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Exploring circulating micro-RNA in the neoadjuvant treatment of breast cancer

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Mini Review

Breast cancer is the most frequently diagnosed malignancy amongst females worldwide. In recent years the management of this disease has transformed considerably, including the administration of chemotherapy in the neoadjuvant setting. Aside from increasing rates of breast conserving surgery and enabling surgery *via* tumour burden reduction, use of chemotherapy in the neoadjuvant setting allows monitoring of *in vivo* tumour response to chemotherapeutics. Currently, there is no effective means of identifying chemotherapeutic responders from non-responders. Whilst some patients achieve complete pathological response (pCR) to chemotherapy, a good prognostic index, a proportion of patients derive little or no benefit, being exposed to the deleterious effects of systemic treatment without any knowledge of whether they will receive benefit. The identification of predictive and prognostic biomarkers could confer multiple benefits in this setting, specifically the individualization of breast cancer management and more effective administration of chemotherapeutics. In addition, biomarkers could potentially expedite the identification of novel chemotherapeutic agents or increase their efficacy. Micro-RNAs (miRNAs) are small non-coding RNA molecules. With their tissue-specific expression, correlation with clinicopathological prognostic indices and known dysregulation in breast cancer, miRNAs have quickly become an important avenue in the search for novel breast cancer biomarkers. We provide a brief history of breast cancer chemotherapeutics and explore the emerging field of circulating (blood-borne) miRNAs as breast cancer biomarkers for the neoadjuvant treatment of breast cancer. Established molecular markers of breast cancer are outlined, while the potential role of circulating miRNAs as chemotherapeutic response predictors, prognosticators or potential therapeutic targets is discussed.

Breast cancer is the most frequently diagnosed cancer among women worldwide, accounting for 23% of total cancer cases.[1] In 2012, worldwide 1.7 million women were diagnosed with breast cancer, representing a >20% increase in incidence since 2008. Concurrently, worldwide more than 520,000 women died from breast cancer, representing a 14% increase in annual breast cancer related mortality and confirming breast cancer as the most common cause of cancer-related deaths amongst women.[1] Within developed countries the incidence of breast cancer continues to rise. This is likely due to the implementation of screening programs and improved imaging techniques, leading to many breast cancers being diagnosed at an earlier stage. Furthermore, our improved molecular understanding of breast cancer and the use of increasingly effective chemotherapeutics has resulted in improved patient outcomes, with mortality decreasing by 2 to 3% per year in developed countries.[2]

Historically, neoadjuvant chemotherapy (NAC) was reserved for locally advanced breast carcinoma, converting technically inoperable tumours into candidates for mastectomy. With the increasing trend toward breast conserving surgery, however, the use of primary systemic therapy was extended to include patients with invasive, early-stage operable tumours. The adoption of NAC has led to increasing rates of breast conserving surgery and provides an opportunity to assess *in vivo* tumour responsiveness to chemotherapeutics.[3] Although previous studies failed to identify any improvement in disease free and overall survival between neoadjuvant and adjuvant therapies, it has been established that patients achieving *complete pathological response* (pCR) to NAC therapy experience improved outcomes, while unresponsive patients or patients with progressive disease during NAC experience worse outcomes.[4–7] Supporting this, it has been shown that the early response to neoadjuvant treatment can predict pCR and therefore may serve as a predictor of long-term outcome.[8,9]

Key words: miRNA, micro-RNA, neoadjuvant chemotherapy, breast cancer, circulating

Abbreviations: ER: oestrogen receptor; Her2: Erbb2 receptor; miRNA: micro-RNA; NAC: neo-adjuvant chemotherapy; NICE: National Institute for Health and Care Excellence; pCR: complete pathological response; PR: progesterone receptor

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Unfortunately at present there is no reliable, clinically validated, method for predicting chemotherapeutic responders from non-responders. While the likelihood of achieving pCR varies greatly by breast cancer subtype (from 7.5% in luminal cancers to 45% in HER2/Triple negative cancers[10,11]), many patients are exposed to the potential morbidity and mortality associated with chemotherapy, without any certainty of benefit from treatment. This has resulted in global efforts to discover breast cancer biomarkers that can predict and detect response to neoadjuvant therapy. Such biomarkers could confer multiple benefits, including tailored patient-care programs, reduced chemotherapy-induced morbidity or mortality and potentially expedite the identification of effective new therapies for the treatment of breast carcinoma. At present, circulating micro-RNAs (miRNAs) represent an important avenue in the search for a non-invasive biomarker for *Breast Cancer Res*ponse prediction and monitoring for neoadjuvant chemotherapy. The evidence for this is discussed further in the following sections.

