

WJCO 5th Anniversary Special Issues (2): Breast cancer**Micro-RNAs as clinical biomarkers and therapeutic targets in breast cancer: Quo vadis?**

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Author contributions: Christodoulatos GS wrote, revised and approved the manuscript; Dalamaga M designed, wrote, revised and approved the manuscript; the authors contributed equally to this paper.

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Received: December 17, 2013 Revised: February 20, 2014

Accepted: March 13, 2014

Published online: May 10, 2014

Abstract

Breast cancer (BC) is the most frequent type of non skin cancer among women and a major leading cause of cancer-related deaths in Western countries. It is substantial to discover novel biomarkers with diagnostic, prognostic or predictive usefulness as well as therapeutic value for BC. Micro-RNAs (miRNAs) belong to a novel class of endogenous interfering RNAs that play a crucial role in post transcriptional gene silencing through mRNA targeting and, thus, are involved in many biological processes encompassing apoptosis, cell-cycle control, cell proliferation, DNA repair, immunity, metabolism, stress, aging, *etc.* MiRNAs exert their action mainly in a tumor suppressive or oncogenic manner. The specific aberrant expression patterns of miRNAs in BC that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis. The detection of circulating extracellular miRNAs in plasma of BC patients may provide novel, non-invasive biomarkers in favor of BC diagnosis and prognosis and,

at the same time, accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. Data from *in vitro* and *in vivo* studies on BC have revealed promising therapeutic approaches *via* miRNA delivery and miRNA inhibition. The purpose of this review is to explore the ontological role of miRNAs in BC etiopathogenesis as well as to highlight their potential, not only as non-invasive circulating biomarkers with diagnostic and prognostic significance, but also as treatment response predictors and therapeutic targets aiding BC management.

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Key words: Biomarker; Breast cancer; Cancer diagnosis; Micro-RNA; Oncogene; Therapy; Tumor suppressor

Core tip: The specific aberrant expression patterns of micro-RNAs (miRNAs) in breast cancer (BC) that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis. The detection of circulating extracellular miRNAs in plasma of BC patients may provide novel, non-invasive biomarkers in favor of BC diagnosis and prognosis and, at the same time, accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. Data from *in vitro* and *in vivo* studies on BC have revealed promising therapeutic approaches *via* miRNA delivery and miRNA inhibition.

Christodoulatos GS, Dalamaga M. Micro-RNAs as clinical biomarkers and therapeutic targets in breast cancer: Quo vadis? *World J Clin Oncol* 2014; 5(2): 71-81 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i2/71.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i2.71>

INTRODUCTION

Breast cancer (BC) is the most frequent type of non skin cancer among women and a major leading cause of cancer-related deaths in women in Western countries^[1-3]. One in eight women has a chance of developing BC once in her lifetime^[4,5]. Early diagnosis and a followed patient-monitored therapy can lead to successful treatment of BC. However, due to the lack of sensitivity and specificity of known biomarkers, especially in early stage disease, there is no available efficient biomarker up to now for screening or early detection of BC^[6,7].

Micro-RNAs (miRNAs or miRs) belong to a novel class of endogenous interfering RNAs that play a crucial role in post transcriptional gene silencing through messenger RNA (mRNA) targeting and are thus involved in many biological processes encompassing apoptosis, cell-cycle control, cell proliferation, DNA repair, immunity, metabolism, stress, aging, *etc*^[8].

Since their initial discovery in 1993 during a study of the gene *lin-4* in *Caenorhabditis elegans*^[9], more than 2000 molecules have been determined in humans so far, regulating the expression of almost 30% of genes^[10]. MiRNAs are short, non-coding RNAs of approximately 20-25 nucleotides in length that are transcribed either from independent genes or from exons or introns of protein-coding genes^[8]. MiRNAs present unique nucleotide sequences that are strongly conserved among species^[11] and possess a specific critical region of 7 nucleotides long, known as the seed sequence, which is responsible for mRNA base pairing^[8]. Their abundant repertoire and the fact that the seed is so short may explain their combinatorial character in regulation: a given miRNA may target different mRNAs and a given mRNA could similarly be targeted by multiple miRNAs^[12].

