Review

Circulating microRNAs for the prediction of metastasis in breast cancer patients diagnosed with early stage disease

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Abstract

Breast cancer is the second most common malignancy diagnosed in women worldwide. The greatest cause of breast cancer mortality is development of metastasis. For many women metastasis is an early event in breast cancer which goes undetected until its presentation, thus there is an urgent need for the development of biomarkers to predict those patients at greatest risk. The expression of a group of small non-coding RNAs, termed microRNAs, has been shown to be altered in tumours. Furthermore, microRNAs identified as being highly expressed in breast cancer tumours can also be detected in the circulation. Circulating microRNAs are an emerging field of biomarker research which have the benefit of being able to be obtained non-invasively and analysed rapidly and relatively cheaply. Here the potential use of circulating miRNAs to detect metastasis in discussed and the current barriers to their progression to the clinic.

Keywords: microRNA; Breast cancer; Metastasis; Prognosis; Biomarker

Introduction

Breast cancer is the second most common malignancy diagnosed worldwide, accounting for approximately 23% of all cancer diagnoses per year and 465,000 cancer-related deaths [1]. These cases will include early stage disease, local recurrence or metastasis in patients previously treated with a curative intent, and advanced stage disease, characterised by the significant spread of the cancer within the breast or systemically [1,2]. The metastatic cascade initiates with localised invasion of surrounding tissue, followed by systemic spread via the blood and lymphatic system and finally dissemination of tumour cells to other organs [3]. At the point of diagnosis up to 5% of patients present with metastatic disease and of those patients who show no lymph-node involvement at diagnosis, approximately 30% will develop metastases [4,5]. This subset of individuals who potentially have metastasis of early disease (MED) represent an at risk group, who may benefit from additional monitoring and augmented treatment strategies. We currently need markers to distinguish these patients from those who do not go on to develop disease recurrence.

Clinical staging and molecular classification

Clinical staging is essential for establishing surgical approaches and treatment regimes. The current TNM system assess primary tumour size and extent of invasion (T), the absence or presence of palpable axillary lymph nodes and indications of local invasion (N), and evidence of distant metastasis (M) [6]. The TNM system is further supplemented by the allocation of stages I–V depending on size and metastatic spread [6]. Molecular factors are also employed to assist prognosis and directing treatment strategies. Breast cancers are categorised based on the expression of oestrogen and progesterone receptors (ER and PR) and the receptor tyrosine kinase erbB-2 (ERBB2 or HER2). Further categorisation defines four molecular subtypes [Refs. 33, 34]: luminal A which accounts for ~50–60% of cases, luminal B accounting for 10–20%, HER2[+ve] represents a further 15–20% and between 10 and 20% of cases are classified as a basal subtype [7,8].

The Nottingham Prognostic Index (NPI) provides further prognostic information based on tumour size, lymph-node (LN) stage and tumour grading [9]. The NPI index categorises patients into 6 groups ranging from the very poor prognostic group (NPI > 6.4) to the excellent prognostic group (NPI < 2.4) [10,11]. The development of the NPI + index is set to further improve stratification of patient outcome, by combining assessment of the molecular class of tumours (7-classes defined) with clinicopathologic variables [12]. Current web based tools such as Adjuvant! Online and Predict combine NPI and receptor status to provide both prognostic information and projected benefits of adjuvant therapy based on population data.

Recurrence and metastasis

Recurrence are defined as local: within a conserved breast or chest wall, locgo-regional: often in the axilla or supraclavicular fossae, or systemic (metastatic): common sites are bone, lungs, liver and brain [3]. Risk of recurrence is linked with stage, hormone receptor–negative status, or poorly differentiated histologic tumour grade [13], recurrence peaks at 2–3 years and again at 7–10 years post-surgery, though this second peak has not been consistently detected in all studies [13–15]. Metastasis is believed to often be an early event in breast cancer, with disseminated cancer cells remaining dormant for many months or years, providing a window of opportunity for detection prior to the re-initiation of cancer cell growth and detectable metastatic tumours [16]. Of the individuals diagnosed with breast cancer only a minority present with metastatic disease, nevertheless nearly 30% of individuals initially diagnosed with early stage disease will develop metastatic lesions [4]. This subset of individuals who potentially have metastasis of early disease (MED) represent an at risk group, who may benefit from additional monitoring and augmented treatment strategies. Whilst models such as Adjuvant! Online exist to provide prognostic information and projected treatment benefits, these models rely on population-based data, lacking the means to provide individual specific information on recurrence.

