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Pathogenic and therapeutic role of miRNAs in breast cancer

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1. ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNA molecules present in all cell types, with sizes that vary from 18 to 28 nucleotides. miRNAs play significant roles in several biological processes, including development, differentiation, metabolism, initiation, and progression of cancer. In recent years, considerable research has been directed towards identifying miRNAs in peripheral blood from circulating tumor cells and disseminating tumor cells. Because these circulatory miRNAs are very stable and reproducible, their identification could be useful as prognostic markers as well as therapeutic agents for many cancers such as breast cancer. In this article, we review the role of specific circulatory miRNAs in breast cancer, with particular emphasis on their clinical importance.

2. INTRODUCTION

MicroRNAs (miRNAs) are small and abundant class of noncoding RNA molecules, which are known to control several genes, and that they are found within in all cell types (1-6). miRNAs represent approximately 1%-2 % of the total genome, and are very stable in blood, after freezing, and in preserved tissue samples. They regulate several biological processes, and their role in altering mRNAs in a stage-specific manner during progression of tumorigenesis has been well documented (7-16). In addition, they target the 3'-untranslated regions (3'-UTRs) of mRNAs and translationally repress specific signal transduction pathways. Biogenesis of miRNAs is a multi-step process. Its simplified adaptation is shown in Figure 1 (17-25). RNA polymerase II transcribes the precursor miRNAs (pri-miRNAs) of either independent genes or