MiRNA biogenesis is initiated in the cell nucleus with the generation of primary miRNA (pri-miRNA), usually by RNA polymerase II. The next step is the conversion of pri-miRNA into a smaller hairpin precursor miRNA (pre-miRNA) which is arbitrated by the microprocessor complex^[8]. The latter consists of the ribonuclease Drosha and the DiGeorge syndrome critical region in gene 8 protein that recognizes the hairpin loop of the pri-miRNA, ensuring nonfaulty cleavage by Drosha. Pre-miRNAs are transported to the cytoplasm by the receptor exportin 5, whereas new cleavage occurs by the RNase III enzyme Dicer along with the transactivation response RNA-binding protein, resulting in a double stranded miRNA about 22 nucleotides long^[8,13]. One strand of the miRNA/miRNA duplex represents the mature miRNA which in conjunction with the Argonaute (Ago) protein and other proteins form the RNA-induced silencing complex (RISC)^[13]. Within RISC, mature miRNA is guided mainly to complementary sequences in 3' or 5' untranslated region of mRNA targets, open reading frames and promoter regions^[14]. Depending on the perfect or partial base pairing between miRNA and mRNAs molecules, the consequence is mRNA degradation or translational repression at both pre-initiation and post-

initiation stages, respectively; leading, thus, to a negative regulation of gene expression^[15]. However, studies have also shown a possible miRNAs involvement in positive regulation of their target genes (transcriptional and translational activation)^[16].

MiRNAs, as major gene-expression regulators, are implicated in the pathogenesis of many diseases, including cancer^[17]. The specific aberrant expression patterns of miRNAs in several cancer types, such as BC, that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis^[18]. MiRNAs exert their action mainly in a tumor suppressive or oncogenic manner^[19].

The purpose of this review is to explore the ontological role of miRNAs in BC etiopathogenesis as well as to highlight their potential, not only as non-invasive circulating biomarkers with diagnostic and prognostic significance, but also as treatment response predictors and therapeutic targets aiding BC management.

ROLE OF MiRNAs IN BC ETIOPATHOGENESIS

Aberrant expression of miRNAs in BC

MiRNA expression profiling studies have detected aberrations with specific signatures of miRNA expression in breast carcinoma^[20]. Certain miRNA expression signatures have been associated with tumor classification, stage and prognosis, while others have been useful in detecting the primary site of tumors of unknown origin^[21].

Accumulating evidence has revealed that miRNAs may act either as oncogenes, commonly named oncomirs, by suppressing the expression of tumor suppressor genes or genes responsible for apoptosis; or as tumor suppressors or oncosuppressors by inhibiting genes that promote carcinogenesis and therefore controlling apoptosis and differentiation. Nevertheless, this miRNA categorization in oncogenes with an upregulated profile and tumor suppressors with a downregulated profile may be inaccurate, as many studies have shown that miRNAs may present a dual function with oncogenic or tumor suppressive properties based on tumor type and cellular context^[22].

In Figure 1, we have included a list of the most important miRNAs acting as oncogenes or tumor suppressors in BC. MiR-21 appears as a significant BC-related intracellular and extra-cellular biomarker and a therapeutic target with upregulated expression detected in human BC tissues and cell lines, playing a key role in all phases of BC pathogenesis^[23,24]. In BC clinical specimens, miR-21 increased expression was associated with advanced clinical stage, lymph-node positivity and shorter survival^[20]. Another example of a miRNA associated with an oncogenic potential in BC is miR-155, whereas the upregulation of miR-155 was correlated with advanced grade, clinical stage, estrogen receptor (ER) negative tumors, lymph node invasion, metastasis and poor prognosis^[25]. However, miR-21 and miR-155 increased expression do not characterize only BC but also colorectal and lung