Individual-specific assessment

The advent of high-throughput sequencing and molecular profiling techniques has led to a rise in personalised treatment and management of cancer. The effective implementation of highly specific targeting therapies requires the parallel development of methods to assess an individual's future disease risk and response to treatment.

Oncotype Dx

Oncotype Dx is a currently available test to predict the recurrence risk for ER-positive, HERer-2 negative breast cancer. The test examines the expression of sixteen signature genes and utilises five housekeeping genes for normalisation. The test calculates recurrence scores within a range of 1–100. Patients with scores of 1–17 are considered to be at low risk of recurrence and are predicted to respond well theto hormonal therapy but derive little benefit from chemotherapy [17,18]. Patient scores of 31–100 are categorised as being at high risk of recurrence and likely to benefit greatly from chemotherapy. The predicted benefit of chemotherapy is currently unclear for patients whose scores fall between these categories (18–30) [17,18]. The National Institute of Health TAILORx study (Trial Assigning IndividuaLised Options for Treatment Rx) has been designed to ascertain the benefits of chemotherapy for this midrange patient group, a report of the trials results are expected in 2015 [18].

Mammaprint

Mammaprint, similar to Oncotype Dx, ranks the expression of breast cancer associated genes to allocate patients into high or low recurrence groups and can predict the patient-specific benefit of chemotherapy [17]. Mammaprint analyses a signature of 70 genes and can be used for both ER positive or negative, node positive or negative breast cancers up to 5 cm in size [18]. Unlike Oncotype Dx which utilises formalin fixed paraffin embedded material, Mammaprint requires fresh-frozen tissue which has limited its use [18].

Oncotype Dx and Mammaprint will likely make significant contributions to the management of breast cancer, but both depend upon the analysis of primary tumours to predict recurrence and do not indicate the anatomical site or presentation of potential metastases.

Circulating markers

Circulating tumour cells

Tumour cells have been observed to circulate within the blood of breast cancer patients, and the presence of these circulating tumour cells (CTCs) correlates with metastatic development [19,20]. However, their causal nature is yet to be determined [21]. Despite a lack of certainty over the CTC-metastasis causality, there has been significant growth in studies aimed at identifying circulating markers that may provide prognostic and/or predictive information regarding disease progression, treatment response and metastasis.

The capability of tumour cells to disseminate to distant organs at early stages of tumour progression has recently been demonstrated [19]. Cancer cells transitioning via the peripheral circulation are termed circulating tumour cells (CTCs) and their detection is associated with decreased progression-free survival [22]. Methods of detection have been challenging due to the low ratio of CTCs to haematological cells ($1:10^6$ respectively) [20]. Isolation using affinity purification via CTC surface-antigens is one method which has proved effective and has resulted in the first FDA-approved assays system. Alternative methods include sedimentation, high-throughput microscopy and qRT-PCR [23–25]. Analysis of CTC RNA profiles has uncovered surrogate markers of CTCs, such as miR-200b, which may prove easier to detect and beneficial in distinguishing CTC-positive and negative patients [26].

Circulating nucleic acids

Tumour and healthy tissue contribute circulating nucleic acids (ctNA) into the blood stream, sources include the primary tumour, CTCs, disseminated tumour cells and micrometastases, hematopoietic cells, stromal cells and other non-cancer cell types [27]. It is estimated that up to 3.3% of tumour DNA enters the circulation daily and the dynamic nature of these molecules has garnered much interest in their potential as biomarkers [28].

Circulating DNA (ctDNA) may prove to be a useful biomarker to monitor response to treatment and minimal residual disease. Studies have shown that within 24 h of surgical resection, the levels of tumour specific ctDNA detectable in patient plasma

decrease dramatically, in accordance with the significant decrease in tumour burden [29]. Furthermore, the persistence of tumour specific ctDNA following mastectomy has also been found to associate with prognostic and histological parameters which indicate the potential presence of undetectable residual disease [30]. Detection of ctDNA has been demonstrated to have a larger dynamic range and greater correlation with changes in tumour burden than CTCs or the cancer antigen 15-3 (CA 15-3) serum biomarker [31]. Such specific and sensitive detection of changes in tumour burden will likely have significant advantages over current imaging technologies which rely on visible changes to tumour size.

