

Critical analysis of the potential for microRNA biomarkers in breast cancer management

Carrie R Graveel¹
 Heather M Calderone²
 Jennifer J Westerhuis²
 Mary E Winn³
 Lorenzo F Sempere²

¹Breast Cancer Signaling and Therapeutics Team, Program in Molecular Oncology and Pre-clinical Therapeutics, Center for Cancer and Cell Biology,
²Laboratory of microRNA Diagnostics and Therapeutics, Program in Skeletal Disease and Tumor Microenvironment, Center for Cancer and Cell Biology,
³Bioinformatics and Biostatistics Core, Program for Technologies and Cores, Van Andel Research Institute, Grand Rapids, MI, USA



Correspondence: Lorenzo F Sempere
 Laboratory of microRNA Diagnostics and Therapeutics, Program in Skeletal Disease and Tumor Microenvironment, Center for Cancer and Cell Biology, Van Andel Research Institute, 333 Bostwick Ave NE, Grand Rapids, MI 49503, USA
 Tel +1 616 234 5530
 Email lorenzo.sempere@vai.org

Abstract: Breast cancer is a complex and heterogeneous disease. Signaling by estrogen receptor (ER), progesterone receptor (PR), and/or human EGF-like receptor 2 (HER2) is a main driver in the development and progression of a large majority of breast tumors. Molecular characterization of primary tumors has identified major subtypes that correlate with ER/PR/HER2 status, and also subgroup divisions that indicate other molecular and cellular features of the tumors. While some of these research findings have been incorporated into clinical practice, several challenges remain to improve breast cancer management and patient survival, for which the integration of novel biomarkers into current practice should be beneficial. microRNAs (miRNAs) are a class of short non-coding regulatory RNAs with an etiological contribution to breast carcinogenesis. miRNA-based diagnostic and therapeutic applications are rapidly emerging as novel potential approaches to manage and treat breast cancer. Rapid technological development enables specific and sensitive detection of individual miRNAs or the entire miRNome in tissues, blood, and other biological specimens from breast cancer patients. This review focuses on recent miRNA research and its potential to address unmet clinical needs and challenges. The four sections presented discuss miRNA findings in the context of the following clinical challenges: biomarkers for early detection; prognostic and predictive biomarkers for treatment decisions using targeted therapies against ER and HER2; diagnostic and prognostic biomarkers for subgrouping of triple-negative breast cancer, for which there are currently no targeted therapies; and biomarkers for monitoring and characterization of metastatic breast cancer. The review concludes with a critical analysis of the current state of miRNA breast cancer research and the need for further studies using large patient cohorts under well-controlled conditions before considering the clinical implementation of miRNA biomarkers.

Keywords: miRNA, miR, blood, formalin-fixed paraffin-embedded tissues, diagnostics FFPE tissues

Introduction

Breast cancer is a highly heterogeneous disease that has multiple subtypes with distinct clinical outcomes. Clinically, breast cancers are classified by hormone receptor status, including estrogen receptor (ER), progesterone receptor (PR), and human EGF-like receptor 2 (HER2) receptor expression, as well as by tumor grade. In the last decade, gene expression analyses have given us a more thorough understanding of the molecular heterogeneity of breast cancer. Breast cancer is currently classified into six molecular intrinsic subtypes: luminal A, luminal B, HER2⁺, normal-like, basal, and claudin-low.^{1,2} Luminal cancers are commonly dependent on hormone (ER and/or PR) signaling and have the best outcome. Basal and claudin-low cancers significantly overlap with the immunohistological subtype referred to as triple-negative breast cancer (TNBC), which

lacks ER, PR, and HER2 expression. Basal/TNBC cancers have the worst outcome and there are currently no approved targeted therapies for these patients.^{3,4}

Breast cancer is a forerunner in the use of targeted therapeutic approaches. Endocrine therapy is standard treatment for ER⁺ breast cancers. The development of trastuzumab (Herceptin[®]) treatment for HER2⁺ breast cancers provides clear evidence for the value in combining prognostic biomarkers with targeted therapies. Even though early detection and targeted therapies have significantly lowered breast cancer-related mortality rates, there are still hurdles that need to be overcome. The most significant of these are: 1) improved detection of neoplastic lesions and identification of high-risk individuals (Tables 1 and 2); 2) the development of predictive biomarkers for carcinomas that will develop resistance to hormone therapy (Table 3) or trastuzumab treatment (Table 4); 3) the development of clinical biomarkers to distinguish TNBC subtypes (Table 5); and 4) the lack of effective monitoring methods and treatments for metastatic breast cancer (MBC; Table 6). In order to make advances in these areas, we must understand the heterogeneous landscape of individual tumors, develop predictive and prognostic biomarkers that can be affordably used at the clinical level, and identify unique therapeutic targets. In this review, we discuss recent findings on microRNAs (miRNAs) research aimed at addressing these challenges.