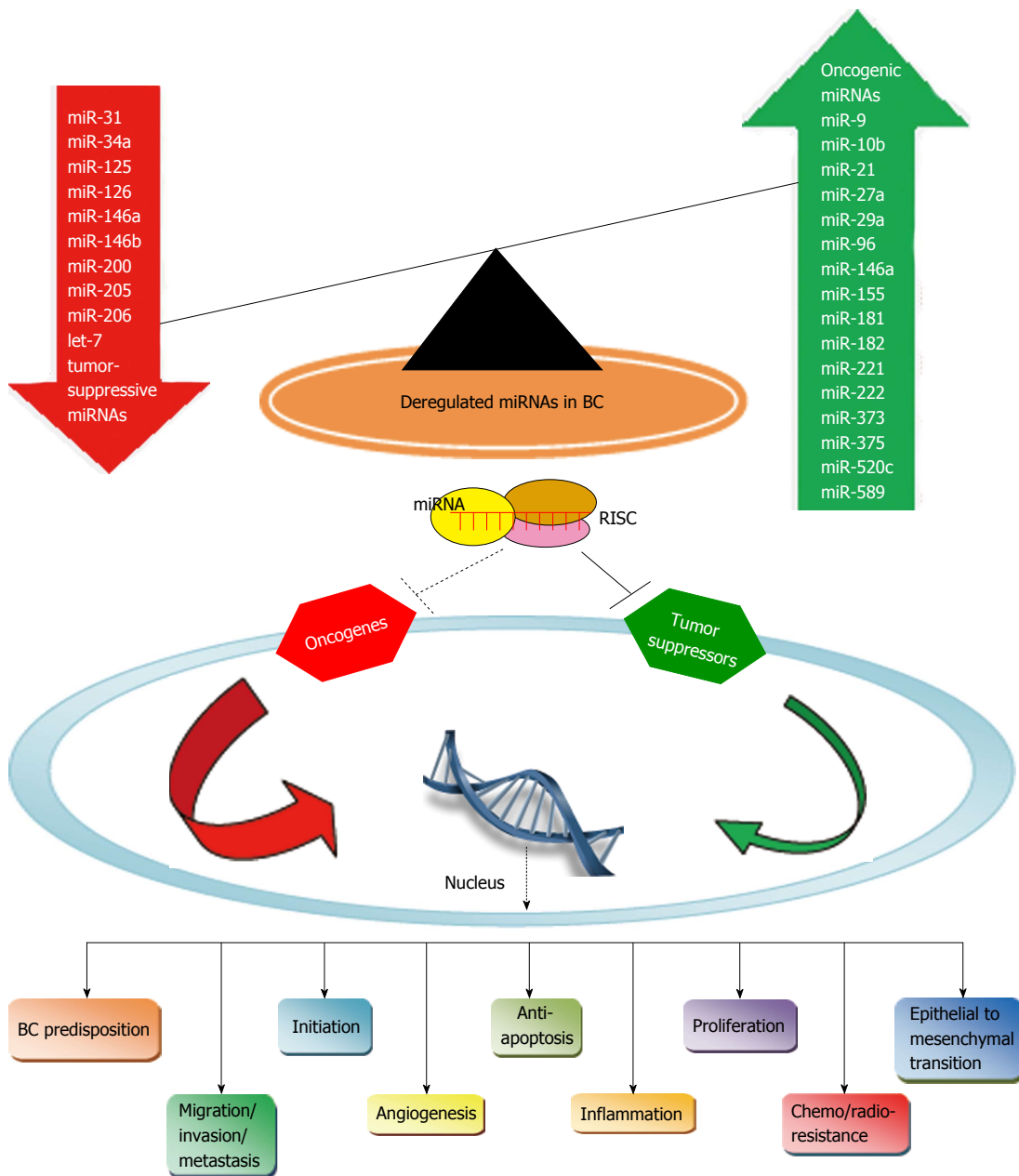


Figure 1 Dysregulation of micro-RNAs in breast cancer pathogenesis. The downregulated/tumor suppressive micro-RNAs (miRNAs) exert decreased inhibition on putative oncogenes in breast cancer (BC). The upregulated/oncogenic miRNAs show enhanced inhibition on tumor suppressors. These mechanisms may lead to increased oncogene-induced gene and decreased tumor suppressor-mediated transcription respectively. Both mechanisms lead to aberrant gene expression that play a significant role in BC predisposition, initiation, cell proliferation, resistance to apoptosis, invasion, angiogenesis, inflammation and metastasis in BC cells. RISC: RNA-induced silencing complex.

cancer as well as leukemia^[25,26].

Examples of overexpressed miRNAs in BC include miR-9/10b/21/27a/29a/96/146a/155/181/221/222/373/375/520c/589, where some of these have been validated in BC clinical specimens, highlighting their potential role in BC diagnosis, prognosis and therapeutics^[18,23]. Concerning downregulated miRNAs, miR-30a/31/34a/125/126/146a/146b/200/205/206 and let-7 emerge in BC pathogenesis through the loss of their tumor suppressor properties^[18,23]. Generally, it is important to mention that miRNA downregulation represents a more frequent event in BC pathogenesis, allowing oncogenes to be activated during BC development.

Dysregulation of miRNAs in BC predisposition, initiation, progression and metastasis

Aberrant expression of miRNAs in cancer-associated genomic regions: MiRNA genes are often situated in cancer-associated genomic regions and are subject to deletions, rearrangements, breakpoints and loss of heterozygosity^[27]. Approximately half of all annotated human miRNA genes are situated in fragile sites or genomic areas that have been associated with cancer^[27,28]. For example, miR-15a and miR-16-1, which are often downregulated in cancer, occupy the most frequently deleted genomic region and may harbor germline mutations in familial cases of B-cell chronic lymphocytic leukemia and

BC^[22,28].