Circulating RNA (ctRNA) is typically contained within exosomes and vesicles or protein bound complexes, which are shed from non-cancerous cells and tumour cells into the circulation (illustrated in Fig. 1) [32,33]. Packaging complexes protect RNA from degradation, making the ctRNAs relatively stable [32]. Messenger RNA transcripts (mRNA) may prove useful within the context of other diagnostic or prognostic markers, e.g. the level of *CCND1* mRNA (encoding cell-cycle protein 1) can be used to delineate patients with poor-overall survival within good prognosis groups [34]. The use of ctRNAs will likely be further advanced through the development of assays to detect tumour specific transcripts. In addition to mRNAs, circulating populations of microRNAs have also attracted significant attention as prospective biomarkers for breast cancer.
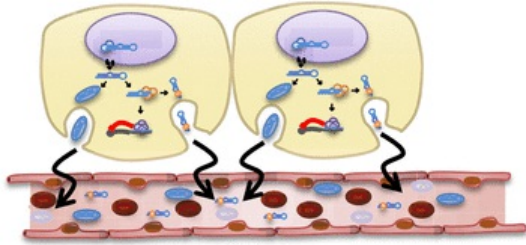

Fig. 1 Entry of microRNAs into the circulation. MicroRNAs (miRNAs) secreted from their cell of origin enter the circulation packed into exosomes or associated with RNA binding proteins, both of which allow them to enter the blood stream protected from degradation.

MicroRNAs

MicroRNAs (miRNAs) are short non-coding RNAs of typically 22 nucleotides in length. MiRNAs are able to silence gene expression by targeting partially complementary regions of mRNAs and inhibiting protein translation [35]. As illustrated in Fig. 2; miRNAs are transcribed as RNA precursors known as pri-miRNAs, these are cleaved by the endonuclease Drosha into pre-miRNAs of around 80 nucleotides in length, which are then transported into the cytoplasm via the exportin 5 pathway [36]. Once in the cytoplasm the pre-miRNA is cleaved by a second endonuclease Dicer creating double stranded RNA segments [35]. One strand of the RNA duplex assembles with an Argonaute protein and forms part of the RNA induced silencing complex (RISC) [35]. The miRNA recruits the RISC complex to mRNAs that exhibit accessible, fully or partially complementary target sequences, resulting in post-transcriptional silencing [35,37]. These highly conserved miRNA molecules function degenerately to negatively regulate a plethora of biological functions and have the potential to contribute to characteristics of cancer such as rapid proliferation, migration and resistance to treatment [38].
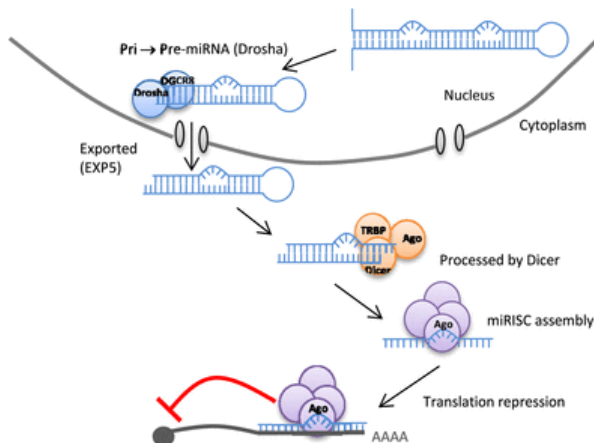

Fig. 2 Biogenesis of microRNAs (miRNAs) from transcription to silencing. The transcribed pri-miRNA is processed in the nucleus into ~ 70-nt stem loops (pre-miRNA) by Drosha and its interacting partner DGCR8. The pre-miRNA is actively transported into the cytoplasm by exportin-5. Once in the cytoplasm, the pre-miRNAs are further processed by Dicer and TRBP into short RNA duplexes, one strand of the duplex is incorporated into the RISC complex by Argonaute (Ago) proteins. The resulting complexes then target mRNAs to silence translation.

Several miRNA types have been reported as potential breast cancer prognostic markers, with many of those showing specificity and sensitivity to indicate metastatic disease, others correlate with treatment response and may have significant utility as

predictive markers [39–45]. This review aims to evaluate recent studies of circulating miRNAs (ct-miRNAs) to identify their potential to distinguish the MED sub-group of breast cancer patients.