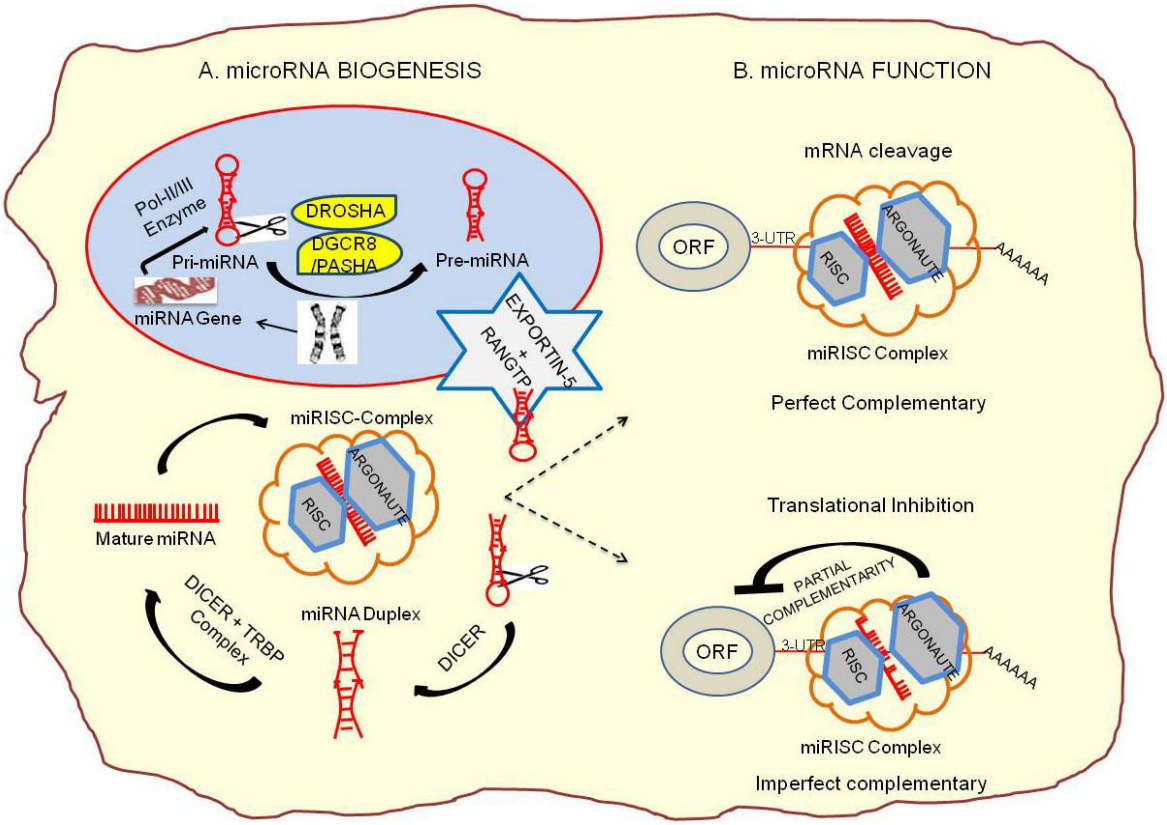

Figure 1. microRNA biogenesis and function. Primary transcripts (pri-miRNAs) are generated by polymerase II and are 5'capped. Drosha (RNase III endonuclease) and DGCR8 (a double-stranded RNA binding protein) recognize the precise secondary hairpin structures of the pri-miRNA and cleave exactly at the base of the stem loop freeing approximately 70-nucleotide pre-miRNA structures allowing Exportin 5–mediated cytoplasmic export. Dicer, a second RNase III endonuclease, cleaves subsequently 22- nucleotides from the Drosha cleavage site to yield the mature miRNA. The mature miRNA then gets disentangled and a single strand is integrated into the RNA-induced-silencing-complex (RISC) which either represses mRNA translation or destabilizes mRNA transcripts through cleavage or deadenylation. Dysregulation of this process leads to perturbation in miRNA genesis that may initiate oncogenesis.

introns of protein coding genes into mature miRNAs. Hairpin-shaped pri-miRNAs primarily are processed by the specific RNAse III enzyme family DROSHA/PASHA. DROSHA cleaves pri-miRNAs into pre-miRNAs, which are then transported from the nucleus into the cytoplasm, facilitated by exportin 5 and RAN-GTP. In the cytoplasm, the DICER and TRBP complexes cleave and process pre-miRNAs further into mature miRNAs, which are incorporated into the miRNA-induced silencing complex (miRISC). Mature miRNAs, together with the other components of the complex [miRISC and Argonaute (AGO)], target and inhibit the function of mRNAs either by translational repression or deadenylation and mRNA cleavage (Figure.1A and 1B). In this review, we focus on the role of circulating miRNAs in breast cancer, and discuss their possible role as a biomarker for cancer, as well as their role in the early diagnosis and prognosis of this disorder.

3. MicroRNA PROFILES IN BREAST CANCER

Early detection and effective treatment for breast cancer remains a challenge because current chemotherapeutic modalities are sometimes only partially effective (26-31). However, recent studies suggest that miRNAs may serve as novel diagnostic, prognostic, and therapeutic agents for breast cancer (32-43). Several miRNAs are found to be expressed in a stage-specific manner during breast cancer progression involving ductal hyperplasia, atypical ductal hyperplasia, ductal carcinoma in situ with microinvasion (DCIS-MI), and invasive ductal carcinoma (IDC) (44, 45). The following miRNAs have been extensively studied in breast cancer *in vitro* and *in vivo* (Figure 2) (Table 1).

3.1. MicroRNA-21

Higher *in vivo* expression of miR-21 has generally been found in breast cancers such as ductal hyperplasia, DCIS, and invasive breast carcinoma stages (44, 45). The miR-21 has been shown to be upregulated in more than 89% of breast cancer patients and is associated with both aggressiveness of the disease (46) and poor patient survival (47). Earlier studies reported that miR-21 targets and down-regulates the tumor suppressor genes, tropomyosin 1-alpha (TPM1), phosphatase tensin homolog (PTEN), and programmed cell death 4 (PDCD4), while simultaneously