Numerous *in vitro* and *in vivo* models have demonstrated that dysregulation of individual miRNAs influences signaling networks involved in breast cancer progression. These studies suggest potential applications for miRNAs as both disease biomarkers and therapeutic targets for clinical intervention. Here, we provide a brief overview of miRNA biogenesis and detection methods with implications for breast cancer management. We also discuss the potential clinical applications for miRNAs in early disease detection, for prognostic indications and treatment selection, as well as diagnostic opportunities in TNBC and metastatic disease.

miRNA biogenesis and regulatory mechanisms of gene control

miRNAs are short non-coding regulatory RNAs that generally regulate gene expression at the post-transcriptional level.⁵ The main molecular mechanism for this regulatory mode consists of mature miRNA (18–24 nt) binding to partially complementary sites on the 3'-UTR (untranslated region) of target mRNAs.^{5,6} The mature miRNA is associated with the Argonaute-containing multi-protein RNA-induced silencing

complex (miRISC). miRNA interaction with a target RNA brings the miRISC into close proximity to the mRNA, causing mRNA degradation and/or translational repression. Due to the low specificity of binding, a single miRNA can interact with hundreds of mRNAs and coordinately modulate expression of the corresponding proteins. The extent of miRNA-mediated regulation of different target genes varies and is influenced by the context and cell type expressing the miRNA.⁵

Methods for miRNA detection in blood and tissues

Most miRNAs are transcribed by RNA polymerase II as part of a host gene transcript or as individual or polycistronic miRNA transcripts.^{5,7} As such, miRNA expression can be regulated at epigenetic and transcriptional levels.^{8,9} 5' capped and polyadenylated primary miRNA transcripts are short-lived in the nucleus where the microprocessor multi-protein complex recognizes and cleaves the miRNA precursor hairpin (pre-miRNA; about 70 nt).^{5,10} pre-miRNA is exported out of the nucleus via the XPO5 pathway.^{5,10} In the cytoplasm, the RNase type III Dicer cleaves mature miRNA (19–24 nt) from pre-miRNA. In most cases, one of the pre-miRNA arms is preferentially processed and stabilized as mature miRNA (miR-#), while the other arm is not as efficiently processed or is quickly degraded (miR-#*). In some cases, both arms can be processed at similar rates and accumulate in similar amounts. The initial nomenclature captured these differences in mature miRNA levels as 'miR-#/miR-#*' and 'miR-#-5p/miR-#-3p', respectively. More recently, the nomenclature has been unified to 'miR-#-5p/miR-#-3p' and simply reflects the hairpin location from which each RNA arm is processed, since they may each produce functional miRNAs that associate with RISC¹¹ (note that in this review we present miRNA names as originally published, so those names may not adhere to the newer guidelines). Molecular aberrations that interfere with miRNA processing, export, and/or maturation affect mature miRNA levels and biological activity. Accordingly, most miRNA detection methods focus on the analysis of mature miRNA as it most closely correlates with miRNA activity, is more long-lived, and more resistant to nuclease degradation than a primary miRNA transcript, a pre-miRNA hairpin, or mRNAs.

While the short length of mature miRNA presents advantages as a robust bioanalyte, it also presents challenges for specific and sensitive detection. Capture-probe microarray and bead platforms were major breakthroughs that have enabled high-throughput characterization of miRNA expression in

