

The potential role of miRNAs in treating lung disorders is not confined to *let-7*. Blower *et al.* showed that altering expression levels of *let-7i, miR-16* and *miR-21* in a lung cancer cell line (A549) altered the potency of chemotherapeutic agents up to four-fold (67). Manipulation of the oncogenic *miR-21* in NSCLC has also been investigated as a possible therapeutic strategy. Using transgenic mice, Hatley *et al.* demonstrated that over-expression of *miR-21* was associated with cell proliferation and tumor growth, whilst genetic deletion of *miR-21* partially protected against tumor formation (68). Inhibiting *miR-21* increased tumor sensitivity to DNA-damaging chemotherapeutic agents and could potentially restore the activity of multiple tumor suppressors acting at various critical points of tumorigenesis.

Pulmonary arterial hypertension has also been associated with aberrant miRNA expression and function. Decreased *miR-204* levels in affected lungs have been shown to correlate with disease severity in both animal and human studies (69). Targeted delivery of synthetic *miR-204* to the lungs of affected animals resulted in a significant reduction in disease severity. This illustrates another potential application of miRNA therapeutics.

BREAST CANCER

Since Iorio *et al.* first reported dysregulated miRNA expression in breast tumors in 2005, evidence has accumulated implicating miRNAs as key players in breast tumorigenesis, progression, and metastases and in determining tumor response to existing treatments (70,71). As in other cancers, miRNAs play dual roles as oncogenes or tumor suppressors in this prevalent disease. In a therapeutic capacity, there have been two predominant objectives and approaches to manipulating miRNA expression in breast tumors thus far: knockdown of candidate breast cancer-related 'oncomirs' to suppress tumor growth and inhibit or prevent distant metastases, and modulation of miRNA expression with the

intent of augmenting or altering tumor responsiveness to adjuvant chemotherapeutic or hormonal agents.

'OncomiR' Knockdown

It is widely accepted that metastases are responsible for most cancer-related deaths. However, targeting or interrupting the metastatic process with therapeutics has been largely unsuccessful as a result of our limited understanding of this pathological process (72). Recent endeavours to explore the role of miRNAs in the metastatic cascade have identified potentially key pathways in this process and novel therapeutic targets. Accumulating data have proven that miRNAs exert their effects at multiple steps in the metastatic cascade by influencing cancer cell adherence, migration, invasion, motility, and angiogenesis (73). In their miRNA microarray analysis of paired tumor tissues and metastatic lymph nodes, Baffa *et al.* identified a metastatic cancer miRNA signature inclusive of *miR-10b*, *miR-21*, *miR-30a*, *miR-30e*, *miR-125b*, *miR-141*, *miR-200b*, *miR-200c*, and *miR-205*. *MiR-10b* is implicated in many cancers, including breast cancer, and is thought to promote tumor invasion and metastasis by inhibiting translation of the *HOXD10* gene, thereby resulting in increased expression of the pro-metastatic gene, *RHOC* (74). Weinberg's group has also recently reported exciting findings from work on antagonizing *miR-10b* in metastatic breast cancer cell lines (MDA-MB-231 cells). Silencing *miR-10b* with antisense oligonucleotides was found to inhibit *Twist*-mediated cell migration and invasion. They observed similar anti-metastatic effects after systemic *miR-10b* antagonism in a murine model (75).

Numerous other miRNAs have been implicated in the metastatic pathway. Yan *et al.* performed *in vitro* LNA silencing of *miR-21* in two breast cancer cell lines (MCF-7 and MDA-MB-231), which resulted in significantly reduced cell proliferation and migration. Their subsequent *in vivo* studies resulted in similar inhibition of breast tumor growth following *miR-21* knockdown with antimiRs (76). Zhang *et al.* have been first to report that *miR-1258* inhibits breast cancer brain metastases by negatively regulating the heparanase pathway (77). Again, these results strongly support the potential of miRNAs to be applied to the clinical setting for therapeutic gain.

Augmenting Response to Adjuvant Therapy

Chemotherapeutic drugs, radiotherapy and endocrine agents (aromatase inhibitors and selective oestrogen receptor modulators, SERMs) are the adjuvant therapies used in the routine management of women with breast cancer at present. Despite their success in improving disease-free and/or overall survival, a proportion of women derive no benefit from these treatments or develop resistance to these agents over time (78). The basal subtype of breast cancer (classically ER, PR and HER2/*neu* negative) presents a specific therapeutic challenge, as there are no targeted therapies currently available. Preliminary studies suggest that miRNA modulation in tumor tissue can augment its response to systemic therapies. *MiR-21* is again one of the most studied miRNAs in this setting. Mei *et al.* combined taxol chemotherapy with *miR-21* inhibitor treatment, via a polyamidoamine (PAMAM) dendrimer vector, to evaluate the effects of combination therapy on suppression of breast cancer cells and found that cells treated with this combination demonstrated significantly reduced cell viability and invasiveness compared with cells treated with taxol alone, reflecting an enhanced chemotherapeutic effect of taxol in the presence of decreased *miR-21* levels (79).

There is also experimental evidence that manipulation of *miR-205* levels can improve breast tumors' response to anticancer agents. Based on computational target prediction algorithms, Iorio *et al.* hypothesised that *miR-205* was involved in regulation of the HER3 receptor, a kinase-inactive member of the HER family which plays an important and necessary function in HER2-mediated tumorigenesis. Indeed, their *in vitro* experiments demonstrated that *miR-205*, which is down-regulated in breast tumors compared with normal breast tissue, directly targeted the HER3 receptor and inhibited activation of the downstream mediator Akt (80). Furthermore, reintroduction of *miR-205* in SKBr3 cells was found to inhibit their clonogenic potential and increase the responsiveness of these cells to the tyrosine-kinase inhibitors Gefitinib and Lapatinib, thus overcoming HER3-mediated resistance and restoring proapoptotic activity.

Manipulation of several other miRNAs has been shown to have the potential to augment breast tumors' responsiveness to existing therapies. Inhibition of endogenous *miR-128a*, which is highly expressed in letrozole-resistant breast tumors, overcomes resistance to the aromatase inhibitor letrozole by modulating TGFß signalling (81). *MiR-125b*, *miR-155*, and *miR-342* have also been implicated in regulating chemosensitivity, whilst knockdown of *miR-34a* is associated with increasing cancer cells sensitivity to radiation (82–85).

HEMATOLOGY

Much of the initial data on miRNA expression profiling and function stemmed from studies of hematological malignancies. However, few experiments testing the therapeutic potential of miRNAs in this setting have been conducted.

Leukemia

The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced

deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia (86). Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene *BCL2* (87). *BCL2* inhibition through replacement of these deficient miRNAs is therefore a plausible therapeutic strategy. Similar potential exists for miRNA-based therapeutics in the management of acute myeloid leukemia (AML), for which distinctive patterns of aberrant miRNA expression have been identified (88). Eyholzer *et al.* demonstrated that *miR-29b* expression is decreased in AML patients displaying either CEBPA deficiency or loss of chromosome 7q (89). Data from Calin and Croce's laboratory showed that restoration of *miR-29b* in AML cell lines and primary bone marrow or peripheral blood samples induced apoptosis and dramatically reduced tumorigenicity in a xenograft leukemia model (90).

Lymphoma

The *miR-17-92* cluster is located in a region frequently amplified in B-cell lymphoma (91). This cluster, located at chromosome 13q31-q32, is comprised of seven individual miRNAs that are transcribed as a polycistronic unit (*miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a,* and *miR-92*). He *et al.* clearly illustrated the oncogenic activity *of miR-17-92,* by demonstrating accelerated tumor development and reduced Myc-induced apoptosis in a Eu-Myc transgenic mouse model of human B-cell lymphoma (92). In theory, modulating this cluster of miRNAs could inhibit lymphoma progression. Targeting the *miR-17-92* cluster could also be used to augment response to radiotherapy in human mantle cell lymphoma (MCL). MCL cells over-expressing *miR-17-92* display increased cell survival and reduced cell death following radiotherapy. Knockdown of this miRNA cluster could increase the radiosensitivity of MCL cells, thereby improving prognosis for these patients (93). The miRNA sponge concept would be an ideal therapeutic strategy in this setting, as a single sponge molecule could target the entire miRNA cluster simultaneously.

MIRNA THERAPEUTICS IN OTHER COMMON CONDITIONS

In addition to cancers, miRNAs are known to be dysregulated in a wide range of other disease processes. From a therapeutic perspective, the most promising applications at present for miRNA-based treatments are in the settings of urological, cardiovascular and neurological diseases.