Table. 1. miRNA targets and function in breast cancer

miRNAs	Targets	Functional pathways
125a,b	HER2,HER3	Anchorage-dependent growth
126	IRS-1	Cell cycle progression from G1/G0 to S
34a	CCND1,CDK6,E2F3,MYC	DNA damage, proliferation
206	ESR1	ER signaling
205	ErbB3 and VEGF-A expression	Inhibits tumor cell growth and cell invasion,
31	FZD3,ITGA5,M-RIP,MMP16,RDX,RHOA	Metastasis
335	SOX4,PTPRN2,MERTK,TNC	Metastasis
27b	CYP1B1	Modulation of the response of tumor to anti-cancer drugs
146a/b	NF-κB	NF-κB, and impaired invasion and migration capacity
101	EZH2	Oncogenic and metastatic activity
17-5p	AIB1,CCND1,E2F1	Proliferation
let-7	H-RAS, HMGA2, LIN28,PEBP1	Proliferation, differentiation
145	p53-mediated repression of c-Myc	Suppresses Cell Invasion and Metastasis
200c	BMI1,ZEB1,ZEB2	TGF-beta signaling
21	BCL-2,TPM1,PDCD4,PTEN,MASPIN	Apoptosis
27a	Zinc finger ZBTB10, Myt-1	Cell cycle progression G2-M checkpoint regulation
373/520c	CD44	Metastasis
10b	HOXD10	Metastasis
221/222	$p27^{Kip1}$	Tamoxifen resistance
155	RHOA	TGF-beta signaling

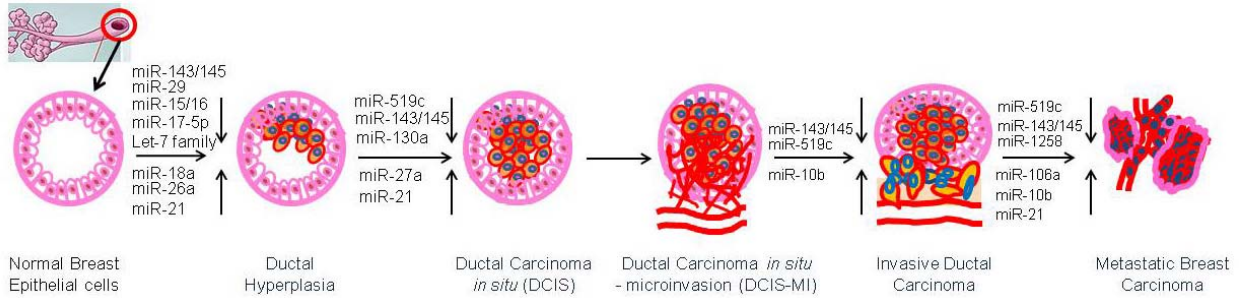

Figure 2. Stage specific miRNAs expression in the progression of breast cancer: During transition from normal breast epithelial cells to ductal hyperplasia, miRNAs (-143/145, - 29, -15/16, -17-5p, and let-7 family) are down regulated and miR-18a, -25a and -21 are up regulated. Similarly, up regulation of miR-27a, & -21 and down regulation of miR -519c, -143/5 & -130a were found in ductal hyperplastic to ductal carcinoma in situ (DCIS) stage. In invasive ductal carcinoma (IDC) stage, miR-10b was upregulated and miR-143/145, & miR-519c were down-regulated. Finally, when breast cancer cells progressed from IDC to metastatic breast carcinoma, miRNAs (-21, -10b and -106a) were highly expressed and miRNAs (-519c,-1258 and-143/145) were found to be down regulated.

enhancing cell proliferation and survival of breast cancer cells (48, 49). In addition, it was reported that downregulation of TPM1 could be one of the possible reasons for the resistance of breast cancer cells to cisplatin (50). Activation of PDCD4 downregulates tissue Inhibitor of Metalloproteinases-2 expression which inhibits invasion of breast cancer (51-62). Expression of PDCD4 appears to be missing in most cancer tissues including breast cancer. In preclinical models specific inhibition of miR-21 significantly blocked cell migration and tumor growth through its targets, ankyrin repeat domain 46 and eukaryotic translation initiation factor 4A1 (63). MiR-21 could be used as a molecular prognostic biomarker because its overexpression correlatedwith poor therapeutic outcomes and survival. Its suppression resulted in increased apoptosis and reduced cell proliferation, and it sensitized the cancer cells to chemotherapy. Hence targeting mir-21 could be helpful to patients who show resistance to traditional chemotherapeutic therapies (64).