Single nucleotide polymorphisms in miRNA genes or miRNA target genes and genetic susceptibility to BC: Single nucleotide polymorphisms (SNPs) in miRNA genes, their processing machinery and their target binding sites could also increase the susceptibility to BC and affect patient prognosis and treatment efficacy^[29]. Despite the fact that SNPs are rare in miRNA genes, they may alter miRNA biogenesis and function as well as miRNA binding sites^[30]. Many SNPs in miRNA genes or in miRNA target genes in germline cells, independently from their possible/putative functional effects, have been analyzed in association (case-control) studies. For example, the SNP rs11614913 in pre-miR-196a-2 has been linked to an elevated BC risk^[31] and the SNP rs895819 in pre-miR-27a has been associated with a decreased BC risk^[32]. Therefore, miRNAs may be used as potential biomarkers for BC predisposition in populations at risk.

Aberrant expression of miRNAs in BC initiation: Cancer stem cells (CSCs) or tumor-initiating cells lead tumor promotion, progression and heterogeneity by proliferating and forming some differentiated tumor cells. CSCs present a self-renewal (symmetric and asymmetric division) potential and the capacity to generate all types of cancer cells within the tumor^[33]. Targeting these initial CSCs may be an effective therapeutic strategy as CSCs are responsible for tumor growth and propagation of cancer and are considered more resistant to chemotherapy and radiotherapy. Breast CSCs were first reported by Al-Hajj *et al.*^[34] and are characterized by the expression of the surface biomarkers CD44⁺, CD24^{-/low}, the epithelial specific antigen and aldehyde dehydrogenase 1^[33]. Under non-adherent conditions for human mammary epithelial cells, only breast CSCs are capable of surviving, proliferating and building mammospheres, which are multicellular formations containing a large number of mammary stem cells^[33]. The Hedgehog signaling pathway activates the self-renewal of breast CSCs *via* the polycomb ring finger oncogene B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1)^[35]. The aberrant expression of miRNAs may play a role in carcinogenesis and breast CSCs self-renewal by acting as oncogenic or tumor suppressive miRNAs and also regulating the stem cell-like phenotype of breast CSCs^[33]. The miRNA triad let-7/miR-200c/miR-30 suppresses the self-renewal of breast CSCs and the spontaneous conversion of immortalized mammary epithelial cells to a stem-like phenotype with less differentiated and mesenchymal properties by targeting Ras, Bmi-1, ubiquitin-conjugating enzyme 9 and integrin b3 respectively^[24,33]. On the contrary, the upregulation of miR-181 family members and miR-495 plays a significant role in modulating breast CSCs and maintaining a stem cell-like phenotype in BC by targeting respectively the tumor suppressor serine/threonine kinase ataxia telangiectasia mutated and E-cadherin and short for regulated in development and DNA damage responses. In particular, over-expression of miR-495 in human BC cells enhances

colony formation *in vitro* and carcinogenesis *in vivo*^[36].

Aberrant expression of miRNAs in BC progression: MiRNAs may be involved in the cell cycle by controlling critical components of the regulatory pathways. In particular, miRNAs could regulate the cyclin/cyclin dependent kinase (CDK) pathway which constitutes a significant pathway in the cell cycle control. The pair miR-17-5p/miR-20a has been shown to attenuate the synthesis of cyclin D1 encoded by the gene *CCND1* in BC MCF-7 cell line, blocking S-phase entry and inhibiting cell proliferation^[37]. MiR-27a, which is also associated with the cyclin/CDK pathway, targets zinc finger and BTB domain containing 10 and Myt-1 which halt BC cell proliferation by suppressing cyclin D1 and cyclin B respectively^[38].

The estradiol (E2)/ER α /Sp1 is another important cell cycle regulatory pathway in BC that aids proliferation by activating cyclin D1, leading to the G1/S phase transition. In ER positive BC, E2 increases the expression of miR-21 and let-7 family members^[24]. The upregulation of let-7 family members in ER positive BC may result in diminished ER α activity and cell proliferation^[18]. BC cells at the stage of ductal carcinoma *in situ* and invasive ductal carcinoma are characterized by let-7 downregulation in comparison to benign lesions^[18]. In contrast to ER positive BC, the E2/ER signaling pathway is inhibited and miR-21 is downregulated in ER negative BC, whereas miR-18a, miR-18b, miR-206, miR-221 and miR-222 are upregulated leading to inhibition of ER expression and induction of other signaling pathways regulating cell growth and proliferation^[39]. The upregulation of the pair miR-221/222 in ER negative BC may lead to reduced p27kip1 levels and continuous BC proliferation^[40].