MiR-34a is known to be under-expressed in prostate cancer cells, which exhibit advanced proliferation and metastatic potential and express the adhesion molecule CD44. Liu *et al.* demonstrated that increasing the expression of *miR-34a* in these CD44(+) prostate cancer cells suppressed tumor progression and metastases and resulted in increased survival in a mouse model. Furthermore, inhibition of *miR-34a* in CD44(−) prostate cancer cells by administering *miR-34a* antagomiR contributed to increased tumor burden and metastases (94). This study provides evidence to support the suggestion that the negative regulatory effect of *miR-34a* on CD44 could be exploited for therapeutic benefits in prostate cancer. Several other miRNAs have been implicated as inhibitors of the metastatic process in prostate cancer, including *miR-16* and *miR-143* (95,96). Bladder cancer is also associated with aberrant expression of miRNAs, such as *miR-203,* which represent ideal therapeutic targets. *In vitro* data have identified pro-apoptotic effects of *miR-203* on bladder cancer cells through its down-regulatory effect on bcl-w, implying that gain-of-function modulation with *miR-203* mimetics has potential utility in the treatment of this malignancy (97).

Altered miRNA expression has also been demonstrated in various cardiovascular diseases, including heart failure, arrhythmias, and fibrosis, unveiling further opportunities for miRNA-targeted therapies. Cardiac hypertrophy and its associated arrhythmias may be suppressed by *miR-1* and *miR-133* over-expression through post-transcriptional repression of HCN2 and HCN4 genes (98). *MiR-208a* and *miR-100* have also been implicated as a modulator of cardiac hypertrophy and electrical conduction (99,100). Myocardial infarction can be complicated by fibrin deposition in the damaged muscular wall, the adverse consequences of which include stiffening of the ventricular walls, diminished contractility, and abnormalities in cardiac conductance. Increasing the expression of *miR-29b* in cardiac fibroblasts has been shown to decrease the expression of collagen transcripts in these cells, hence reducing collagen production (101). This knowledge highlights *miR-29b* as a potential therapeutic agent for fibrotic diseases.

An important role of miRNAs in neurological conditions has also been identified. This work has stimulated the expectation that miRNAs hold potential as therapeutic agents for the treatment of debilitating neurodegenerative conditions such as Huntington's, Parkinson's, and Alzheimer's diseases, for which no disease-modifying treatment strategies exist currently (102). Animal model studies have shown that loss of neural miRNAs may be involved in the development and progression of these neurodegenerative diseases. *In vitro* experiments provide further support for miRNAs as therapeutic agents in these conditions; they have been shown to partially preserve miRNA-deficient neurons when over-expressed in these cells. A serious limitation of many novel drugs in this setting to date has been their inability to cross the blood-

brain barrier. Indeed, this will remain one of the major challenges in developing miRNA-based therapeutic strategies for neurological diseases.

POTENTIAL CHALLENGES

The rush to identify novel miRNAs with a role in specific disease processes continues in an effort to expedite the transition of miRNA-based therapeutics from bench to bedside. While significant advances have been made in this field to date, various challenges remain to be overcome before miRNA therapies become a reality. The development of therapeutic strategies involving disease-specific miRNAs is subject to identification and validation of their multiple mRNA targets and to elucidating the complex pathways which they partly or wholly regulate. A significant amount of functional work remains to be performed in order to achieve this. Thus far, predicting gene targets of miRNAs has been largely computationally governed, and miRNA targets are predicted by sequence complementarity rather than in a gene-specific manner. This complex approach to target identification, along with the fact that individual miRNAs have multiple potential targets, leads to difficulty in predicting the spectrum of side effects and toxicity profiles which may be associated with miRNA-based therapeutics. Only *in vivo* investigations followed by carefully designed early phase clinical studies will identify these issues and help overcome them.

Another obstacle which must be surmounted before miRNA-based therapies become a reality is the issue of site-specific, safe, and effective delivery. The two main approaches at present for delivering miRNA therapies to target tissues, direct delivery of miRNA mimics or antigomirs and viral vector-encoded miRNA delivery, have specific limitations. The direct delivery approach, made possible by conjugating the oligonucleotide to cholesterol or coating it with liposomes or polycationic agents, avoids the immunogenic safety issues intrinsic to viral vector delivery. But it is challenged by the need for repeated dose delivery to achieve therapeutic effect. This becomes a critical issue if the route of delivery necessitates an invasive procedure. The viral vector-encoded miRNA delivery approach has the potential to simplify the delivery of multiple miRNA mimics/antagomirs in a single dose, due to the small size of the miRNA coding sequence, but is limited by its potential to trigger a host immune response, transient expression, and poor integration into the host genome (103,104). Future work must focus on developing more efficient delivery systems which minimize the number of healthy cells exposed to these therapies, promote good uptake/integration into the target tissue, and reduce the potential for off-target effects.

CONCLUSION

MiRNAs and their role in disease processes is a rapidly evolving field, as evidenced by the increasing body of literature. Their intricate involvement in the pathogenesis of many common diseases, including cancers, makes them ideal candidates for novel therapeutic strategies. In this review, we have highlighted the ways in which miRNAs can be used as disease modulators and drug targets. We have detailed the evidence to date that specific miRNAs can be associated with and manipulated for the treatment of liver disorders, diseases of the lung, breast cancer, and hematological malignancies. Given the increasing global interest in miRNAs, coupled with advances in molecular biochemistry and pharma technologies, miRNA-based therapeutic strategies look set to become the next generation of individualised targeted therapy adopted by the pharmaceutical and medical fields.

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CHAPTER 13

MiRNAs AS POTENTIAL THERAPEUTIC TARGETS IN CANCER

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ABSTRACT

Aberrant mi(cro)RNA expression is associated with most pathological disease processes, including carcinogenesis. The knowledge that miRNAs have dual roles, as oncogenes or tumour suppressors, unveils their remarkable potential in novel cancer therapeutics. Additionally, the ability of miRNA expression profiles to classify tumours according to clinico-pathological variables highlights their potential as cancer biomarkers. This may contribute to improved patient selection for adjuvant therapies and help monitor response to treatment. MiRNAs may be exploited as therapeutic agents in various ways: firstly through antisense-mediated inhibition of over-expressed miRNAs, secondly through 'replacement' of under-expressed miRNAs with either miRNA mimetics or viral vector-encoded miRNAs, and thirdly by modulating miRNA expression to augment a patient's response to

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existing treatments. This chapter will outline the specific mechanisms and applications for each of these therapeutic strategies. We will also outline the progress achieved to date in this field, and the challenges that remain to be addressed before miRNA-based therapies become the next generation of cancer treatments.

1. INTRODUCTION

Mi(cro)RNAs are a class of small, non-coding RNA fragments that play important roles in most biological processes by regulating gene expression. They were first discovered almost 20 years ago, and have since become the focus of much scientific and translational research. MiRNAs are known to play functional roles in both the normal and pathological state. Aberrant miRNA expression has been described in several pathological processes, including carcinogenesis (Calin and Croce 2006). Indeed, investigation into their altered expression in cancer unveiled their dual roles, as oncogenes and tumour suppressor genes. MiRNAs have been identified as potential novel therapeutic targets, or agents, to employ in cancer treatment strategies.

MiRNA expression profiles can classify tumours by type or clinico-pathological characteristics, which has potential utility in cancer diagnostic, prognostic and predictive settings. This would permit more appropriate treatment selection, allow close surveillance of response to treatment and even spare those patients who have early disease or will not respond to systemic treatment, from the toxicities associated with adjuvant chemotherapy.

MiRNAs may be exploited as potential therapeutic targets in cancer by several approaches. Firstly, depleted miRNAs may be replaced by either miRNA mimetics or viral-vector encoded miRNAs; secondly, over-expressed miRNAs can be inhibited by miRNA masking, miRNA sponges or anti-miRNA oligonucleotides (AMOs) and thirdly, patient response to current treatment modalities can be enhanced by altering miRNA expression (Heneghan et al. 2010).