3.2. MicroRNA-125b

It was initially found that overexpression of miR-125b conferred resistance to paclitaxel treatment by inhibiting apoptosis in breast cancer cells. The anti-apoptotic activity of miR-125b was due to inhibition of the pro-apoptotic protein BAK1 (65). Le et al., (66) reported that miR-125b was also capable of downregulating p53 function by binding to the 3'-UTR of p53. Single nucleotide polymorphisms (SNPs) are variations in the DNA sequence that occur in more than one percent of the population. Regulatory regions, exons, or exon-intron boundaries of genes contain these SNPs which may affect expression of the genes or may control the protein function or splicing sites (67-69). The BMPR1B gene, which is a target of miR-125b, contains such SNPs in its 3' UTR, which confer a better prognosis (70). In addition, in several other miRNA profiles, downregulation of miR-125b led to augmented expression of oncogenes such as HER-2 and HER-3 (71). Negatively regulating the expression of miR-125b should therefore be important in the development of targeted therapeutics for suppressing resistance in a variety of breast tumors.

3.3. MicroRNA-206

MiR-206 has been reported to be overexpressed in estrogen receptor (ER)-negative tumors (72). It represses ER-mRNA expression which in turn inhibits ER-induced cell proliferation in ER-positive breast cancer cells (73). Inhibition

of miR-206 in ER-positive MCF-7 cells resulted in cell cycle arrest in G1. The overexpression of miR-206 also inhibited DNA synthesis and repair genes such as DNA polymerase alpha in ER-cells (74). Conversely, miR-206 also acts as a tumor suppressor by downregulating Notch 3 expression and inducing apoptosis in cervical cancer (HeLa) cells (75). Considering the contrasting functions of miR-206, it appears that it plays a dual role based on its ER status. Nonetheless, it is clear that miR-206 could be used as a predictive marker if these results are confirmed using *in vivo* models.

3.4. MicroRNA-221/222 cluster

It has been hypothesized that miR-221/222 cluster may be involved in the transition of ER-positive breast tumors to ER-negative tumors (74). Gene expression studies suggested that miR-221/222 inhibits the expression of caveolin 1 and 2 and transforming growth factor-β, leading to malignant transformation in breast cancer cells (74). Overexpression of this cluster confers resistance to tamoxifen by downregulating cell cycle inhibitor $p27^{KIP1}$ expression in ER-positive breast cancer cells. When compared to HER2/neu-negative breast cancer cells, MiR-221/222 cluster was also found to be significantly overexpressed in HER2/neu-positive breast cancer cells, resulting in resistance to drug therapy (76). These studies suggested that miR-221/222 cluster could be a potential target for clinical therapy and raises the possibility that its expression could be a predictive marker for hormonal resistance in breast cancer.

3.5. Let-7 Family

Let-7 microRNAs were the first evolutionarily conserved miRNAs identified in C. elegans. They were shown to be part of a much larger class of noncoding RNAs termed microRNAs (77, 78). Overexpression of the let-7 family of miRNAs (79) in breast cancer cells as well as in nonobese diabetic/severe combined immunodeficiency mice resulted in attenuated cell proliferation, mammosphere formation, increased amount of *in vitro* undifferentiated cells, and metastasis formation. In contrast, decreasing let-7 miRNAs by miRNA inhibitors enhanced self-renewal of non-tumor initiating cells *in vitro,* and downregulated the targets H-RAS and HMGA2. H-RAS silencing reduced self-renewal with no effects on differentiation, while silencing of HMGA2 enhanced differentiation with no effects on self-renewal (79). The results describe multiple functions for let-7, which confer stem cell-like characteristics to breast cancer cells. The let-7 family of miRNAs are downregulated in the DCIS and IDC stages of breast carcinoma when compared to the benign stage, with ER-α expression inversely correlated with expression of the let-7 family of miRNAs (79). Lowery et al. (80) reported that isoforms of let-7 microRNAs can be used to identify differences between clinicopathological features such as the proliferation index (miR-let-7c and miR-let-7d), PR status (miR-let-7c), and metastatic (miR-let-7f-1 and a-2& a-3) breast cancers (80). Together, the available data suggests that the let-7 miRNA family plays an important role in regulating breast cancer progression and metastasis.