Micro-RNAs

Micro-RNAs are a naturally-occurring class of short, non-coding RNA molecules ~19 to 25 nucleotides in length. miRNAs have been demonstrated to regulate gene expression at the post-transcriptional level, *via* binding primarily to 3' or 5' untranslated regions of target messenger RNAs (mRNA), leading to inhibition of translation or mRNA degradation.[12] Interestingly in addition to their inhibitory role, miRNAs have recently been demonstrated to facilitate increases in transcript levels, under certain conditions.[13,14]

Since their discovery in 1993, knowledge of the role of miRNAs in regulating gene expression across a spectrum of pathological processes has grown exponentially.[15] It is now recognized that certain miRNAs are highly specific for tissue and developmental stages, exerting a regulatory effect on a myriad of cellular processes including cell development, differentiation, proliferation and apoptosis.[12,14,16] Many miRNA are expressed in tissue- and disease-specific patterns and are known to correlate with clinicopathological features and prognostic indices across a spectrum of pathologies.[17–22] However, to date few studies have investigated the effect of neoadjuvant chemotherapy on miRNA expression patterns in breast cancer.

miRNA biosynthesis and mechanisms of action

miRNA generation is a complex process that commences in the nucleus. miRNA genes are transcribed by RNA polymerase II/III as primary miRNAs (pri-miRNAs). These pri-miRNAs are processed by the Drosha-DGCR8 complex, becoming pre-miRNAs. Pre-miRNAs are transported into the cell cytoplasm by the nuclear export protein Exportin 5, where they are cleaved by the RNase III enzyme Dicer, with either TRBP (Trans-activator RNA-binding protein) or PACT (protein activator of PKR), into a double stranded miRNA duplex. One strand of the duplex represents a mature miRNA and is incorporated into the RNA-induced silencing complex (RISC), while the other strand is degraded. The miRNA:RISC complex (miRISC) then targets mRNA containing complementary sequences to the mature miRNA, inhibiting translation or inducing mRNA degradation (Fig. 1).[12,16,23]

Further to their intracellular function, it has been recognized that miRNAs function at an intercellular level, transmitting information from one cell population to another and inducing changes through this novel extracellular signaling mechanism.[24,25] Although small RNAs were detected in the circulation as early as 2004,[26] it was in 2008 that the presence of miRNAs in the circulation was confirmed, with significant differences in expression patterns detectable between patients with cancer compared to controls.[27,28] These circulating miRNAs were found to be present with remarkable stability, indicating that they must be protected from the digestive action of circulatory RNases. Recent studies have confirmed that miRNAs are transported by a variety of mechanisms that shield them from this RNase degradation, including packaged into membrane-derived vesicles, such as exosomes, bound to lipoproteins and as part of ribonucleoprotein complexes.[24,25,29–31] While some mechanisms regulating the packaging and export of membrane-bound miRNA are understood,[32–34] the process of non-membrane-bound miRNA export from cells remains unclear.[35,36] The exact sources of all circulating miRNA remains unconfirmed. It appears there are two, complimentary, sources of circulating miRNA: (*i*) miRNA released passively into the bloodstream following tissue injury and cell death and (*ii*) miRNA actively exported from cells into the bloodstream. However, in both cases miRNA could be either protein bound "un-encapsulated" miRNA or miRNA protected inside membrane coated vesicles (such as exosomes). Of note, it has been demonstrated that an estimated ≥90% of circulating miRNAs are bound to argonaute-2 (Ago2) containing complexes, with only a minority being transported packaged in vesicles.[30,37] However, controversy regarding the proportions of free and membrane-bound circulating miRNA remains, due to a lack of standardization of sample processing techniques, preventing data from individual studies from being directly compared.

Breast Cancer and Neoadjuvant Chemotherapy

The first successful chemotherapeutic regimen for operable breast cancer was described in 1976 by Bonadonna *et al.*[38] The combination adjuvant therapy with *cyclophosphamide, methotrexate* and *fluorouracil* (CMF) was shown to significantly reduce post-operative recurrence rates. By the early 1990s, *anthracycline*-containing regimens were introduced and are now recognized as superior to treatment with CMF alone.[39] Importantly, it was during this time that administration of chemotherapy in the preoperative or neoadjuvant period began. The *National Surgical Adjuvant Breast and Bowel Project B-27* and the *Aberdeen trial* recognized the addition of a *taxane* to an *anthracycline*-based chemotherapy further reduced the risk of recurrence, and in the neoadjuvant setting improved the rates of complete pathological remission and therefore overall outcome (Fig. 2).[4,40,41]

In recent years, it is recognized that breast cancer is a heterogeneous disease characterised by discrete breast cancer subtypes.[42] While the exact number of subtypes remains to be elucidated, in a landmark paper, Sorlie *et al.*[43] described