2. miRNA FUNCTION AND MECHANISMS OF ACTION

MiRNAs have been demonstrated to play key roles in almost every aspect of the cell cycle. A mature miRNA strand is incorporated into a miRNA-associated RNA-induced silencing complex (miRISC) (Lowery et al. 2008). It is in this arrangement that a specific miRNA interacts with its target mRNA and exhibits its function at a post-transcriptional level. Understanding the mechanism of miRNA sequence-specific modulation is critical in realizing their application as therapeutic targets in the cancer setting. The ‘seed

sequence', the important region on a miRNA for target recognition, is located on the five prime tail of the miRNA extending from bases two to eight (Bartel 2004). There are two potential mechanisms of action for each miRNA, determined by the degree of base-pair complementarity between the seed sequence of the miRNA and its mRNA target. If there is perfect complementarity, the RNA-mediated interface pathway is activated and the mRNA is cleaved. However, more commonly, there is imperfect base pair matching which results in repression of protein translation. The miRNA seed sequence binds with partial complementarity to sequences frequently located in the three prime untranstranslated region (UTR) of the mRNA target. It is worth noting however, that miRNAs are also capable of binding to the five prime UTR and the coding region of mRNAs (Jackson and Standart 2007). The mechanism of action responsible for translational repression as a consequence of imperfect base pairing has been proposed by Filipowicz et al. (Filipowicz et al. 2008). The efficacy of translation is decreased as a result of 'bulges' which are created in the central region (less commonly the three prime UTR) due to miss-matching of the base pairs. These miss-matched bulges can have an effect on cleavage of mRNA that is Argonaute (AGO)-mediated. To date, over 1,500 human miRNAs have been described. Each individual miRNA has the ability to bind with several mRNA targets, and exert a multitude of effects through imperfect base pairing. In this way, it is estimated that miRNAs govern in excess of 30% of protein coding genes (Miranda et al. 2006). This highlights the potential influence of miRNAs on practically every biological pathway. These potent regulatory molecules are involved in differentiation, apoptosis, proliferation, and cell-fate determination (Mann et al. 2010).

3. THE ROLE OF miRNAs IN CANCER

MiRNAs function at the initiation, promotion and metastatic stages of many malignancies. They are intricately involved in tumourigenesis and so, the recent ability to manipulate miRNAs has become an appealing anti-cancer treatment strategy. In the cancer state, there is a large body of evidence demonstrating how miRNAs function as oncogenes and tumour suppressor genes. Interestingly, a single miRNA can act dually, as both an oncogene and a tumour suppressor gene, depending on the specific cancer type. For example, *miR-125b* is known to have varying expression levels in different tumours; down-regulation is observed in anaplastic thyroid and serous ovarian carcinoma, indicating a potential tumour suppressor role (Nam et al. 2008, Visone et al. 2007) while oncogenic properties (over-expression) are noted in prostate cancer (Ozen et al. 2008). This is an important concept, as it highlights how each cancer must be considered a distinct disease entity when developing cancer-specific miRNA-based therapeutic strategies.

4. miRNA IDENTIFICATION AND PROFILING IN CANCER

Cancer comprises a heterogeneous group of complex diseases, several of which share fundamental pathological processes. Our improved understanding of miRNA function has allowed us to better understand the complexity of human malignancies, particularly the pathways involved in tumour initiation and progression. Indeed miRNA expression profiling of a range of human tissues, both normal and pathological, enhances our understanding of the developmental stages of several cancers. Specific profiles of dysregulated miRNAs can be created for almost every type of tumour. The use of miRNA expression profiles has several advantages over their predecessors, mRNA profiles, predominantly in permitting the classification of poorly differentiated tumours (Lu et al. 2005, Volinia et al. 2006). Unique miRNA signatures highlight the potential of miRNAs as cancer-specific diagnostic biomarkers, and also unveil the potential therapeutic strategy of restoring the miRNA expression profile to normal as an anti-cancer treatment approach. There are a range of different techniques available for high-throughput miRNA expression profiling. Here we describe some of the most common high-throughput miRNA profiling techniques.

4.1 Oligonucleotide miRNA-Microarray

Oligonucleotide based miRNA-microarray analysis was first described in 2004 (Liu et al. 2004). It the most commonly used method for genome-wide assessment of miRNA expression in human cancers, particularly in large studies. The microarray chip is printed with the sense strand, the miRNA gene specific oligonucleotide probe on the microarray hybridizes with labelled cDNA of the miRNA targets. The cDNA is synthesized by reverse transcription. The results are obtained after staining with Streptavidin-Alexa 647 conjugates and detected by laser scanning. This method is easily standardized and can be applied reliably to a large sample size. It is also cheaper to perform than some of the more novel approaches, permitting simultaneous analysis of over 300 miRNAs. However, drawbacks include a lack of specificity in detecting structurally similar miRNAs.

4.2 Bead-based Flow Cytometric Technology

Bead-based flow cytometric technology is a highly specific method of miRNA expression profiling. The technique was first described by Lu et al. in 2005 (Lu et al. 2005). Polystyrene beads are coated with antisense

oligonucleotide probes which hybridize with biotin-labelled PCR amplicon dsDNA (target). Staining for streptavidin–phycoerythrin is performed before results are obtained from signal detection using flow cytometry. Bead-based technologies are technically more challenging to perform, allowing only small numbers of miRNAs to be studied at a time. In addition it has been suggested that bias may be introduced to this technique; the small RNA sample must be enriched prior to commencing by fractionation. Bias could also be introduced during PCR. However, this method permits higher miRNA specificity than oligonucleotide-based microarrays.

4.3 Tag-based Sequencing Methods

Other technologies in this field include the tag-based sequencing methods; miRNA serial analysis of gene expression (miRAGE or SAGE) (Cummins et al. 2006) and RNA-primed array-based Klenow enzyme (RAKE) assay (Nelson et al. 2004). SAGE involves the serial analysis of miRNA expression by small RNA purification, tagging and cloning. However its widespread use is restricted due to these laborious and expensive steps. The RAKE assay includes on-slide application of the Klenow fragment of DNA polymerase I. This assay is sensitive and specific; additionally it permits the discovery of novel miRNAs. However it is also labour intensive and requires a large amount of RNA.

4.4 Deep Sequencing Technology

Recent advances in scientific technology permit genome wide sequence analysis; deep sequence technology is one such large-scale platform. The application of this technology permits the simultaneous sequencing of millions of sequences on a single sample. Deep sequencing is free from many of the limitations confronted by other methods; there is no cross-hybridization and no prior sequence knowledge is required. Deep sequencing can be considered 'unbiased' in this regard and is an excellent choice for diseases in which molecular profiling has previously been unfruitful. The major difficulty associated with this method of miRNA expression profiling is the large amount of data that confronts the researcher. Advances in bioinformatic tools have permitted informative results to be deduced from the data produced during such experiments (Friedlander et al. 2008, Hackenberg et al. Wang et al. 2009, Yang et al. 2009). Indeed, some researchers consider this complex data analysis and large numbers of generated sequences to be a disadvantage associated with deep-sequencing. In addition, this method is expensive to perform.

4.5 The Future of miRNA Expression Profiling Techniques

The techniques discussed above can only be applied to samples or specimens that have been archived, as cell lysis or fixation is required. However, it is well known that miRNA expression patterns are likely to be transient, and reflect the host or disease micro-environment at that particular time. It could be that a miRNA profile of a specific cancer state represents a stage in the natural history of tumour development, constantly evolving with tumour progression. The next step in miRNA expression profiling would be to devise profiling techniques which could be applied *in vivo*. Molecular imaging is one such technique which permits the evaluation of the dynamic functioning of miRNAs within living cells. This remarkable advance supports repeated quantitative imaging of tumour and stem cells. Molecular imaging is superior in this regard as it permits remarkable insight into miRNAs that are dysregulated in carcinogenesis for consideration as novel treatment strategies.

The high-throughput technologies outlined above aid in the identification of novel disease-specific miRNA targets and miRNA signatures for diagnostic and therapeutic approaches. It is, however, necessary to validate these findings using additional techniques. The most commonly employed validation methods are northern blotting and quantitative real time polymerase chain reaction (qRT-PCR). Northern blotting requires large amounts of total RNA, is time consuming and requires handling of radioactive material (if conducted according to standard protocol). RT-PCR requires less RNA, is a simple technique to perform, and is more commonly employed in validation experiments.

5. miRNA AS THERAPEUTIC TARGETS IN CANCER

MiRNAs are rapidly becoming the next generation of disease-specific therapeutics. There is an increasing body of knowledge and experience in miRNA profiling and functional analyses, particularly in cancer. These tiny RNA molecules are critical to almost every aspect of the cell cycle, and appear to be key players in carcinogenesis in which they mediate specific gene silencing. The ability to safely manipulate specific miRNA expression levels as a treatment modality, and in doing so to change the natural history of a cancer, would revolutionize both the pharmacologic and medical fields.

Given the dual functionality of miRNAs in cancer, it stands to reason that a tumour's growth could potentially be halted or even reversed, by returning the disease-specific dysregulated miRNA to 'normal' expression levels. This could be achieved through replacement of down-regulated miRNAs, or knock-down/inactivation of miRNAs which are over-

expressed. Several molecular and pharmacological approaches have been developed as potential treatment strategies.

5.1 Potential Treatment Strategies for Oncogenic miRNAs

Over-expressed 'oncogenic' miRNAs need to be knocked-down or deactivated in order to restore their normal expression level and reverse the mRNA inhibition that results from these aberrantly expressed miRNAs. Several strategies of RNA interference exist, some of which were originally developed for mRNA silencing but have been tailored for miRNA manipulation. The main approaches include anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking.