Circulating microRNAs as markers and predictors of metastasis

The potential use of ct-miRNAs as biomarkers has been popularised by the ease of sampling via the blood and their stability once sampled. Global and focussed screening approaches have been used to identify ct-miRNAs which are differentially regulated between patient subgroups. In patients with metastasis elevated miR-10b was the first significantly altered miRNA to be reported [40]. Subsequent studies confirmed this finding and also identified elevation of miR-34a and miR-155 in patients with metastatic disease, and increased miR-10b and miR-373 expression in lymph-node positive patients compared to patients with negative nodal involvement or healthy controls [39,46]. More recently, evidence for miR-10b as a biomarker specific for brain and bone metastasis has been presented [45,47]. In addition, dramatic changes in circulating miR299-5p, miR-411, miR-215 and miR-452 expression have been reported in metastatic patients and increased expression of miR-20a, miR-214 and miR-210 in lymph-node positive patient subgroups [42,48,49].

The correlation between the presence of CTCs and breast cancer progression has also promoted the search for surrogate markers to detect patient subsets with a CTC component. Madhavan et al. identified eight miRNAs (miR-375, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, miR-801) with significantly elevated expression in CTC-positive patients and found miR-200b as a potential prognostic marker for progression-free and overall survival [26]. The miR-200 family of miRNAs has also been shown to promote metastatic development in xenograft breast cancer models, through the transfer of extracellular vesicle bound miR-200 family members from highly metastatic to low/non-metastatic breast cancer cells [50].

A study of stage II-III locally advanced and inflammatory breast cancer patients determined groups of ct-miRNAs which associate with ER/PR/HER2 status and two miRNAs, miR-375 and miR-122, which strongly correlated with clinical outcomes. Progression of the study with independent patient cohorts demonstrated the specific prediction of metastatic recurrence in stage II and III patients from elevated circulating miR-122 levels [51]. The triple-negative breast cancer (TNBC) sub-type is associated with early tumour recurrence and poor prognosis [52]. Analysis of circulating miRNAs in the serum of 60 treatment naive primary ductal TNBC patients gave rise to the development of a signature of 4 miRNAs (miR-18b, miR-103, miR-107 and miR-652) capable of distinguishing tumours from patients with early relapse from those without recurrence, making this signature a potential risk predictor for MED [53]. Interestingly, three of the four miRNAs have been linked with tumour mechanisms in breast cancer, miR-103 and miR-107 have been implicated in DNA repair and epithelial to mesenchymal transition, whilst ER is a target of miR-18b [54–57].

MicroRNAs expressed in metastatic breast cancer

A significant correlation is yet to be established, between miRNA biomarkers detected in either metastatic samples or primary tumour tissues from patients with metastatic disease and those detected in serum or plasma samples from similar patient subsets (summarised in Table 1) [58–64]. There are a few individual examples of detected miRNAs which correlate between studies and sample types, of note are miR-373, miR-375 and miR-107. The elevated expression of miR-375 was detected in both the primary tumour and serum samples of node-positive patients [39,48,61]. In two recent studies of TNBC, miR-107 was reported to be elevated in both serum and primary tissues samples in patients who subsequently relapsed with distant metastases [53,60]. Molecular studies of miR-373 in breast cancer cell lines linked miR-373 expression with tumour invasion and metastasis, by demonstrating an enhanced migratory phenotypes via the suppression of CD44 [58]. Analysis of matched primary breast cancer samples and lymph node metastases determined higher levels of miR-373 in metastases, adding further evidence to the metastasis promoting effects of miR-373 [58]. However, Wu et al., recently reported correlations between decreased miR-373 expression and disease relapse in stage II and III locally advanced breast cancer patients, and resistance to pathologic complete remission in HER2[+ve] patients [51]. These contradictory findings may be the result of the molecular sub-groups analysed or other elements of the study designs. However, these studies highlight the current lack of correlation in detected miRNA expression between tissue (primary and metastases) and circulating sources.

Table 1 A summary of microRNAs associated with metastatic disease.