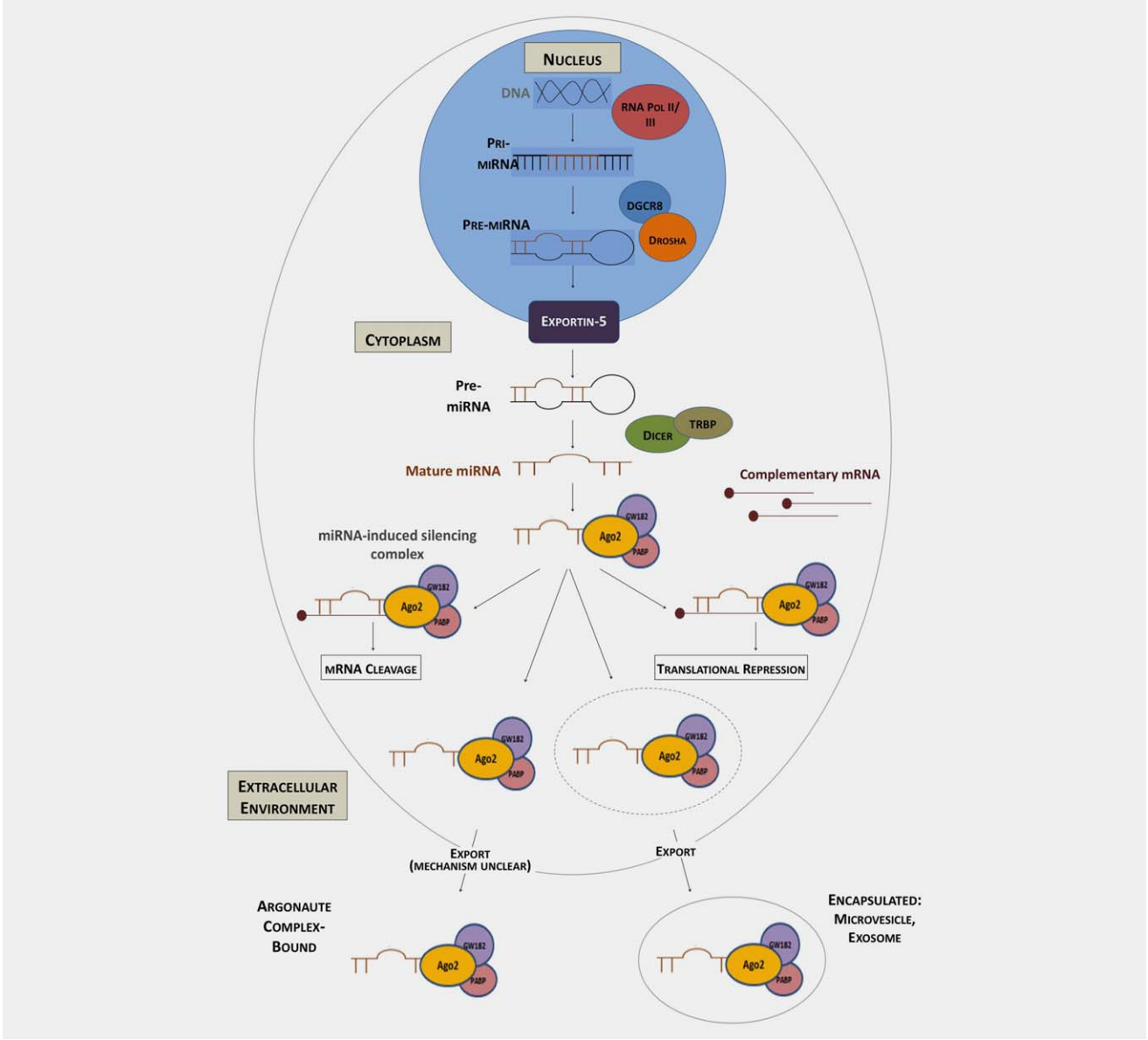

Figure 1. Model of miRNA biogenesis and cellular export.

Luminal A, *Luminal B*, *Basal* (also known as "*triple negative*" tumours) and *HER2* over-expressing (Table 1). More recently, 10 distinct breast cancer subtypes have been proposed, although this stratification is not yet applied clinically.[4]5 Currently utilized breast cancer subtypes have known distinct clinical behaviors and responses to therapy and are stratified according to presence or absence of the *oestrogen receptor* (ER), *progesterone receptor* (PR) and the *human epidermal growth factor receptor 2* (HER2) (discussed further in Current and Potential Molecular Markers Used to Guide the Administration of Chemotherapy section). While no gold-standard chemotherapeutic regimen currently exists for breast cancer, it is generally accepted that an *anthracycline*-based regimen be utilized, with the addition of a *taxane*.[46,47] For patients with breast cancer overexpressing the HER2 receptor, targeted therapy with the humanized monoclonal antibody *Trastuzumab* is recommended by NICE clinical guidelines[48] (Fig. 2; Table 2).