3.6. MicroRNA-10b

Compared with normal mammary epithelial cells, miR-10b is highly expressed in breast cancer cell lines that are metastatic, where it regulates migration and invasion (44, 45). MiR-10b expression is controlled by the transcription factor TWIST (an epithelial mesenchymal transition marker), which directly binds to the putative promoter of miR-10b. Metastatic tumor formation induced by miR-10b initiates translational inhibition of the homeobox D10. This results in overexpression of a pro-metastatic gene, Ras homolog gene family member C, which leads to increased metastasis (81, 82). Inhibition of miR10b failed to inhibit growth of primary mammary tumors, but significantly suppressed formation of lung metastases in a sequence-specific manner in breast cancer models (81, 82). These results suggest that miR-10b could be used as a metastatic marker for breast cancer and that the miR-10b specific antagomiRs could be developed as new anti-metastatic agents.

3.7. MicroRNA-9

The proto-oncogene, MYC activates miR-9 by binding specifically to the miR-9-3 locus in breast cancer cells which target e-cadherin-encoding mRNA, CDH1. Overexpression of miR-9 leads to enhanced cell motility and migration, while activating β-catenin signaling (83). The higher levels of miR-9 correlate with tumor grade and metastatic status *in vivo*. However, epigenetic inactivation by aberrant hypermethylation leads to subsequent transcriptional downregulation of miR-9-1, an isoform of miR9, supporting the progression of preinvasive and ductal invasive breast cancers (84). Hence, the levels of miR-9 and its isoforms could be used as biomarkers to characterize the progression of metastatic breast cancer.

3.8. MicroRNA-378 and -378*

MiR-378, that originates from the sense strand of pre-miR-378 and is unlike miR-378* that originates from the anti-sense strand, was found to be overexpressed in breast cancer. The overexpression and subsequent activation of miR-378 is influenced by the C-MYC transcription factor. The oncogenic activity of miR-378 results from targeting and attenuating a transducer of ERBB2-2, a member of the anti-proliferating BTG family that transcriptionally represses the proto-oncogene, cyclin D1 (85). PPARGC1b (peroxisome proliferator-activated receptor gamma, coactivator 1 beta) encodes for PGC-1β, a transcriptional regulator of oxidative metabolism. MiR-378*, which is found within the PPARGC1b gene, targets and regulates ERBB2 expression, acting as a molecular switch to control the Warburg effect via the bioenergetics transcriptional pathway in breast cancer. MiR-378* decreases expression of estrogen-related receptor gamma and GA binding protein transcription factor, alpha subunit. These are the partners of PPARGC1B that lead to decreased tricarboxylic acid cycle gene expression, decreased lactate production, and increased cell proliferation (86). In summary, both miR-378 and miR-378* appear to be important in regulating cancer cell proliferation using different physiological processes.

4. CIRCULATING MiRNAs IN BREAST CANCER AND THEIR CLINICAL SIGNIFICANCE

Circulating tumor miRNAs in the serum and plasma were found to be clinically viable and very stable, reproducible, and reliable among individuals of the same

species (87-100). The miRNAs could therefore be isolated from circulating tumor cells (CTCs) or disseminating tumor cells (DTCs) (101-105) and be used as biomarkers, especially in the early stages of breast cancer detection and management (106). Previous studies have reported that circulating miRNAs can be used as definitive predictors in patients with favorable outcome vs. unfavorable outcome, and the levels of circulating miRNAs may correlate with tumor load (107-109). In an adjuvant systemic therapy setting or in a metastatic setting, circulating miRNAs can also be measured frequently to predict the response of anti-tumor therapy. Assessing circulatory miRNAs in relation to tumor load can overcome the resistance response and might augment the patient's prognosis. Chemoresistance has been one of the major problems in treatment and management of breast cancer, so development of therapies to prevent this resistance has been a high priority. Consistent with this objective, neoadjuvant chemotherapy has used circulating miRNAs such as miR-221, which was found to be overexpressed in breast cancer. In breast cancer hormone receptor (HR)-negative patients, the higher levels of miR-221 were correlated with their HR status, overall response rate, and pathologic response rate (110). Circulating plasma miR-221 could therefore be used as a predictive biomarker for sensitivity to neoadjuvant chemotherapy in patients with breast cancer. In the other chemoresistant circulating miRNA, miR-125b, expression was found to be higher in 56 breast cancer patients with IDC, and higher expression of miR-125b was associated with non-responsiveness to anticancer drugs (111). However, reducing levels of miR-125b ectopically resulted in the opposite effect (111). Hence, miR-125b could be used as another chemotherapeutic resistance biomarker to predict clinical outcomes in breast cancer patients with preoperative neoadjuvant chemotherapy (111).