In BC, miR-31 acts as a tumor suppressor targeting the human frizzled transmembrane receptor frizzled-3 and is downregulated in all interactions with the Wnt signaling transduction pathway^[41]. The oncosuppressor miR-34a, which is downregulated in triple negative and mesenchymal-type BC cell lines, has been shown to inhibit BC proliferation and migration *via* downregulation of B-cell lymphoma 2 (Bcl-2) and sirtuin 1^[18]. The tumor suppressor miR-205 targets human epidermal growth factor receptor 3 (HER3) directly, a receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) family, inactivating thereby the downstream mediator Akt, suppressing the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and inhibiting BC proliferation with improved response to targeted therapies^[42]. Finally, the overexpression of the oncogenic miR-146a may be responsible for the altered expression of the breast cancer type 1 susceptibility protein (BRCA1), which is a negative regulator of BC growth associated with the hypophosphorylated form of Rb, and the BC metastasis suppressor 1 (BRMS1), which reduces the metastatic potential of BC cells^[43].

MiRNAs also interfere with the apoptotic process in BC cells. MiR-21 has been shown to play an anti-apoptotic role by indirectly targeting Bcl-2 in MCF-7 BC cells^[44]. On the contrary, the oncosuppressor miR-145 targets

Rhotekin (RTKN), the gene coding for the Rho effector, which activates B-cell lymphoma extra large *via* the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway^[45]; hence, promoting apoptosis. The oncogenic miR-155 is an effective suppressor of the execution phase of apoptosis *via* its actions in caspase 3 and its negative regulation of the tumor suppressor gene suppressor of cytokine signaling 1^[18]. The members of forkhead box protein O family (FOXO) which are often the target of miRNAs, represent transcription factors characterized by a distinctive forkhead DNA binding domain and play an important role in promoting the cell cycle arrest at the G₁/S checkpoint, apoptotic responses *via* the pro-apoptotic factor Bcl-2 homology domain 3-only molecule Bcl-2-interacting mediator (Bim) of cell death and cellular metabolism^[43]. In BC, the up-regulation of the oncogenic triad of miR-27a, miR-96 and miR-182 which targets FOXO1 may contribute to BC progression and maintenance of the oncogenic state^[46]. In addition, the oncogenic miR-155 may induce BC cell survival and anti-apoptosis by blocking FOXO3a^[47].

Aberrant expression of miRNAs in BC migration, invasion and metastasis: After initiation and progression, tumor cells proceed to invasion and metastasis, which are enabled by the epithelial to mesenchymal transition (EMT). BC cells in carcinoma *in situ* lose cell adhesion mediated by E-cadherin repression, become more motile and break through the basement membrane with increased invasive properties, progressing to invasive BC. EMT, which is essential in cancer invasion, metastatic dissemination and resistance acquisition to cancer therapy, is characterized by the loss of the epithelial phenotype marker E-cadherin^[18,43]. EMT is activated by tumor necrosis factor- α , hepatocyte growth factor and transforming growth factor- β (TGF- β), while many transcription factors, including zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2, can repress E-cadherin directly or indirectly^[43]. In BC, the expression of the miR-200 family is downregulated, resulting in the overexpression of ZEB1 and ZEB2, which are crucial EMT activators by inhibiting E-cadherin expression^[48]. MiR-9 directly targets the E-cadherin coding gene CDH1, leading to an enhanced cell motility and invasiveness of SUM149 human BC cells^[49]. Ras homolog gene family member (Rho) A, a prometastatic gene regulating EMT in a multiphasic manner, is the target of both oncogenic miRNAs such as miR-155, which mediates the TGF- β induced EMT, and tumor suppressor miRNAs such as miR-31^[41].

For the invasion and metastatic processes, attachment of BC cells to matrix components must take place. Metalloproteinases (MMP) degrade the extra-cellular matrix (ECM) which mediates cell attachment, while the tissue inhibitor of metalloproteinases suppresses the MMP activities^[50]. MiRNAs may induce cell migration and invasion through ECM destruction or disruption of recognition between ECM and cells. Later, circulating BC cells exit the bloodstream to form micro-metastases, undergoing mesenchymal to epithelial transition for clonal

outgrowth at the metastatic sites.

MiR-21 inhibits TIMP3 expression in BC, promoting ECM disruption, BC invasion in multiple cell lines *in vitro* and metastasis^[51]. Furthermore, the overexpressed miR-21 directly blocks the tumor suppressor programmed cell death protein 4 (Pdc4), tropomyosin 1 and maspin, enhancing the promotion of invasion and metastasis^[52]. The prometastatic miRNAs miR-373 and miR-520c may promote cell migration and invasion *in vitro* and *in vivo* by inhibiting the expression of CD44, which is a cell surface receptor for ECM components and cell to cell interactions with ECM^[53]. The basal-like subtype-specific miRNAs miR-221 and miR-222 are associated with increased cell migration and invasion, aiding in the progression of the clinically aggressive basal-like BC^[39]. Interestingly, miR-223 transferred from macrophages to BC cells through exosomes may lead to enhanced invasiveness of BC cells; highlighting the important role of exosomal communication between BC cells and macrophages^[54]. The oncogenic miR-10b, which is overexpressed in metastatic BC, may initiate tumor invasion and metastasis *in vivo* and *in vitro* by interrupting the homeobox D10 (HOXD10) expression (a transcription factor that maintains a differentiated phenotype in epithelial cells), resulting in an increased expression of Ras homolog gene family member C which leads to BC cell invasion and metastasis^[55]. The upregulation of miR-375 contributes to breast lobular neoplasia and invasive lobular breast carcinoma progression^[18].