Current and Potential Molecular Markers Used to Guide the Administration of Chemotherapy

The current gold standard molecular markers for *Breast Cancer Res*ponse prediction are the ER, PR and HER2 receptors and the proliferation marker Ki67. The *American Society of Clinical Oncology* guidelines mandate that the immunohistochemical markers ER, PR and HER2 be assessed in all cases of invasive breast carcinoma to guide management decisions, including choice of chemotherapeutic regimen.[53] Further markers such as Claudin and specific miRNAs have been proposed and are presently undergoing further validation (Fig. 3).[54]

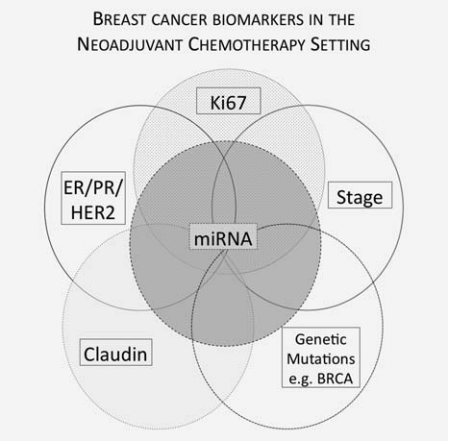

Figure 2. Breast cancer biomarkers in the neoadjuvant chemotherapy setting. Currently used clinical biomarkers (solid lines); new potentially clinically relevant biomarkers (dotted lines).

ER/PR receptor

ER/PR expression, as identified by immunohistochemistry, provides an index for sensitivity to endocrine treatment and acts as a marker of chemosensitivity. Approximately two thirds of tumours display ER-positivity, correlating with improved responsiveness to endocrine therapy and improved patient outcomes. PR expression is strongly dependent on ER expression, with $<1\%$ of breast cancers displaying sole PR-positivity. In this instance, limited benefits from endocrine therapy have been described.[55] Associating chemotherapeutic treatments and ER/PR status, ER positivity correlates with poor tumour response.[56] However, ER-negative tumours are more likely to achieve complete pathological remission and thus experience improved outcomes with chemotherapy.[57]

HER2 (ERBB2)

HER2 is a membrane tyrosine kinase receptor that upon activation affects cell proliferation and survival.[58] It is located on chromosome 17q12 and is an oncogene, amplified in $\sim$15 to 20% of breast cancer cases. Initially identified as a prognostic marker, HER2 overexpression is associated with increased relapse rates, increased incidence of metastases and worse overall survival.[59,60] However, the development of therapies specifically targeting HER2 has resulted in significant improvements in outcomes for patients with HER2-positive breast cancer.[61] Risk of relapse and death are reduced by approximately 50% and 30% respectively, improving disease-free and overall survival.[62] In 2013 the addition of Trastuzumab to neoadjuvant chemotherapy in patients with HER2-positive tumours was found to double complete pathological remission rates (compared to chemotherapy alone) and was associated with a longer event free survival.[63] Most recently, use of neoadjuvant followed by adjuvant HER2 has demonstrated sustained benefit in event-free survival and a strong association with complete pathological remission.[64]

Basal/triple negative breast cancers

"Basal" and "triple negative (TNBC)" breast cancer subtypes overlap greatly in terms of their immunophenotype (ER, PR & HER2 negative), aggressive clinical behavior and increased prevalence in younger, African-American patients. However, they are not synonymous, as not all basal cancers determined by gene expression analysis lack ER, PR and HER2 and not all triple-negative cancers show a basal phenotype by expression array analysis.[65] Due to a more aggressive clinical pathology, both of these subtypes are associated with a higher risk of mortality. Both subtypes lack all known effective biomarkers and therefore targeted therapies. However, both subtypes are highly sensitive to neoadjuvant chemotherapy.[66–68] The highest rates of complete pathological remission have been achieved in TNBC tumours utilising a neoadjuvant regimen of docetaxel, doxorubicin and cyclophosphamide, while the addition of bevacizumab is expected to increase this rate further.[63]

Ki67

Ki67 is a nuclear non-histone protein utilized as a marker of proliferation, as it is absent in quiescent cells, yet universally expressed among proliferating cells. Immunohistochemical staining of, Ki67 expression levels is associated with the percentage of tumour cell nuclei positively stained and are used to determine a Ki67 score. In early and advanced breast cancer the Ki67 score can predict the response to chemotherapy.[69] A high pre-treatment score is associated with a good chance of complete pathological remission to therapy and therefore improved long-term outcome.[70] Lee *et al.*[71] describe a significant decrease in Ki67 index following neoadjuvant chemotherapy, a finding that is recognized as a strong predictor of recurrence-free and overall survival. Of concern regarding the use of Ki67 is the lack of standardization of analytical practice, with laboratories utilising differing cut-off points to differentiate between "high" and "low" Ki67. Further to this, as with all of the above described markers, determination of expression levels requires tumour tissue, mandating invasive sampling techniques, which further emphasizes the need for a non-invasive breast cancer biomarker.