5.1.1 Anti-miRNA oligonucleotides (AMOs)

Anti-miRNA oligonucleotides are synthetic oligonucleotides which represent a new application of the antisense concept which was originally applied to mRNA. AMOs for miRNA use are designed with perfect reverse

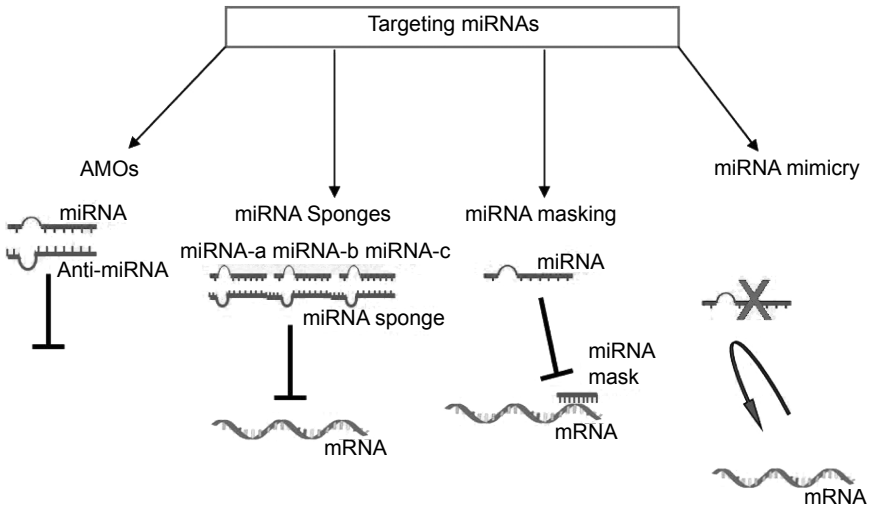

Figure 1. Potential miRNA-based therapeutic strategies. Oncogenic (over-expressed) miRNAs can be knocked-down or inactivated in an effort to restore 'normal' expression levels by anti-miRNA oligonucleotides (AMOs), miRNA sponges or miRNA masking. MiRNAs with reduced expression in the cancer state (tumour suppressor genes) can be replaced by miRNA mimicry. Other replacement techniques include induction of miRNA over-expression by viral vectors and reversal of epigenetic silencing using small molecules. *Reproduced with permission from Pharmaceutical Research.*

Color image of this figure appears in the color plate section at the end of the book.

base pair complementarity and competitively inhibit binding between the miRNA and its mRNA target by binding with the miRNA molecule. Unmodified DNA oligonucleotides were the first AMOs investigated. DNA AMOs were microinjected into *Drosophila* embryos, resulting in inactivation of *miR-2* and *miR-13* (Boutla et al. 2003). However, DNA AMOs are susceptible to degradation by nucleases and this experiment was likely to be successful due to direct microinjection of the AMO. RNA AMOs have distinct advantages over DNA AMOs; binding affinity is higher for duplex formation with miRNA target (Freier and Altmann 1997); modified RNA AMOs are less susceptible to degradation by nucleases and other enzymes. The most commonly used types of chemically modified AMO are two prime-O-methyl AMOs, two prime-methoxyethyl AMOs and locked nucleic acids (LNAs) (Weiler et al. 2006).

Chemical modifications of RNA AMOs permit enhancement of the above properties. For example, substituting a sulphur atom for a non-bridging oxygen atom in the phosphate backbone, called the phosphorothioate (PS) modification reduces the ability of enzymatic degradation. However, PS modifications also reduce binding affinity (Lennox et al. 2006) and these linkages have been deemed necessary for *in vivo* studies to permit cellular uptake and enhancement of pharmacokinetics. The safety of PS application has been reviewed and has been deemed acceptable (Levin 1999). However, a drawback of using AMOs with PS modifications lies in their activation of the immune system and non-specific interaction with proteins. The two-O-methyl- and two-O-methoxyethyl-AMOs are modified to provide enhanced binding affinity and enhanced resistance to nucleases, the latter in particular.

LNAs are a newer class of AMOs. They are chemically modified with the furanose ring in the sugar-phosphate backbone is locked in an RNA mimicking N-type configuration, as a consequence of a two prime-O, four prime-C methylene bridge (Petersen et al. 2000). LNAs have several advantages over the more traditional AMOs; they do not require a vector, have a lower toxicity profile and have superior thermal stability (Elmen et al. 2008). In addition, in comparison to other AMOs, LNAs display excellent mismatch discrimination as base pairing occurs in keeping with Watson-Crick complementarity and as such they provide potent antisense based gene silencing. LNAs are employed in most current *in vivo* studies in the miRNA field. LNAs are however, not the ideal therapeutic modality, they have several associated weaknesses, namely their inability to target more than one specific miRNA at a time and their short duration of action.

5.1.2 miRNA masking

MiRNA masking is another approach to anti-miRNA antisense oligonucleotides, with potentially important clinical utility. A miRNA mask is typically a single stranded two prime-O-methyl-modified oligonucleotide which exhibits perfect complementarity not to the miRNA itself, but rather to the miRNA binding site on the three prime UTR of a protein coding mRNA gene (Wang 2011). In this way, miRNA masking has the ability to provide gene-specific and miRNA-specific miRNA targeting, as it does not directly interact with the miRNA, but prevents binding of the miRNA. In addition, this approach avoids off-target effects and provides a better platform to study the effects of a specific gene on miRNA function. MiRNA masking technology has been successfully applied to *in vivo* studies. Choi et al. used a zebrafish model to investigate the role of *miR-430* on regulating expression transforming growth factor beta (TGF-ß) nodal agonist *squint* and antagonist *lft2* (lefty), which are important in zebrafish development namely of mesendoderm induction and left-right axis discrimination (Choi et al. 2007). They disrupted the specific miRNA-mRNA pairing by introducing miRNA masks which successfully inhibited the repressive action of *miR-430* on the target mRNAs *squint* and *lft2.*

5.1.3 miRNA sponges

MiRNA sponges provide an alternative to antimiR inhibition of miRNA function. First described in 2007, miRNA sponges can be expressed in cells as RNA molecules produced from transgenes and have the advantage of being able to bind multiple over-expressed miRNAs at the same time, a remarkable challenge to have overcome (Ebert et al. 2007). This is particularly attractive in the cancer setting, where multiple miRNAs are simultaneously deregulated and function together, particularly those within the same miRNA family. These competitive inhibitors display numerous and tandem binding sites complementary to the miRNAs of interest. When a sponge is present at high levels, it specifically deactivates the effects of the family of miRNAs sharing the common seed sequence. The miRNAs become bound to the miRNA sponge and are unable to exert their effects at the mRNA level. To ensure that the sponge transcript is present in high enough levels to maximize sponge to miRNA ratio, the sponges are frequently under the control of potent promoters, such as CMV. Typical sponge constructs contain between four and ten binding sites, each separated by a few nucleotides. Interestingly, increasing the number of binding sites is not beneficial,

as it increases the likelihood of sponge degradation. The most effective structure for a miRNA sponge is that which contains 'bulged-sites' that are miss-paired opposite miRNA positions nine to twelve (Ebert et al. 2007, Gentner et al. 2009), this presumably forms a more stable sponge-miRNA interaction, one that is less susceptible to Ago2-mediated endonucleolytic cleavage. MiRNA sponges offer several advantages over AMOs. Firstly, the model of producing dominant negative transgenes is perhaps simpler than knock-outs such as AMOs. Secondly, all miRNA family members with the same seed sequence are targets, irrespective of their site of encoding or where they exert their functionality (Ebert and Sharp 2010). MiRNA sponges target only mature miRNAs and therefore do not exert any effect on other miRNA precursors within the same cluster. It is also plausible to suggest that sponges could be modified to include regulatory elements such that they may be induced by specific drugs or even by specific tissues. Delivery of miRNA sponges can be achieved by viral vectors. The efficacy of miRNA sponges has been demonstrated by Ebert et al. in an *in vitro* model. Cultured cells were transiently transfected with vectors encoding miRNA sponges resulting in a reduced level of miRNA to at least that achieved by AMOs (Ebert et al. 2007, Lu et al. 2005). *Drosphila* miR-SP is a novel miRNA sponge technology which aims to elucidate the functional outputs of miRNA-mRNA target interactions (Loya et al. 2009). It is designed to inform about the functional output of miRNAs with precise *in vivo* spatial resolution, while overcoming the lack of tissue specificity associated with more traditional miRNA sponges. This study by Loya et al. was the first to produce stable, germline sponge expression in an animal model organism. The sponge constructs consist of five Upstream Activation Sequence (UAS) elements, ten bulged miRNA binding sites in the three prime UTR and a fluorescent reporter system. These transgenic miRNA sponges are synthesized by placing modified miRNA complementary oligonucleotides downstream of recurring UAS. This remarkable system provides insight into the contribution of specific miRNAs to complex biological processes, and permits insight into their interactions with other genes.