MicroRNA		Reference
Circulating microRNAs (serum)		
	miR10b	[40,45,47]
	miR-34a, miR-155	[46]
	miR10b, miR-373	[39]
	miR-299-5p, miR-411, miR-215, miR-452	[42]
	miR-20a, miR-214	[48]
	miR-210	[49]

	miR-375, miR-122	[51]
	miR-18b, miR-103, miR-107, miR-652	[53]
Primary tumour and metastases (Tissue)		
	miR-126, miR-335	[63]
	miR-21, miR-139-5p, miR-486-5p	[59]
	Let7i, miR-16, miR-26a, miR-27a, miR-143, miR-196a, miR-375, miR-503, miR-519a, miR-519b-3q, miR-361-5p	[61]
	miR-27b-3q, miR-107, miR-103a-3p	[60]
	miR-22	[62]
	miR-373	[58]
	miR-21	[64]
Circulating tumour cells and surrogate microRNAs (Serum)		
	miR-375, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, miR-801	[26]

Potential for circulating microRNAs to predict MED

In order for miRNAs to be useful markers in a clinical setting, they must be detectable at a stage of the disease which allows therapeutic intervention to occur. In the case of MED, the majority of the studies described above identified miRNAs in patients with clinically detectable metastatic disease, these miRNAs may not be differentially expressed when the disease is not clinically detectable. This is important, as if there is not a great enough sojourn time for the disease then a biomarker may not be of any worth. Secondly, the miRNAs found to be differentially expressed in metastatic disease may be a different set of miRNAs to those expressed during the sojourn time when the disease is in an initiating or dormant state, as has been seen with other investigations [65,66]. Studies focussed on early stage patients have shown the potential to provide markers capable of predicting clinical outcomes including MED. Demonstrated by the identification of miR-122, as a predictor of metastatic recurrence in stage II-III locally advanced breast cancer patients, and the development of a four ct-miR signature, which can distinguish early recurrence in TNBC patients [51,53]. However, a lack of reproducibility between studies has posed a major barrier to the progression of such promising findings.

A review of 15 independent studies of ct-miRNAs in breast cancer observed very little concordance between the differential miRNAs reported. The review examined 10 quantitative-PCR based studies conducted by nine independent groups. These ten studies analysed 25 miRNAs in 541 cases and 326 control samples (serum and plasma), identifying 16 differentially expressed miRNAs of which only two miRNAs (miR-21 and miR-155) were substantiated by independent studies [67]. The review also compared the data from 5 studies using a genome-wide approach. These studies identified 158 differentially expressed miRNAs, but only 16 of the 158 miRNAs were found in one of the other studies – no miRNAs were featured in 3 or more of the 5 studies [67].

This reported lack of concordance may be the result of a lack of standardisation over sample collection resulting in technical variance. Currently it is unclear what effect different sample handling techniques and long-term storage has on the ct-miRNAs profile [68,69]. The choice of serum orf plasma may further complicate analysis of previous studies as current evidence supports intra-individual difference between these two sources [70]. Contamination with blood cellular components may also be resulting in artefacts, pre-designated filtering of blood-cell specific miRNAs may help to remove these artefacts but as yet a robust list for filtering has not been created [67]. Finally, perhaps one of the biggest issues currently facing this field of research is the lack of a universal house-keeping miRNAtarget for the normalisation of ct-miRNAs measurements [69,71].

Conclusion

Considerable advancements have been made in our understanding of breast cancer progression, yet there is still 78% mortality within 5-years among breast cancer patients who develop metastatic disease [2]. There is a clear need for biomarkers capable of detecting MED and for more personalised predictions of treatment response, for which miRNAs may be one such potential biomarker.

Several studies have emerged reporting miRNA markers which can distinguish metastatic disease and potentially predict recurrence in early stage disease (MED) (summarised in Table 1). Current issues surrounding a lack of consensus between studies are still masking the potential value of these findings. These issues may yet be resolved through reductions in technical variance and perhaps most importantly, determination of a universal normalisation protocol. However, biological difference at distal metastatic sites cannot be ruled out as source for some degree of variability, and miRNAs indicative of site specific metastases, such as the potential link between miR-10b with brain and bone, may be determined

[45,47].

Significant further research in this area is clearly needed to deliver on the potential that ct-miRNAs have in not only detecting MED, but providing a better understanding of the current molecular sub-types and improving monitoring of treatment response [44,49,72]. The recently emerging area of research identifying the potential role of miRNAs in intercellular communication between cancer cells and stromal cells and between heterogeneous cancer cell populations [50,73], has reignited enthusiasm for ct-miRNAs as predictive and prognostic biomarkers. There is a need for in-depth longitudinal analysis of ~~circulating~~ ct-miRNAs ~~levels~~ from diagnosis to post-treatment follow-up to predict miRNAs associated at all stages of the metastatic cascade, whether this takes the form of global analysis or directed by functional *in vitro* and *in vivo* studies remains to be determined~~., to predict miRNAs associated at all stages of the metastatic cascade remains to be determined~~.

Conflict of interest statement

None declared.

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