Serum miRNAs, such as miR-16, miR-25, miR-222, and miR-324-3p were used as a "four-miRNA signature" in the discovery and validation stages of breast cancer, and may serve as noninvasive and predictive biomarkers (112). MiR-34a and miR-145 were found to be significantly downregulated in whole blood samples of 19 breast cancer patients, along with colorectal cancer cases, in a cohort study. However, there was no correlation between levels of miR-34a and the tumor stage or grade (113). Although not specific for breast cancer, miR-34a and miR-145 could still be used as biomarkers, because their levels were significantly altered (113). In addition, miR-195 and let-7a were found at higher levels in postoperative whole blood samples of 83 breast cancer patients when compared to whole blood samples of 44 control patients (114). Tumor-associated circulating miRNAs such as miR-10b, miR-34a, and miR-155 in whole blood of 89 patients could distinguish between patients with primary breast cancer and/or with early tumor stages and/or patients with advanced breast cancer (87). A large number of circulating miRNA-based biomarkers for early detection of breast cancer were found in the plasma of Caucasian-American (CA) and African-American (AA) patient populations (115). Among the CA and AA patients, miR-181a and miR-1304 were found to be the differentially expressed. MiR-589 and let-7c were specific for the CA group and miR-425* and let-7d* were specific for the AA group (115). Krutzfeldt et al. reported that circulating miRNAs could be used to monitor drug targets (pharmacodynamics markers) because their expression levels could be determined at various time points (e.g., in the blood, after the anti-oncomiR, or after antagomiR treatments) (116). These findings suggest that systemic miRNAs can be used as biomarkers for different stages of breast cancer development and progression, for pre- and postoperative breast cancer, and for detection of chemoresistance during the treatment of breast cancer.

5. CONSIDERATIONS IN DETECTION OF CIRCULATING miRNAs

There are limitations in the detection of circulating miRNAs because of differences in the patient populations, in the definitions of CTCs and DTCs, and/or in differences in sensitivity and/or specificity of the above mentioned technologies (117, 118). Another consideration in detecting circulating miRNAs is the lack of stringent experimental criteria in the detection of CTCs/DTCs (117, 118). Changes in the expression patterns of circulating miRNAs during pre- and post-treatment regimens or surgery can also pose a problem (119119, 120). Unless subgrouping and simultaneous but independent analysis is performed, size and detection of small metastasizing cells that might evade the current expression profiling methods can present a problem. Hopefully, genome-wide miRNA microarray profiling will be an appropriate method to efficiently detect differences between circulating miRNAs and their mutant forms.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Breast cancer is still the leading cause of cancer-related death in women worldwide, so effective treatment strategies still need to be developed for this disorder. The use of miRNA profiles, especially circulating miRNAs from the CTCs/DTCs, could be a tool in development of more effective therapeutic treatments.

The circulating miRNAs have great potential to be used as diagnostic and therapeutic tools in the management of breast cancer. An important advance in the treatment of breast cancer would be to comprehensively assess the biological function of circulating miRNAs that are diverse and are presently not fully understood. Circulating miRNAs can be easily obtained, and are sensitive to changing disease parameters that would provide information about early breast cancer development, progression, and metastasis. Together with current technologies, miRNA microarrays could ascertain and distinguish potential differences between normal and breast neoplastic disease phenotypes, and could identify novel and clinically important circulating miRNAs that are disease-specific, prognosis-specific, and targeted therapy-specific.

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Abbreviations: MIRNA: MicroRNA; 3'-UTRs: 3'-untranslated regions; MIRISC; MiRNAinduced silencing complex; DCIS-MI: Ductal carcinoma *in situ* with microinvasion; IDC: Invasive ductal carcinoma; SNPs: Single nucleotide polymorphisms; AGO: Argonaute; DTCs: Disseminating tumor cells; CTCs: Circulating tumor cells; CA: Caucasian-American; AA: African-American

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