The miRNA pair miR-126 and miR-335 has been associated with the capacity of BC cells to metastasize to bones and lungs through blocking the expression of tenascin c, a ECM component^[56]. The downregulation of the tumor suppressive let-7 contributes to BC metastasis. Let-7 modulates the repressive action of Raf kinase inhibitory protein, a BC suppressor gene inhibiting NF- κ B, mitogen-activated protein kinases and G protein-coupled receptor kinase-2 signaling pathways in BC metastatic cells^[57]. The tumor suppressive miR-31, which is undetectable in metastatic BC cells, has been shown to inhibit the expression of multiple prometastatic genes blocking BC metastasis^[18]. The tumor suppressor miR-146a/b diminishes the expression of EGFR, inhibiting metastasis^[58].

Distant BC metastases need tumor-induced formation of new blood vessels (angiogenesis) in order to allow expansion of the primary breast tumor and to obtain sufficient oxygen and nutrients. The angiogenic factor vascular endothelial growth factor (VEGF) represents the most important inducer of angiogenesis and may be regulated by several miRNAs. In particular, miR-126 has been shown to target VEGF expression in BC whereas the VEGF/PI3K/Akt signaling cascade is activated^[59]. On the contrary, miR-9 may promote angiogenesis by enhancing VEGF-A expression in BC and downregulating E-cadherin^[49]. In hypoxic conditions within the tumor microenvironment, hypoxia inducible factor-1 may also mediate the expression of VEGF in BC cells in a miR-20b-dependent way^[60]. Also, the downregulation of the oncosup-

pressor miR-125a is associated with the overexpression of a stress-induced HuR protein in the cytoplasm, which in turn could increase the invasiveness of BC cells and angiogenesis *via* VEGF-A expression^[61]. In addition to angiogenesis, miRNAs may constitute feedback mechanisms that link inflammation to BC. In particular, the upregulation of miR-155 in BC could lead to stimulation of signal transducer and activator of transcription 3 *via* the Janus kinase pathway and activation of BC cells by interleukin-6, interferon γ and lipopolysaccharide^[62].

Finally, miRNAs may also represent key regulators of the epigenetic interaction that takes place in BC cells with DNA methylation and histone modifications. The expression of the oncosuppressor miR-200 was shown to be epigenetically modulated by DNA promoter methylation and histone modifications^[53]. The downregulation of the pro-apoptotic and tumor suppressor miR-34c, which inhibits invasion, occurs through hypermethylation of the promoter region and may lead to enhanced self-renewal and EMT of breast CSCs^[63].

MI RNAs AS EXTRACELLULAR CIRCULATING BIOMARKERS IN BC

Several blood-based profiling studies have tried to elucidate the role of extracellular miRNAs in BC biology and pathogenesis^[64]. It should be also noted that miRNAs have been detected in breast milk^[22]. MiRNAs could be used as promising diagnostic, prognostic and predictive biomarkers in BC, presenting the following advantages: (1) their remarkable stability in plasma due to their association not only with RNA binding proteins [Ago2 protein, high density lipoprotein or nucleophosmin 1 (NPM1)] but also exosomal vesicular transportation^[65,66]; (2) miRNAs represent a non-invasive diagnostic approach as a liquid biopsy in contrast to the existing tissue-dependent biopsy; and (3) miRNAs may be regarded as tumor-derived molecules that have been present early into circulation, therefore reflecting tumor status.