Claudins

More recently, a potential fifth subtype of breast cancer has been described, classified as "Claudin-low."[72,73] Claudins are a family of proteins that function in tight-junctions and cell-cell adhesion, including Claudin 3, 4, 7 and E-cadherin. A new subtype of breast cancer, "Claudin-low" has recently been described, characterised by lack of expression of the Claudin proteins.[72,74] Claudin-low tumours are typically triple-negative and display high expression of epithelial-to-mesenchymal transition (EMT) markers.[75] The expression of EMT markers has known associations with resistance to therapeutics and higher metastatic potential.[76] Claudin-low cancers display lower complete pathological remission rates following NAC. Overall Claudin-low cancers were found to

Table 1. Breast cancer subtypes with receptor status and prevalence[44]

Breast cancer subtype	ER	PR	HER2	Prevalence (%)
Luminal A	+	±	−	40
Luminal B	+	±	+	20
HER2	−	−	+	15–20
Basal/triple negative	−	−	−	10–15

This table outlines the prevalence and receptor status of current clinically utilized breast cancer subtypes.
Abbreviations: ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

have an intermediate prognosis, worse than basal-like breast cancer, but better than luminal cancer.[72] However, the "Claudin-low" breast cancer subtype remains poorly described.[77] Further definitive characterization is required before it is fully accepted into clinical practice.

miRNA

In 2005, genome-wide miRNA expression analysis enabled identification of miRNAs that were differentially expressed in breast cancer tissue.[78] The panel of 29 miRNAs identified differentiated tumours from normal tissues with an accuracy of 100%. Importantly, miRNA expression correlated with distinct tumour phenotypes, ER and PR expression and tumour stage. More recently, a miRNA expression pattern of 31 miRNAs evaluated in 93 tumour samples was found to predict hormone receptor status, and thus classify tumours by genetic subtype.[79] These findings were independently corroborated by Lowery *et al.*[20] who profiled 453 miRNAs in 29 early-stage breast cancer specimens, identifying a distinct panel of miRNAs corresponding to expression of ER, PR and HER2.

Translating these tissue findings into the circulation, it was found that miR-195 expression was significantly elevated in breast cancer patients ($n = 148$) compared to controls ($n = 44$), and that these levels reduced postoperatively.[80] Furthermore, high levels of circulating miR-21 and miR-10b were found to be associated with ER negativity, thus a poorer prognosis.[80] In a further study, elevated miR-155 expression was associated with PR positivity.[81]

The ability of miRNA expression profiles to classify breast tumours by biopathologic variables currently utilized to determine responsiveness to neoadjuvant chemotherapy highlights the potential of miRNA signatures as novel predictive and prognostic biomarkers that could allow individualization of breast cancer treatment and improved selection of patients for neoadjuvant chemotherapy (Fig. 3).

miRNA as Novel Biomarkers of NAC Response

The role of miRNA in neoadjuvant chemotherapeutic response prediction and monitoring has been investigated across a variety of pathologies. In colorectal carcinoma an association between miRNAs and tumour response to neoadjuvant chemoradiotherapy was proposed.[82] This association was confirmed by identification of a distinct miRNA expression signature that could effectively predict colorectal cancer response to neoadjuvant chemoradiotherapy.[83] In human gastric cancer, decreased let-7i expression was found to have a significant association with a poorer response to chemotherapy and shorter overall survival.[84] In breast cancer the ability of a panel of miRNAs to predict response of triple negative breast carcinoma to neoadjuvant chemotherapy was investigated.[85] Although study numbers were limited (11 patients), results indicated higher miR-200b-3p and miR-190a expression and lower miR-512-5p expression was associated with a better pathologic response to chemotherapy.

While all the above findings involved miRNA analysis from tumour samples, some peri-neoadjuvant studies of circulating miRNA have also been conducted. One study investigated miRNA extracted from the sera of stage II-III locally advanced and inflammatory breast carcinoma patients preneoadjuvant chemotherapy.[86] A two-gene signature of miR-375 and miR-122 was identified with the ability to predict metastatic disease relapse with a sensitivity of 80% and specificity of 100%. Patients relapsing following neoadjuvant chemotherapy were found to have significantly up-regulated expression of miR-122 while patients with higher circulating miR-375 experienced a good clinical outcome. Fluctuation within a panel of miRNA was also found in patients with primary operable or locally advanced breast cancer receiving neoadjuvant chemotherapy.[87] Of a panel of eight miRNAs, miR-221, miR-195 and miR-21 were noted to decrease most significantly with the administration of chemotherapeutics, although correlation with response to systemic therapy was not conducted. A further study recognized expression of two particular miRNAs to be induced by treatment with chemotherapeutics, namely miR-34a and miR-122. Elevated expression of these miRNAs was detected both in tumour tissue and serum, and was particularly associated with anthracycline-based regimens in patients achieving partial response to neoadjuvant chemotherapy.[88]

Some interesting *in vitro* findings that show promise for translation to the clinical setting have been conducted. Investigating targeted therapies for triple negative breast cancers, the overexpression of miR-181a/b was found to associate with more aggressive breast cancer subtypes.[89] Utilizing a range of cell lines (MDA-MB-231, HEK 293GP, MDA-MB-468, SUM159PT, OVCAR, HT29, PANC1 and Sk-Br-3) the overexpression of miR-181a/b was found to dampen the DNA damage response, thus increasing the sensitivity of the triple negative cells in which it was expressed to poly (ADP-Ribose) polymerase-1 (PARP-1) inhibition. It was proposed that profiling miR-181a/b expression in patients with triple negative breast cancer could identify patients for PARP-1 inhibition or platinum-based chemotherapy.