5.2 Potential Treatment Strategies for Tumour Suppressor miRNAs

In the cancer state, miRNAs with decreased expression are regarded as tumour suppressor genes. The fundamental aim of the therapeutic approach in this case is to restore miRNA expression to a normal level. This can be achieved by miRNA mimicry, the induction of miRNA over-expression by viral vectors, or by reversing the epigenetic silencing of specific miRNAs

using small molecules. These strategies are desirable as therapeutic approaches for cancer, as in general miRNA levels are found to be decreased in the cancer state (Lu et al. 2005).

5.2.1 miRNA mimicry

MiRNA oligonucleotide mimics are double stranded RNA molecules that contain a 'guide strand' that is identical to the mature miRNA, and is designed to mimic its function. The 'passenger strand', the second RNA strand, is usually perfectly complementary to the guide strand. The duplex formation provides better loading onto the mRISC and therefore improves the biological activity (Behlke 2008). MiRNA oligonucleotide mimics require chemical alteration to reduce degradation by nuclease. In addition, modifications can be made to enhance target organ specific uptake, for example cholesterol can be added to increase hepatic uptake. In theory, as miRNA mimics have the same functionality as the endogenous miRNA, this technique should have reduced off-target effects. MiRNA mimics have successfully been applied to both *in vitro* and *in vivo* studies, the details of which will be discussed later in this chapter.

5.2.2 Viral vector delivery of miRNAs

Increasing the level of tumour suppressor miRNAs can also be achieved by DNA plasmid delivery of the pri-miRNA, usually with the aid of a viral vector. This form of gene therapy typically exploits adenoviral or lentiviral vectors. The vector is based on the adeno-associated virus (AAV) and the specific miRNA is incorporated into this system to enable tissue delivery. Adeno-associated viral (AAV) vectors are thought to have reduced insertional mutagenesis compared to other viruses, and as such are particularly promising (Schnepp et al. 2003). These viruses are appealing as they do not stably integrate, unlike retro- or lenti-viruses which may randomly integrate and cause gene disruption, even perhaps resulting in *trans*-activation of oncogenes. MiRNAs are particularly suited to AAV delivery techniques as they are small. Indeed AAV miRNA replacement approaches have been studied *in vivo* and obtained encouraging results (Trang et al. 2010). MiRNA therapeutics companies such as Mirna Therapeutics and Asuragen have also shown interest in this strategy. AAV vectors have been reported to be utilized with minimal toxicity and transduction efficiency. However, this approach is not free from serious adverse effects. A study by Grimm et al. in 2006 highlighted the danger associated with this type of miRNA replacement. A delivery vector based on duplex-DNA-containing adeno-associated virus

type eight (AAV8) was utilized to systemically administer short RNAs to adult mice. This resulted in down-regulation of crucial hepatic miRNAs, and the mice suffered morbidities and even fatalities (Grimm et al. 2006). It was hypothesized that the fatalities were due to over-saturating endogenous miRNA pathways, a consequence that can be avoided by monitoring and optimizing dose and sequence carefully.

6. OBSTACLES TO THE CLINICAL UTILITY OF miRNA THERAPEUTICS

Over the past two decades we have witnessed major scientific advances, from miRNA discovery to their application as novel treatment tools. Progress has been made but there are still several issues which must be overcome before we can safely move each of the above methods of miRNA suppression and miRNA replacement to the clinical setting.

Many of the studies to date regarding miRNAs and cancer have focused in the identification of disease-specific miRNA profiles. However, there is an extensive gap in our knowledge on the precise function and metabolic pathway involvement of these miRNAs, both in health and disease. It would not be safe to launch a miRNA treatment strategy without first characterizing the role of each therapeutic miRNA in other tissues and biological pathways. The complexity of the situation is augmented by the knowledge that miRNAs can have dual functionality, with the potential to act both as an oncogene and tumour suppressor gene depending on the tissue and cancer state in question, and in addition, a single miRNA can have multiple gene targets. Gene target prediction is currently conducted computationally in a sequence specific manner, with software programmes such as miRBase, TargetScan and Pictar capable of providing numerous potential targets. Yet it seems that sequence complementarity is not all that governs an efficient interaction between a miRNA and its mRNA target, as not all predicted targets are functional. Validation of such predicted targets and *in vivo* functional analysis need to be carried out in order to accurately predict the wide range of side effects and toxicities which may be expected from a miRNA treatment approach. It would be prudent to identify all existing miRNAs, and their functions, before miRNA therapeutics can safely be employed.

Standardization of miRNA analysis should also be enforced before these platforms are approved for clinical utility. Academic research and industrial laboratories employ different methods of identifying and validating the specified miRNA, or indeed its precursor. To ensure reproducible results across laboratories and continents it will also be necessary to share methods of miRNA analysis as well as detailed information on the type of specimen

evaluated and the ideal storage conditions. If patients are to be treated on the basis of their individual tumour's miRNA profile, it is necessary to ensure an international standard of tumour miRNA expression is achieved.

Another challenge which must be overcome before miRNA-based therapeutics can become a clinical reality is delivery of these remarkable technologies. In order for this strategy to be safe, effective and tissue-specific, improvements in delivery modalities must be achieved. Delivery systems employed to date include direct, indirect and viral vector delivery. However, all of these methods have inherent weaknesses, as discussed earlier. The cell membrane is a natural barrier to exogenous miRNA-based drugs; it limits uptake of oligonucleotides into cells. To overcome this obstacle, methods of delivery focus on the specific target site, or on methods of enhancing uptake of the drug into the target cell. Oligonucleotides have been administered by several routes in *in vivo* studies including subcutaneous injections, intravenous injections, inhalation, oral intake, intraocular and topical applications. Enhanced cellular uptake can be achieved by either improving overall uptake or by permitting tissue-specific or disease-specific uptake by conjugation or coating. Conjugating the oligonucleotide to cholesterol, for example, improves hepatic uptake. Liposomes and lipoplexes (cationic lipid DNA complexes) have also been reported to improve cellular uptake (Akhtar et al. 2000, Jaaskelainen and Urtti 2002). Specific cells or tissues can be targeted by endocytosis techniques, which opportunistically exploit the cell membranes natural process of receptor mediated endocytosis as a consequence of protein (or antibody) cell surface binding. Coupling miRNA therapeutics with antibodies or other proteins that recognize tumour specific antigens, proteins which are uniquely expressed or over-expressed in the cancer state, would provide the ideal scenario of targeted treatment delivery. Potential targets, include the folate receptor and transferrin receptor (both over-expressed in some cancer cells), the ERBB2 receptor (breast and ovarian cancers) and GM-CSF (leukaemic blast cells) (Derycke and De Witte 2002, Frankel et al. 2002, Gabizon et al. 1999, Noonberg and Benz 2000). Such advances unveil ligand targeted therapeutics (LTT) as a promising method to create selective toxicity in neoplastic cells, while sparing already compromised patients from the immunogenic safety issues inherent to viral vector delivery systems. The main advantage of viral vector delivery is the potential to deliver more than one miRNA therapeutic agent at any one particular time. This would have remarkable potential in the cancer setting where multiple miRNAs are dysregulated. The ability to simultaneously replace deficient tumour suppressor genes while reduce oncogenes would theoretically result in a more powerful anti-tumour effect, particularly considering the complexity of most cancer states with multiple oncogenic pathways. However, viral vectors also have several associated weaknesses; they are likely to trigger an immune response, their effect is

usually transient, and in the case of retro- or lenti-viruses, may even stably integrate into the patient's genome and cause disruption or even oncogenic activation. Advanced delivery technologies and increased knowledge on miRNA functionality will aid the reduction in off-target effects. Optimal dosing of miRNA is a further pharmacokinetic challenge. *In vivo* animal studies are likely to be most informative in this setting.

The future of miRNA therapeutics is exciting but these obstacles must be overcome so that disease- or tissue- specific uptake can be achieved. This is critical in order to minimize off-target effects and so reduce the exposure of healthy cells to potential side effects.

7. DISEASE SPECIFIC APPLICATION OF miRNA TREATMENT STRATEGIES

MiRNA-based treatment approaches have been successfully applied to a number of common cancers to date. We will now elaborate on the rationale and advances made in this setting, through both *in vitro* and *in vivo* work.