Genome-wide expression profiling studies of extracellular miRNAs have investigated whether serum samples could be used to identify differentiated miRNA expression levels between BC patients and healthy individuals; thus, distinguishing normal from diseased state. Wu *et al.*^[67] showed that serum miR-29a and miR-21 levels were significantly increased in 20 BC patients compared to healthy controls. Kumar *et al.*^[68] also demonstrated an overexpression of miR-21 and miR-146a in plasma samples of BC patients. Using microarray-based expression profiling followed by real time quantitative polymerase chain reaction (RT-qPCR), Zhao *et al.*^[69] found deregulated expression levels of 49 miRNAs in plasma from 20 women with early stage BC compared to 20 matched controls. Furthermore, the authors showed that both up-regulated ($n = 26$) and downregulated ($n = 23$) miRNAs could discriminate patients from controls with acceptable specificity and sensitivity scores. Let-7c and miR-589 were significantly decreased and increased respectively in

BC patients. In a study by Chan *et al.*^[70], 4 (miR-1, miR-92a, miR-133a and miR-133b) of the 7 miRNAs that were differentially expressed in a set of serum samples from a cohort of 132 Asian BC patients and 101 healthy controls were validated and identified as the most significant diagnostic markers. Interestingly, only 7 miRNAs out of the total 20 were overexpressed in both tumor and serum of BC patients, indicating that miRNAs could be released into serum selectively. Profiling results of another study have indicated that the combination of circulating miR-145 and miR-451 seems capable of predicting BC patients from normal individuals^[71].

Other investigations have shown a correlation between systemic miRNA levels and various clinicopathological features of BC. MiR-10b and miR-373 were related to lymph node status^[72], while the upregulated miR-21 was associated significantly with visceral metastasis^[73]. MiR-21, miR-126, miR-155, miR-199a and miR-335 levels were closely correlated with histological grade and hormone receptor expression. A significantly higher relationship of miRNA expression levels between BC tumor tissues and sera was also found^[74]. Roth *et al.*^[75] revealed that circulating miR-10b, miR-34a and miR-155 levels were significantly related to the presence of overt metastases. Serum levels of miR-182 in ER and progesterone receptor (PR) positive BC patients were lower when compared with patients suffering from ER-PR negative BC^[76]. On the other hand, miR-155 expression levels were higher in serum of women with hormone sensitive BC (PR positive)^[77]. Similarly, in a prospective study, levels of miR-195 and let-7a were significantly correlated with ER and lymph nodal status and decreased interestingly in BC patients postoperatively following curative tumor resection^[78]. Cuk *et al.*^[79] showed that a panel of 7 circulating miRNAs, including miR-127-3p, miR-148b, miR-409-3p, miR-652 and miR-801, presents a substantial diagnostic potential, not only as a screening method for benign and malignant breast tumors, but also for the detection of early BC stage. Notably, another study has linked exosomal miRNAs to poor prognosis in BC through the maintenance of dormant BC cells in the bone marrow stroma^[80]. Sieuwerts *et al.*^[81] have highlighted the diagnostic potential of detecting tumor specific miRNAs in circulating tumor cells (CTCs) in the bloodstream in an attempt to discriminate BC patients with CTCs from patients with no detectable CTCs and healthy volunteers. Accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. For example, downregulated miR-34, miR-17 and let-7a were associated with chemosensitivity to fluorouracil, adriamycin and cyclophosphamide, respectively^[82]. Studies in BC cell lines have also related targeted miR-21 downregulation with increased sensitivity to topotecan and taxol^[44,83], whereas other investigations have indicated that restoration of the oncosuppressor miR-205 expression levels was associated with improved response to tyrosine-kinase inhibitors gefitinib and lapatinib through abrogating the HER3-

mediated resistance^[42]. A growing number of studies have demonstrated a correlation between circulating miRNA expression levels and patterns of chemoresistance or chemosensitivity. Zhao *et al*^[84] have found that plasma miR-221 could be a predictive biomarker for neoadjuvant chemotherapy sensitivity in BC patients. In other studies, circulating miR-210 and miR-125b were associated with sensitivity to trastuzumab and neoadjuvant chemotherapeutic resistance respectively; underlining the possibility of using them as indicators of treatment response^[85,86].