miRNA and chemoresistance

It is recognized that specific miRNA expression signatures are associated with resistance to all forms of breast cancer treatment, including chemotherapy, anti-endocrine therapy and radiotherapy.[90–94] Regarding chemotherapeutic

Table 2. Chemotherapeutics used to treat breast cancer

Drug class	Mechanism of action	Example	Reference
Anthracyclines	Inhibition of DNA and RNA synthesis Disruption of DNA damage response Inhibition of Topoisomerase II	Doxorubicin Epirubicin Mitoxantrone	49
Taxanes	Disruption of microtubule function	Docetaxel Paclitaxel	50
Alkylating Agent: Nitrogen Mustard	Interference with DNA replication	Cyclophosphamide	40
Anti-metabolites	Prevention of folate use for DNA generation	Methotrexate Fluorouracil Capecitabine	51
Anti-HER2/EGFR	Tyrosine kinase inhibition Arrest of cell cycle Suppression of angiogenesis	Trastuzumab Pertuzumab Lapatinib	52

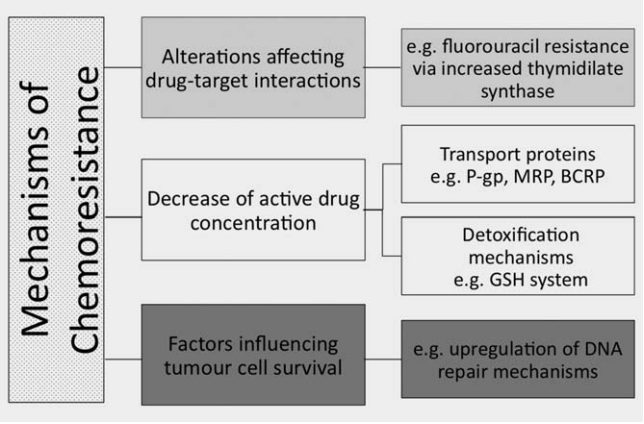

Figure 3. Mechanisms of chemoresistance.

resistance, several recent reports have revealed the key regulatory role of miRNAs affecting drug resistance proteins and targeting proteins involved in apoptosis (Table 3, Fig. 3).

In the circulation, an association between serum miR-125b levels from patients with invasive ductal breast carcinoma receiving NAC and chemoresistance has been described.[105] Increased expression of miR-125b was found to have a significant association ($p = 0.008$) with non-response to chemotherapy. Further to this, forced miR-125b overexpression in breast cancer cells *in vitro* increased chemotherapeutic resistance, with subsequent reduction in miR-125b levels sensitizing the cells to chemotherapy once more. Similar results were observed regarding miR-210, where increased plasma miR-210 levels correlated with a reduced sensitivity of HER2 breast carcinoma to *Trastuzumab* therapy. High pre-treatment circulating mir-210 was found to be associated with lower pCR rates and lymph node metastasis. Recently the radiological and clinical response of breast cancer to either neoadjuvant chemotherapy or hormonal therapy was assessed using a low density miRNA array. Significantly increased Let-7a was found in the plasma of patients achieving a radiological response following neoadjuvant chemotherapy, but not hormonal therapy.[117]

In breast cancer tissue, down-regulation of miR-200c was found in patients who were non-responsive to NAC.[110] Subsequently, up-regulation of miR-200c in human breast cancer cell lines enhanced chemosensitivity and decreased expression of multi-drug resistance (MDR) proteins P-glycoprotein (P-gp) and MDR-associated protein (MRP-1).

Utilizing breast cancer cell lines, differential miRNA expression has been noted to correlate with resistance to chemotherapeutics. Using MCF-7/MX100 cell miR-328 was identified as a negative regulator of breast cancer-resistance protein (BCRP), with higher miR-328 levels facilitating an improved Mitoxantrone response.[118] Up-regulated miR-19 (in MCF-7/TX200, MCF-7/VP-17, MCF-7/MX100 and MCF-7/WT cell lines) correlated with overexpression of three MDR-related transport proteins (MDR-1, MRP-1 and BCRP). Importantly, miR-19 inhibitors decreased the expression of these MDR proteins.[96] MiR-451 was also found to regulate expression of MRP-1, with up-regulation of miR-451 in doxorubicin-resistant MCF-7 cells returning chemotherapeutic sensitivity.[92] Mir-326 exhibited the same effect on MRP-1, inducing sensitivity to doxorubicin in MDR MCF-7 cells.[119] Further miRNAs noted to target MRP-1 include miR-345 and miR-7, which were found to decrease cellular levels of MRP-1.[95]

In BT474, SKBR3, and MDA-MB-453 breast cancer cell lines miR-21 conferred resistance to Trastuzumab, *via* down-regulation of its target PTEN.[97] Subsequently, this pathway was also found to modulate resistance to doxorubicin in doxorubicin-resistant MCR-7 cell lines.[98] In addition, miR-137 was found to be down-regulated in MDR MCF-7 cells.[107]