7.1 Hepatocellular Carcinoma

The major advances made in the realm of miRNA therapeutics have been in liver disorders and hepatocellular carcinoma (HCC). HCC is amongst the most commonly diagnosed cancers and is one of the leading causes of cancer-related death worldwide (Parkin et al. 2005). The majority of HCC arises in the setting of pre-existing chronic liver disease. On a worldwide scale this is largely related to infection with viral hepatitis B or C (Bosch et al. 2005). In this context, methods of preventing or treating viral hepatitis and liver fibrosis, will prevent the development of HCC and hence warrants discussion here. Our current understanding of the molecular mechanisms underpinning viral hepatitis, hepatic fibrosis and subsequent HCC remains fragmented, however it is clear that miRNAs play an intricate and complex role in this pathway. The introduction of a viral component affects miRNA-mediated RNA silencing pathways which impact on viral-host cell interactions (Gottwein and Cullen 2008). Host miRNAs are exploited and in addition, viruses even have the capacity to encode their own miRNAs (Lin and Flemington 2010, Nair and Zavolan 2006). These miRNAs evolve at a rapid pace and regulate both viral life-cycle and viral-host interactions. Some miRNAs were found capable of enhancing or inhibiting viral replication by directly acting on viral RNAs (Cullen 2009).

7.1.1 Viral hepatitis B

Hepatitis B Virus (HBV) is a small, enveloped DNA virus which belongs to the Hepadnaviridae family of viruses. HBV can cause acute or persistent infection, and is strongly associated with chronic cirrhosis which predisposes to HCC (Yang et al. 2008). There is robust evidence to support a role for miRNAs in the development of, and host response to, HBV infection (Bala et al. 2009). Jin et al. further examined the miRNA-encoding potential of HBV. By employing computational analysis, they identified that HBV encodes only one putative candidate pri-miRNA. Using a database of three prime UTR from the human genome, the authors also deduced that this viral miRNA could not target any host mRNA, but only viral mRNA. In this way, it appears that HBV has evolved to use viral miRNAs (vmiRNAs) to its advantage to regulate its own gene expression (Jin et al. 2007). This statistically derived conclusion was later examined *in vitro* by Gao et al. HepG2.2.15 cells were transfected with three artificial miRNA-HBV plasmids. HBV antigen secretion was detected using time resolved fluoroimmunoassays (TRFIA). Fluorescence quantitative-PCR was used to evaluate HBV DNA replication, while HBV mRNA was detected by real-time PCR (RT-PCR). In this study, vector-based artificial miRNA successfully inhibited viral replication and expression (Gao et al. 2008). The *in vivo* utility of this potential miRNA-centred treatment approach was confirmed by Ely et al. RNA polymerase II promoter cassettes that transcribe anti-HBV pri-miRNA-122 and pri-miR-31 shuttles were generated and studied in a murine hydrodynamic injection model resulting in reduced HBV expression (Ely et al. 2008). Zhang et al. aimed to identify whether host cellular miRNAs affect HBV replication by creating a loss of function approach. HepG2.2.15 cells were transfected with anti-miRNA oligonucleotides of 328 human miRNAs. Compared to controls, *miR-199a-3p* and *miR-201* transfection was associated with a reduction in HBV surface antigen without impacting on HepG2.2.15 cell proliferation, reflecting decreased viral replication (Zhang et al. 2010).

7.1.2 Viral hepatitis C

Hepatitis C Virus (HCV) is a member of the *Flaviviridae* family. Vaccination for this particular virus is not yet available. This highly dynamic virus replicates in human hepatocytes and is translated as an approximately 3,000 amino acid polyprotein which undergoes post-translational processing, by both viral and human enzymes, to result in ten viral structural and non-structural proteins (Moradpour et al. 2007). HCV is a major cause of chronic

hepatitis, liver cirrhosis and HCC. The role of miRNAs in HCV replication and treatment is not as well documented as HBV, however further investigation is certainly warranted. Human host cells appear to modulate HCV replication through RNA interference (RNAi) (Randall et al. 2007). *MiR-122*, the first described liver-specific cellular miRNA which comprises over 70% of miRNAs expressed in the liver, is crucial for HCV replication and accumulation in cultured Huh-7 cells. In fact *miR-122* has two potential binding sites for HCV. Replication of HCV is enhanced by the presence of *miR-122* as it targets the five prime viral end of the non-coding region (Jopling et al. 2005). Jopling et al. highlighted a potential miRNA derived antiviral treatment strategy. This group inactivated *miR-122* by transfection with a two prime-O-methylated RNA oligonucleotide that displayed a complementary sequence to *miR-122* and reported an 80% reduction in HCV replication (Jopling et al. 2008). This *in vitro* model was later followed by an *in vivo* model using chimpanzees. Krutzfeld et al. elegantly showed that intravenously delivery of LNA-mediated oligonuclotides could successfully inhibit *miR-122* for 23 days (Jopling et al. 2008). The mechanism by which *miR-122* supports HCV infection may involve the Heme-Oxygenase-1 (HO-1) pathway. This pathway has the potential to inhibit HCV duplication and *miR-122* down-regulates this particular pathway. It therefore stands to reason that combination therapy with *miR-122* antagonism and HO-1 stimulation may act as a potent anti-HCV treatment approach (Shan et al. 2007). However, a study by Sarasin-Filipowicz et al. revealed that human *miR-122* expression and its role in natural HCV infection may not correlate with experimentally derived findings (Sarasin-Filipowicz et al. 2009). Further *in vivo* analysis is necessary before miRNA based treatment approaches are designed and approved for this disease.

7.1.3 Hepatic fibrosis

Liver fibrosis occurs as a response to chronic tissue injury. The most common insults are viral hepatitis (particularly HBV and HCV), autoimmune hepatitis, alcohol and metabolic diseases (resulting in iron or copper overload). Similar to other organs, the liver is comprised of numerous cell types. These include hepatocytes, an endothelial lining, Kuppfer cells (macrophages) and perivascular hepatic mesenchymal cells, known as Stellate cells (Friedman 1998). The Hepatic Stellate cells (HSCs) are critical to the process of fibrosis. Injury and inflammation causes these cells to undergo 'activation', a process which transforms these cells into highly proliferative, contractile and fibrogenic fibroblasts. Activated HSCs secrete large amounts of extracellular matrix (ECM), with a different composition which then undergoes repeated remodelling (Friedman 2000). Liver fibrosis

is reversible, unlike cirrhosis which occurs as a consequence of longstanding fibrosis. However, once cirrhosis has occurred, there is an increased risk of developing HCC. There are currently no FDA-approved therapeutic strategies for the treatment of liver fibrosis, although a select cohort of patients with HCV may respond to interferon therapy, with regression in fibrosis and a viral response (Shiratori et al. 2000). Surgical intervention with resection or liver transplantation remains the only option for certain cases, however this is not ideal as the number of patients requiring treatment for hepatic fibrosis dramatically exceeds the number of organs available for transplantation. It is in this context that miRNA therapeutic approaches hold great promise. Several miRNAs have been studied in fibrosis and liver fibrosis in particular. Down-regulation of *miR-29* family members has been observed in fibrosis, including cardiac and renal fibrosis (Jiang et al. 2010). Roderburg et al. conducted a microarray on murine livers and concluded that *miR-29* family was also significantly down-regulated in liver fibrosis. This study also revealed that *miR-29* played a regulatory role in TGF-ß and NF-κß pathways. Moreover, over-expression of *miR-29b* caused down-regulation of collagen expression in murine HSCs (Roderburg et al. 2011). It is clear from this work that the *miR-29* family could have a future application as an anti-fibrotic miRNA-based agent. *MiR-27a* and *miR-27b* have also recently been evaluated in hepatic fibrosis. These miRNAs are typically over-expressed during inflammation. Down-regulation of these miRNAs in rat HSCs *in vitro* was associated with the HSCs returning to their normal state, with reduced proliferation (Ji et al. 2009).

7.1.3 Hepatocellular carcinoma

Seminal advances in the realm of miRNA therapeutics have been based on their application to HCC, a disease with limited successful management approaches thus far. *MiR-122*, a liver specific miRNA which was previously discussed with respect to HCV, also has a critical role to play in HCC (Gramantieri et al. 2007). This miRNA is known to act as a tumour suppressor in HCC; expression profiling has revealed that *miR-122* is down-regulated in approximately 50% of human HCC cases (Kutay et al. 2006). Restoring *miR-122 in vitro* in metastatic Mahlavu and SK-HEP-1 cells has been shown to inhibit migration and invasion. Furthermore, replenishing *miR-122* levels in an *in vivo* model reduces tumourigenesis, angiogenesis and metastases (Tsai et al. 2009). Additional studies have confirmed similar findings *in vitro*, with cells displaying reduced viability and increased apoptosis (Bai et al. 2009, Lin et al. 2008). Young et al. applied this knowledge, in an effort to test the feasibility of *miR-122* replacement as a therapeutic strategy for HCC. They were the first to attempt small molecule inhibition and activation of *miR-122*,

by delivering miRNA modifiers (named 1–3) which act at the transcriptional level. Small molecule *miR-122* inhibitor two inhibited HCV replication and small molecule miR-122 inhibitor three resulted in an increased expression of *miR-122*, caspase activation and ultimately reduced cell viability in the HepG2 cell line for HCC (Young et al. 2010).