MI RNAs AS PROMISING THERAPEUTIC TARGETS IN BC

The pivotal role of miRNA as oncogenes or tumor suppressors has stimulated scientists to manipulate their expression; an effort that indicates their emerging role as therapeutic targets and replacement therapies for BC treatment. Depending on a given miRNA that is up or downregulated, various methods exist in order to inhibit or increase its expression, including miRNA inhibition *via* antisense targeting with oligonucleotides (anti-miRs) or miRNA replacement *via* viral or liposomal delivery (miRNA mimics), respectively^[87,88]. Functional analyses using knockdown mouse models and BC cell lines have revealed great therapeutic potential for the studied miRNA molecules. Potentially, every miRNA could serve as a possible therapeutic target. Si *et al*^[44] showed that inhibition of miR-21 expression using anti-miR-21 oligonucleotides resulted in reduced MCF-7 BC cell growth and tumor growth in the xenograft mouse model due to decreased proliferation and increased apoptosis. In agreement with these findings, Yan *et al*^[89] showed that knockdown of miR-21 inhibited growth and migration of MCF-7 and MDA-MB-231 BC cell lines *in vitro* and tumor growth in nude mice *in vivo*. MiR-21's potential therapeutic relevance is also supported by its capacity to sensitize BC cells to anticancer therapy. MiR-21 suppression has been reported to increase sensitivity of BC cells to topotecan and taxol^[44,83], whereas its tumor suppressive gene target phosphatase and tensin homolog has been shown to be a regulator of sensitivity to trastuzumab^[90]. These findings suggest that the combination of anti-miR-21 with classical chemotherapy may result in overcoming drug resistance and in individualizing therapy in BC patients. Furthermore, Kong *et al*^[47] demonstrated that knockdown of miR-155 led to apoptosis and increased chemosensitivity by upregulation of its direct target FOXO3a, suggesting that miR-155 inhibition could present a promising therapeutic potential for BC. Additionally, let-7 could contribute to cancer therapeutics due to its association with self-renewal ability and tumorigenicity of BC cells^[91]. Let-7 also regulates apoptosis and CSC differentiation^[92]; thus, targeting let-7 in BC could serve as an effective treatment option.

Recent studies have targeted miR-205 for inhibiting the metastatic nature of BC. Wu *et al*^[93] demonstrated that ectopic expression of the downregulated miR-205 effec-

tively hinders cell proliferation, anchorage-independent growth and cell invasion, supporting its use as a possible therapeutic target. MiRNA delivery *via* nanoparticles is also a promising technique. Jin *et al*^[94] have recently used nanoparticles to deliver anti-miR-10b for targeting the overexpressed miR-10b which is related to BC cell migration and invasion through inhibition of HOXD10 target synthesis. A RNA poly L-lysine complex has been developed; it releases concentrations of anti-miR-10b into the cytoplasm of BC cells with sustainable effectiveness.

Further therapeutic potential is likely *via* targeting breast CSCs with miRNA manipulation. Certain miRNAs seem to be responsible for breast CSCs behavior, including self-renewal characteristics, increased chemotherapeutic resistance and EMT^[33]. Thus, anti-CSC therapy with miRNAs could combat breast CSCs' positive effect on tumorigenesis. Additionally, a potential mesenchymal stem cell-mediated anti-miR delivery directly to the tumor area was proposed based on mesenchymal stem cells ability to migrate^[95].

Unmasking the precise role of miRNAs in the regulation of breast CSC renewal and the potential for combination of stem cell and novel miRNA-associated targeted therapies may represent effective therapeutic strategies of significant clinical benefit, probably when combined with the classic anticancer agents.

CONCLUSION

MiRNAs constitute a novel class of dysregulated molecules that could provide new avenues for diagnosing and classifying tumor-specific malignancies such as BC but also different phases of BC development from initiation to progression, migration, invasion and metastasis. The scientific interest is mainly concentrated on two basic aspects in regard to the clinical utility of miRNAs: their extracellular presence in body fluids, particularly blood, and their potential therapeutic applications either by miRNA replacement or miRNA inhibition. In BC, both aspects appear promising, with miRNAs being used as potential circulating non-invasive biomarkers detected in serum and plasma samples, and as therapeutic targets for cancer under current investigation, respectively. Although the available recent data are almost exclusively pre-clinical evidence, the application of miRNAs in BC therapy as adjuvant tools or targets appears exciting and very promising. A better understanding of the complex network of genes and cellular signaling transduction pathways regulated by miRNAs would enrich our knowledge of BC etiopathogenesis and hence would improve the therapeutic outcome of BC patients.

The diagnostic potential of circulating miRNAs as BC biomarkers is based mainly on their non-invasive detection in serum and plasma and on their high resistance and stability under difficult environmental conditions that could degrade the majority of RNAs, such as extended storage, frequent freeze-thaw cycles, extreme PH variations, boiling, preservation in archived human blood samples

for several years, transport, *etc.* Several methodologies are available for establishing miRNA signatures in BC, such as RT-PCR, miRNA microarrays and next-generation sequencing, with several limitations regarding their cross-comparison, various reference genes used to normalize miRNA levels and differences in blood collection.

Nonetheless, crucial issues need to be resolved before establishing extracellular miRNAs as biomarkers and therapeutic tools for BC. The lack of larger prospective clinical trials with robust and standardized analyzing methods, the necessity for clarifying the real origin of circulating miRNAs as they may be confused with by-products of normal tissues or dead cells, and the validation of a well-characterized BC-specific signature of circulating miRNAs are important limitations that need to be overcome when bringing miRNAs from bench to bedside. However, taking into account the important pace of evolution in understanding the main ways of miRNA effects on BC pathogenesis, these small molecules will amaze the scientific world with more revelations in the near future.

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