Cells lines can provide conflicting data however. MCF-7 and MDA-MB-231 cells with acquired docetaxel resistance, showed increased expression of miR-34a, with miR-34a inhibition enhancing chemotherapeutic response in docetaxel-resistant MCF-7 cell lines.[102] However, decreased levels of miR-34a were described in the Adriamycin-resistant MCF-7 cell line,[104] with forced overexpression of miR-34a found to increase chemotherapeutic sensitivity of Adriamycin-resistant MCF-7 cell lines.[103] The exact role of miR-34a in breast cancer chemotherapeutic response is not fully understood and requires

Table 3. miRNAs with a validated involvement in chemotherapeutic resistance in breast cancer

miRNA	Expression	Target(s)	Drug Ass[n]	Source (# patient samples)	Reference
miR-7	Down-regulation	MDR-1	Cisplatin	Cell line	95
miR-19	Up-regulation	MDR-1, MRP-1 and BCRP *via* PTEN	Paclitaxel Mitoxantrone VP-16	Cell Line	96
miR-21	Up-regulation Up-regulation	PTEN PTEN	Doxorubicin Trastuzumab	Cell Line	97, 98
miR-25	Up-regulation	Inhibits autophagic cell death	Epirubicin	Cell Line	99
miR-30c	Down-regulation Down-regulation	YWHAZ TWF1, IL-11	Doxorubicin Paclitaxel Doxorubicin	Cell Line Human breast tissue (n = 51) Cell Lines	100 101
miR-34a	Up-regulation Down-regulation Down-regulation	BCL-2, Cyclin D1 Notch-1 E2F3, PXR	Docetaxel Adriamycin Doxorubicin	Cell Line Cell Line Cell Line	102 103 104
miR-125b	Up-regulation Up-regulation	E2F3 Bak 1	5-Fluorouracil Paclitaxel	Blood Serum Cell Line	105 106
miR-137	Down-regulation	P-glycoprotein, *via* YB-1	Vincristine Doxorubicin Paclitaxel	Cell Line	107
miR-149	Down-regulation	NDST1	Adriamycin	Cell Line	108
miR-155	Up-regulation	FOXO3a	Doxorubicin VP-16 Paclitaxel	Human Breast Tissue (n = 126)	109
miR-200c	Down-regulation Down-regulation	P-glycoprotein MDR mRNA TrkB, Bmi1	Doxorubicin Doxorubicin	Human Breast Tissue (n = 39) Cell Line Cell Line	110 111
miR-210	Up-regulation	Not studied	Trastuzumab	Blood Serum (n = 43) Cell Lines	91
miR-221	Up-regulation	Not studied	Adriamycin	Blood Plasma (n = 125)	112
miR-288	Down-regulation	MDR-1, P-glycoprotein	Doxorubicin	Cell Line	113
miR-320a	Down-regulation	TRPC5, NFATC3	Adriamycin Paclitaxel	Cell Line	114
miR-345	Down-regulation	MDR-1	Cisplatin	Cell Line	95
miR-451	Down-regulation	P-glycoprotein, MDR-1	Doxorubicin	Cell Line	92
miR-489	Down-regulation	Smad3	Adriamycin	Cell Line	115
miR-663	Up-regulation	HSPG2	Adriamycin	Cell Line	116

This table presents an overview of miRNAs that have a validated role in chemotherapeutic resistance, referencing the drug and source examined and the identified miRNA targets.
Abbreviations: HSPG2: heparin sulfate proteoglycan 2; Smad3: mothers against decapentaplegic homolog 3; NDST1: GlcNAc *N*-deacetylase/*N*-sulfotransferase-1; TRPC5: transient receptor potential channel C5; NFATC3: nuclear factor of activated T-cells isoform C3; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; PXR: pregnane X receptor; BCL-2: B-cell lymphoma 2; YB-1: Y-box binding protein-1; MRP-1: multidrug resistance-associated protein-1; BCRP: breast cancer resistance protein; TWF1: Twinfilin 1; IL-1: interleukin-1; TrkB: tyrosine receptor kinase type 2; Bmi1: B-cell-specific Moloney murine leukemia virus integration site 1; Bak 1: Bcl-2 antagonist killer 1.

further investigation. This seemingly contradictory data highlights the complex nature of miRNAs and their role in chemoresistance, particularly in relation to taxane resistance.[120]

miRNAs as Targeted Therapies in the Neoadjuvant Setting

The use of miRNAs in the treatment of breast cancer includes using miRNAs as therapeutic treatments and manipulating miRNA expression to enhance existing treatments. As miRNAs function as oncomirs and tumour suppressors, two therapeutic potentials exist: overexpression of targeted miRNAs (miRNA replacement therapy) or down-regulation (silencing) of miRNAs.