Other putative tumour suppressors which could have a therapeutic application in HCC include *miR-101* (Baffa et al. 2009, Li et al. 2009), *miR-26a* (Kota et al. 2009) and *miR-223*. *MiR-26a* is down-regulated in human liver cancers. Replacement of *miR-26a* using an AAV vector *in vivo* strongly prevents proliferation and induces tumour-cell-specific apoptosis. This is a remarkable finding as tumour-specific cell death could potentially provide a reduced toxicity and side effect profile. This phenomenon occurs as a consequence of HCC cells entering a state of G1 arrest due to cyclin D2 and E2 down-regulation (Kota et al. 2009). MiRNA-based therapeutic strategies for HCC appear to have potential in the clinical setting.

7.2 Cancers of the Lung

Lung cancer is the leading cause of cancer mortality worldwide and represents a disease in need of new, effective therapeutic strategies. Over 80% of lung cancers are of the non-small cell lung cancer (NSCLC) type (Jemal et al. 2009). The overall five year survival for NSCLC remains low (15%) and the recurrence rate is high, even amongst those with early stage disease (Miller 2005). As with other tumour types, several miRNAs have been found to be dysregulated in NSCLC and indeed various miRNA signatures have been devised to as diagnostic and prognostic tools (Boeri et al. 2011, Shen et al. 2010). MiRNAs have multiple potential therapeutic roles in lung cancer: as direct anti-cancer agents, as inhibitors of invasion and metastases, and in mediating the reversal of chemo- and radio-resistance.

The majority of the work to date in this setting has focused on the *let-7* family. This group of miRNAs are stably expressed in normal lung tissue, but some *let-7* members have been shown to be under-expressed in NSCLC, revealing a potential role as tumour suppressor genes (Johnson et al. 2007). Slack's group has evaluated the *in vitro* and *in vivo* application of *let-7* based miRNA treatment approaches. Esquela-Kerscher et al. conducted *in vitro* experiments on NSCLC cell lines in 2008 and concluded that *let-7* inhibited the growth of multiple human lung cancer cell lines. They also demonstrated the *in vivo* inhibitory effects of *let-7* on lung tumour formation in a murine model of intranasal *let-7* delivery, in which the mice displayed a G12D activating mutation for the *K-ras* oncogene (Esquela-Kerscher et al. 2008). Trang et al. supported the role of the *let-7* family of miRNAs as a potential treatment approach again in 2010 with another study confirming the *in*

vivo application in lung cancer. It was confirmed that loss of *let-7* activity enhances lung tumour formation in a murine model, further evidence to support its tumour suppressor role. Additionally, this group studied the *in vivo* effects of *let-7* delivery on already established non-small cell lung tumours in a mouse model. Immuno-deficient NOD/SCID mice were inoculated with subcutaneous injections of human H460 cells and monitored until palpable tumours had formed. Then *let-7b* or a negative control miRNA (miR-NC) were administered by intra-tumoural injections every three days. Intracellular uptake of these synthetic miRNAs was enhanced by siPORTamine, a lipid based uptake reagent. Those tumors treated with *let-7b* displayed remarkably increased tumoural necrosis and cellular debris than the control group. Intranasal delivery of *let-7b* was then confirmed as a potential delivery approach as effective remission of a *K-ras* activated NSCLC murine model was achieved (Trang et al. 2010). Kumar et al. also explored the value of *let-7* as a therapeutic agent in cancer. A lentiviral miRNA delivery system was utilized in a mouse model to transfect a *let-7g* duplex into *K-ras* (GD12) expressing lung adenocarcinoma cells (LKR 13). This resulted in cell cycle arrest and cell death. Furthermore, these lentiviral vectors were then utilized to deliver *let-7g* in a mouse model, a reduction in tumour burden was observed (Kumar et al. 2008). These studies provide proof-of-concept supporting *let-7* based treatments for use in lung cancer.

Other miRNAs have been studied for therapeutic applications in lung cancer. Wiggins et al. replaced deficient *miR-34a*, both locally and systemically, in a mouse model of NSCLC using synthetic miRNA and a lipid based delivery vehicle. This study design resulted in inhibition of lung tumour growth and interestingly was not associated with any elevation of cytokines, liver or kidney enzymes, implying that it was safely tolerated (Wiggins et al. 2010).

The role of miRNA therapeutics is not confined to direct anti-tumour effects. Reducing the invasion and metastatic capabilities of lung cancer cells is equally as important. *MiR-221* and *miR-222* have been shown to enhance cellular migration by targeting PTEN and TIMP3, by inducing TRAIL resistance in aggressive NSCLC cells (Garofalo et al. 2009). MiRNA-silencing strategies for *miR-221* and *miR-222* could prove advantageous in this regard. Gibbons et al. demonstrated that forced expression of *miR-200* abrogated the ability of metastatic lung cancer cells to undergo EMT, epithelial-to-mesenchymal transition, invade and metastasize, identifying a potential valuable miRNA target for future studies (Gibbons et al. 2009). Finally, miRNAs have a role in targeting genes governing drug sensitivity, resulting in the ability to alter sensitivity of cancer cells to anti-cancer drugs and radiotherapy. The sensitivity to Cisplatin, Etoposide and Doxorubicin *in vitro* was greatly increased following transfection with *miR-134* in a small cell lung cancer cell line (Guo et al. 2010). Down-regulation of *miR-*

200b has been implicated in docetaxel-resistant lung adenocarcinoma cells, with replacement of this miRNA being associated with reversal of this chemo-resistance (Feng et al. 2011). Indeed *let-7* has also been implicated in radio-sensitivity via altered *K-ras* signalling. Two studies have reported that increasing *let-7* expression levels in lung cancer results in increased sensitivity to radiation therapy (Oh et al. 2010, Weidhaas et al. 2007).

7.3 Breast Cancer

Breast cancer is the most common female malignancy among women in almost all of Europe and North America. Each year, more than 1.3 million women worldwide will be diagnosed with this disease, and approximately 4,652,000 women will die as a result of the disease (Garcia 2007). The role of miRNAs in breast tumorigenesis, progression and metastases has been well studied and there is a large body of evidence supporting their role as key players in each of these events (Heneghan et al. 2009). Much of the breast cancer related research in this field to date focuses on miRNA profiling of breast cancer and perhaps it is surprising that relatively few studies have investigated miRNA replacement or knock-down strategies for use in breast cancer. As in other cancer states, miRNAs may be up- or down-regulated in breast cancer and therefore may act as oncogenes or tumour suppressor genes, respectively. As such, miRNA-based treatments have three main approaches in breast cancer: inhibition or loss of function of miRNAs which are over-expressed (oncomiRs), replacement of miRNAs which are under-expressed (tumour suppressor genes) and modulation of miRNAs which are implicated in augmenting response to chemotherapy, hormonal therapy or radiotherapy.

7.3.1 miRNA-based interference with the metastatic cascade

It is widely accepted that chemo-resistance and metastases are responsible for most breast cancer related deaths (Fossati et al. 1998). As such, targeting and interrupting the breast cancer metastatic cascade has formed the focus of miRNA based therapeutic strategies. Baffa et al. conducted a miRNA microarray on paired tumour tissues and metastatic lymph nodes in an effort to identify differentially expressed miRNAs, with a putative role in metastases. Several miRNAs were identified including *miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c* and *miR-105* (Baffa et al. 2009). Indeed *miR-10b* has been implicated in several cancers, particularly in the metastatic setting. *MiR-10b* expression is induced by Twist (a transcription factor) and exerts its pro-metastatic effects by inhibiting

translation of the HOXD10 protein, leading to increased expression of RHOC, a pro-metastatic gene (Ma et al. 2007). Ma et al. confirmed that *miR-10b* positively regulates cell invasion and migration *in vitro*. Furthermore, this group showed that *in vivo* over-expression of *miR-10b* in a murine model of otherwise non-metastatic breast tumours resulted in increased invasion and metastases (Ma et al. 2007). The potential application of *miR-21* in breast cancer management has been evaluated *in vitro*. Yan et al. conducted *in vitro* LNA-mediated *miR-21* silencing in breast cancer cell lines, specifically MCF-7 and MDA-MB-231, which reduced proliferation and cell migration. This group also showed a similar effect in an *in vivo* model (Yan et al. 2011). Indeed, the mechanism by which *miR-21* promotes invasion may be via the regulation of TIMP3 (Song et al. 2010). *MiR-10b* and *miR-21* appear to have oncomiR properties in breast cancer, with a potential function for loss of function or knockdown based treatment approaches. *MiR-145* is under-expressed in breast cancer cells, therefore potentially acting as a tumour suppressor gene, and adenoviral vector deliver of *miR-145 in vitro* and *in vivo* suppresses tumour growth and cellular motility (Kim et al. 2011). Interestingly, a relatively newly described miRNA, *miR-1258*, is reported to have inhibitory properties in the development of breast cancer brain metastases by targeting heparanase (Zhang et al. 2011).

7.3.2 miRNAs to augment response to adjuvant therapies

Adjuvant therapies routinely used for the management of breast cancer include chemotherapeutic drugs, hormonal therapies and radiotherapy. However, a large proportion of women do not derive any benefit from these approaches, or develop resistance to these strategies over time (Gonzalez-Angulo et al. 2007). It has been postulated that miRNAs may have the ability to augment response to these adjuvant therapies and preliminary *in vitro* and *in vivo* studies appear to be promising. This is particularly promising for triple negative or basal breast tumours, which are characteristically hormone receptor negative and HER2/*neu* negative, for which no targeted therapy currently exists. Indeed miRNA signatures capable of predicting hormone receptor and HER2/*neu* status have been described (Lowery et al. 2009). This reveals the potential for inducing ER expression in ER negative disease and permitting targeted hormonal therapy for basal and HER2-overexpressing (ER negative) subtypes. *MiR-21* has already shown promise in augmenting the response to chemotherapy in breast cancer. Combining taxol chemotherapy with a *miR-21* inhibitor was successful in producing reduced cell viability and invasiveness, compared to taxol treatment alone. This study elegantly displays the increased chemotherapeutic effect of taxol in the presence of reduced *miR-21* levels (Mei et al. 2010). Slack's group

demonstrated that *miR-34* is required for the DNA damage response, and is upregulated by p53 following radiation exposure. Moreover, loss of *miR-34 in vivo* in *C. elegans* results in increased radio-sensitivity in the soma of the animals, with radio-resistance of the germline. When applied to breast cancer cells *in vitro* it was observed that replacing *miR-34* post-radiation exposure altered cell survival (Kato et al. 2009). This work highlights the plausible role of anti-*miR-34* treatment strategies in radio-resistant breast cancer in a clinical setting. The manipulation of several other specific miRNAs have been described as single agents with a putative role in augmenting the response of breast tumours to adjuvant chemotherapy. *MiR-128a*, *miR-125b*, *miR-155* and *miR-342* have all been associated with the regulation of chemo-sensitivity (Cittelly et al. 2010, Kong et al. 2010, Masri et al. 2010, Zhou et al. 2010).

7.4 Haematological Malignancies

The initial work on miRNA expression profiling and functional analyses progressed from the studies on haematological malignancies. However, when compared to the solid tumours, very little research has been done on the application of miRNA-based therapeutics to this group of cancers.

7.4.1 Leukaemia

MiR-15a and *miR-16* were the first miRNAs identified as having a role in cancer. A translocation induced deletion at chromosome 13q14.3 in B-cell chronic lymphcytic leukemia (CLL) was identified (Calin et al. 2002). Loss of *miR-15a* and *miR-16* from the locus creates an increased expression of BCL2, an anti-apoptotic gene (Cimmino et al. 2005). A reasonable hypothesis would be to inhibit BCL2 by replacing *miR-15a* and *miR-16* for the treatment of B-cell CLL, but this has yet to be reported. Acute myeloid leukaemia (AML) has been the focus of more promising miRNA-centred treatment approaches. Transfection of synthetic *miR-193a in vitro* produces decreased AML cell proliferation in primary cell lines and primary AML blasts but not in normal bone marrow cells, which correlated with a reduction in the *c-kit* oncogene (Gao et al. 2011). Eyholzer et al. reported that *miR-29b* is under-expressed in AML patients with either loss of chromosome 7q or CEBPA deficiency (Eyholzer et al. 2010). *MiR-29b* mimicry was applied to an *in vitro* cell line and an *in vivo* mouse xenograft model of AML and resulted in reduced growth and increased cellular apoptosis (Garzon et al. 2009).

7.4.2 Lymphoma

The role for miRNAs as therapeutic agents in lymphoma is also relatively understudied. Most of the work in this realm focuses on the *miR-17-92* cluster. This group of miRNAs is comprised of *miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a* and *miR-92*. This cluster is located at chromosome 13q31-q32, an area that is often amplified in B-cell lymphoma (Ota et al. 2004). The *miR-17-92* cluster has been shown to have oncogenic effects in this disease process, with enforced expression associated with accelerated tumour growth and reduced Myc-induced apoptosis in a mouse model of B-cell lymphoma (He et al. 2005). Targeting this cluster and reducing its expression level could reduce the pro-tumour properties of these miRNAs. Additional functions of targeting the *miR-17-92* cluster have been described for increasing radio-sensitivity of Mantle Cell Lymphoma (MCL), as overexpression of this cluster is described in MCL cells with reduced cell death after radiotherapy. It would therefore be plausible that inhibition of this cluster could also sensitise MCL cells to radiation, thus improving the prognosis for these patients. An *in vivo* xenograft MCL murine model demonstrated how inhibition of the *miR-17-92* cluster was associated with reduced tumour growth (Rao et al. 2011).

8. CONCLUSIONS

The application of miRNAs as therapeutic agents and disease targets in cancer is a relatively new but rapidly evolving field, as evidenced by an increasing body of research in this setting. These tiny molecules play important roles of almost all aspects of the cell cycle, both in health and disease. Their individual roles in different cancer states make them ideal tissue-specific and disease-specific therapeutic agents. Moreover, our increasing level of understanding allows us to devise methods of replacing deficient miRNAs and reducing the activity of over-expressed miRNAs. Such technologies have been applied to liver disorders, lung cancers, breast cancer and haematological malignancies. However, several challenges must be overcome before miRNA-based therapeutic strategies can be safely translated to the clinical setting.

9. SUMMARY POINTS

- The application of miRNAs as therapeutic agents and disease targets in cancer is a relatively new and rapidly evolving field.
- MiRNAs are aberrantly expressed in the cancer state where they have dual functionality, acting as oncogenes or tumour suppressor genes.

- Specific miRNA profiles can be generated for almost every tumour type, unveiling the potential for cancer-specific diagnostic biomarkers and therapeutic strategies.
- There are several experimental techniques for miRNA expression profiling available. The most common methods are: Oligonucleotide based-microarray, bead-based flow cytometry technology, tag-based sequencing methods and deep sequencing technology.
- MiRNA-based therapeutics aim to restore the disease-specific dysregulated miRNA to 'normal' expression levels.
- Over-expressed oncogenic miRNAs may be knocked down by anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking.
- MiRNAs with decreased expression in cancer (tumour suppressor genes) can be replaced by miRNA mimicry, induction of overexpression by viral vectors or by reversal of epigenetic silencing of specific miRNAs using small molecules.
- MiRNA-based treatment approaches have been applied to a number of common cancers both *in vitro* and *in vivo* including hepatocellular carcinoma, lung cancer breast cancer and some haematological malignancies.
- However, there are several challenges which must be overcome before miRNA-based treatment strategies can be safely transferred to the clinical setting.

ABBREVIATIONS

AAV	:	Adeno-associated virus
AAV8	:	Adeno-associated virus type 8
AGO	:	Argonaute
AML	:	Acute myeloid leukaemia
AMO	:	Anti-miRNA oligonucleotide
cDNA	:	Complementary DNA
CEBPA	:	CCAAT/enhancer-binding protein alpha
CLL	:	Chronic lymphocytic leukaemia
CMV	:	Cytomegalovirus
DNA	:	Deoxyribonucleic acid
dsDNA	:	Double-stranded DNA
ECM	:	Extracellular matrix
EMT	:	Epithelial mesenchymal transition
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
HBV	:	Hepatitis B virus

HCC	:	Hepatocellular carcinoma
HCV	:	Hepatitis C virus
HO-1	:	Heme-Oxygenase-1
HSC	:	Hepatic stellate cells
LNA	:	Locked nucleic acid
LTT	:	Ligand targeted therapeutics
MCL	:	Mantle cell lymphoma
miRAGE	:	MiRNA serial analysis of gene expression
miRNA	:	microRNA
mRNA	:	Messenger RNA
miR-NC	:	miRNA normal control
miRISC	:	miRNA-associated RNA-induced silencing complex
NOD	:	Non-obese diabetic
NSCLC	:	Non small cell lung cancer
PCR	:	Polymerase chain reaction
PS	:	Phosphorothioate
qRT-PCR	:	Quantitative real-time polymerase chain reaction
RAKE	:	RNA-primed array-based Klenow enzyme
RNA	:	Ribonucleic acid
RNAi	:	RNA interference
SAGE	:	Serial analysis of gene expression
SCID	:	Severe combined immunodeficiency
UAS	:	Upstream activation sequence
UTR	:	Untranslated region
vmiRNA	:	viral miRNAs

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