Oncomir inhibition

Antisense-inhibition of miRNA activity can be achieved by using miRNA antagonist oligonucleotides (anti-miRs),

locked-nucleic acids (LNA), or targeted miRNA silencing (antagomiRs).[121] The efficacy of this targeted therapy has been demonstrated by several studies. MCF-7 cells transfected with anti-miR-21 oligonucleotides were grown *in vitro* and in a xenograft murine model. Anti-miR-21 suppressed both cell growth *in vitro* and tumour growth in the mouse model. In addition, cell growth inhibition was associated with increased apoptosis and decreased cell proliferation.[122]

In the chemotherapeutic setting, down-regulation of mir-21 was seen to increase sensitivity of MCF-7 cells to taxol therapy,[123] with miR-203 knockdown increasing cisplatin sensitivity.[124] Increased sensitivity in response to miRNA knockdown has also been demonstrated, in HS578T cells whereby by miR-155 down-regulation (using antisense-*miR-155* oligonucleotides) increased apoptosis in response to treatment with Paclitaxel, Doxorubicin and VP-16.[109]

miRNA replacement therapy

This strategy involves the reintroduction of function of a tumour suppressing miRNA.

In NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice, SK-3rd cells over-expressing Let-7 or miR-30 displayed significantly reduced tumourigenicity and lung and liver metastasis.[125,126] In a further study in chemoresistant MDA-MB-231 and BT-549 cells, overexpression of miR-200c was found to restore chemosensitivity to microtubule-directed agents.[127]

Although rapid and continual advancements are being made regarding the manipulation of miRNAs for the treatment of breast cancer, this area of research remains in its infancy, with many obstacles to overcome prior to mainstream implementation in breast cancer therapeutics. The majority of studies conducted to date are *in vitro,* examining miRNAs and their effects in various cell lines. To validate these studies, large clinical trials are required to support these preliminary findings. Present obstacles to overcome include the identification of optimal delivery methods and the prevention of off-target effects and safety optimization.

Discussion

The management of breast cancer, in terms of diagnosis, chemotherapeutics and surgical intervention, continues to adapt in line with translational research and evidence. Whilst the use of chemotherapy in the neoadjuvant setting is now acceptable for any patient considered a candidate for adjuvant therapy,[128] there currently exists no clinically validated means of differentiating chemotherapeutic responders from non-responders. While miRNA expression analysis holds significant promise in this setting, further studies investigating miRNAs in the neoadjuvant breast cancer setting are undoubtedly warranted. Presently, as per ClinicalTrials.gov, two clinical trials are currently recruiting breast cancer patients undergoing neoadjuvant chemotherapy for miRNA profiling and analysis.

A further challenge affecting the use of circulating miRNA as breast cancer biomarkers is the lack of accepted standardized protocols for sample collection, handling and processing. As a consequence, many previous studies cannot be easily compared. This is due to intrinsic differences in: Patient cohorts (treatment regimes, timing of sample collection), Biofluid collected and analyzed (whole blood, plasma, serum), Collection methods (EDTA, Paxgene tubes, sample handling), Processing/extraction techniques (diverse extraction kits, timing of extraction), Investigation of miRNA pool (total, free or membrane bound), Use of multiple non-standardized endogenous controls and Detection methods utilized [arrays (which are constantly being updated) or different RQ-PCR platforms].[129–132] The development and adoption of a standardized set of operating and technical protocols would facilitate study comparison, improved reproducibility and the development of improved targeted future studies.

The use of miRNAs as targeted therapies, although in its infancy, holds immense promise, although further evaluation and validation of current findings are required. The identification of a biomarker that could predict or potentially monitor tumour response to neoadjuvant chemotherapy could revolutionize the manner in which chemotherapeutics are administered, bringing us ever closer to personalized breast cancer management.

Future directions

In addition to miRNAs, dysregulation of miRNA machinery can play a crucial role in cancer initiation and progression.[133] miRNA-binding proteins mediate miRNA-dependent cleavage or degradation of target mRNAs, with all miRNAs studied to date assembling into miRNA-silencing complexes. Studies have shown that genes involved in miRNA biogenesis are dysregulated in various breast cancer subtypes.[79] Down-regulation of Drosha and Dicer, two key elements of the miRNA machinery, has been associated with more aggressive breast cancer subtypes.[134,135] Furthermore, increased expression of exportin-5, a pre-miRNA transporting nuclear receptor, has been associated with increased breast cancer susceptibility.[136] In addition, expression of the key miRNA-binding protein Argonaute-2 protein (Ago2), required for miRNA extracellular transport, is elevated in basal-like breast cancer subtypes, with elevated Ago2 levels producing enhanced proliferation, reduced cell-cell adhesion and increased migratory ability,[137] implicating Ago2 in more aggressive breast cancer subtypes. Further to this, single-nucleotide polymorphisms of Ago2 have been associated with disease free and overall survival in breast cancer.[138]

The use of miRNA machinery genes as breast cancer biomarkers is still in its infancy, however, with further investigation required to fully elucidate mechanisms of miRNA maturation, miRNA-machinery gene regulation and the cancer-specific functions of these miRNA machinery genes and their resultant proteins.

Conclusion

Whilst breast cancer management continues to improve the requirement of a breast cancer biomarker that is both predictive and prognostic remains. Investigation of the potential for

miRNAs to fulfill this role holds much promise, although further clinical studies are required, particularly in the neoadjuvant chemotherapeutic setting.

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