Table 1 miRNA signatures in blood for early detection of BC

miRNA(s)	Patient cohort	Sample	Methodology	Clinical observation	Reference
let-7b	102 BC cases, 26 benign breast disease cases, and 37 healthy controls	Serum (pre- and post-surgery [34 only])	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher levels of let-7 separate BC from benign disease and normal breast.	125
miR-1, miR-92a, miR-133a, miR-133b	Training set: 32 BC cases and 22 healthy controls Validation set: 132 BC cases and 101 healthy controls	Serum (and matched frozen tissue)	SYBR green qRT-PCR (Exiqon)	Changes in these miRNAs are the most significant out of 20 miRNA found to be informative for early disease detection.	126
miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-191, miR-382	61 BC cases (Stage I-II [44.3%] vs Stage III [55.7%]) and ten healthy controls	Serum	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA changes separate BC cases from controls.	127
miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, miR-425	Training set: 48 early-stage ER ⁺ cases (LN ⁻ [50%] vs LN ⁺ [50%]) and 24 age-matched healthy controls Validation set: 60 early-stage ER ⁺ cases (LN ⁻ [50%] vs LN ⁺ [50%]) and 51 healthy controls	Serum	SYBR green qRT-PCR (Exiqon)	miRNA changes separate BC cases from controls.	128
miR-18a, miR-19a, miR-20a, miR-30a, miR-103b, miR-126, miR-126,* miR-192, miR-1287	20 BC cases and 30 healthy controls	Serum (samples were pooled)	SOLiD sequencing	miRNA changes separate BC cases from controls.	129
miR-18a, miR-181a, miR-222	Training set: 410 participants in sister study (205 eventually developed BC and 205 stayed cancer-free) Validation set: five BC cases and five healthy controls	Serum	Affymetrix arrays (Discovery study); SYBR green qRT-PCR (Qiagen NV)	miRNAs with highest changes between participants that developed cancer and those who stayed cancer-free. Signature did not validate in independent cohort.	130
miR-19a, miR-24, miR-155, miR-181b	63 early-stage BC cases and 21 healthy controls	Serum (pre- and post-surgery, and after first cycle of adjuvant treatment)	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA changes separate BC cases from controls.	29
miR-21	89 BC cases (ER ⁺ [77.6%] vs ER ⁻ [22.4%]; Stage I-II [55%] vs Stage III-IV [45%]) and 55 healthy controls	Serum	SYBR green qRT-PCR assay (Hoffman-La Roche Ltd)	Increased circulating levels of miR-21 in BC cases.	131
miR-21, miR-92a	100 primary BC patients and 20 healthy controls	Serum (and matching tissue)	SYBR green qRT-PCR assay (Takara Bio Inc.)	miRNA changes separate BC cases from controls.	132
miR-27a, miR-30b, miR-148a, miR-451	129 BC cases and 29 healthy controls	Serum	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA changes separate BC cases from controls.	133
miR-30a	100 BC cases (ER ⁺ [77%] vs ER ⁻ [23%]; LN ⁻ [69%] vs LN ⁺ [31%]; Stage I-II [77%] vs Stage III-IV [17%]) and 64 age-matched healthy controls	Plasma (pre- and post-surgery)	TaqMan qRT-PCR (Thermo Fisher Scientific)	Decreased circulating levels of miR-30a in BC cases.	134
miR-92b,* miR-568, miR-708*	20 BC cases before surgery (ER ⁺ [60%] vs ER ⁻ [40%]; Stage I-II [85%] vs Stage III-IV [15%]), 20 BC cases after surgery (ER ⁺ [75%] vs ER ⁻ [25%]; Stage I-II [95%] vs Stage III-IV [5%]), ten cases with other cancer types and 20 healthy controls	Plasma	Illumina miRNA arrays	miRNA changes separate BC cases specifically (not present in other cancer types) from controls.	26
miR-107, miR-148a, miR-223, miR-338-3p	24 ER ⁺ early-stage BC patients (LN ⁻ [50%] vs LN ⁺ [50%]) and 24 age-matched healthy controls	Serum (pre- and post-surgery)	SYBR green qRT-PCR (Exiqon)	miRNA changes separate ER ⁺ BC cases from controls.	28

(Continued)

Table 1 (Continued)

miRNA(s)	Patient cohort	Sample	Methodology	Clinical observation	Reference
miR-127-3p, miR-148b, miR-376a, miR-376c, miR-409-3p, miR-652, miR-801	Training set: 127 BC cases (ER ⁺ [81.1%] vs ER ⁻ [19.1%]; LN ⁺ [59%] vs LN ⁻ [41%]; Stage I-II [75.5%] vs Stage III-IV [24.5%]) and 80 healthy controls Validation set: 120 BC cases (ER ⁺ [82.5%] vs ER ⁻ [17.5%]; LN ⁺ [59.1%] vs LN ⁻ [40.9%]; Stage I-II [78.3%] vs Stage III-IV [21.7%]), 30 benign breast disease cases, and 60 healthy controls	Plasma	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA signature separates BC cases from healthy controls. Only changes in miR-127-3p, miR-376a, miR-376c, and miR-409-3p separate BC cases from benign breast disease.	135
miR-133a, miR-148b	Training set: 52 early-stage BC cases, 35 DCIS cases and 35 healthy controls Validation set: 50 early-stage patients and 50 healthy controls	Plasma	SYBR green qRT-PCR (Exiqon)	miRNA changes separate BC cases from controls.	27
miR-138	83 BC cases (ER ⁺ [50.6%] vs ER ⁻ [48.4%]; Stage I-II [85.5%] vs Stage III [14.5%]) and 83 healthy controls	Blood	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher circulating levels of miR-138 separate ER ⁺ BC cases (but not ER ⁻ cases) from controls.	136
miR-148b, miR-376c, miR-409-3p, miR-801	127 BC cases (ER ⁺ [77.1%] vs ER ⁻ [15.7%]; LN ⁺ [58.2%] vs LN ⁻ [34.6%]; Stage I-II [76.3%] vs Stage III-IV [7.8%]) and 80 healthy controls	Plasma	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA changes separate BC cases from controls.	137
miR-155, miR-205	20 BC cases (ER ⁺ [65%] vs ER ⁻ [35%]; Stage I-II [65%] vs Stage III [35%]) and ten healthy controls	Plasma	TaqMan qRT-PCR (Thermo Fisher Scientific)	miRNA changes separate BC cases from controls.	138
miR-182	46 BC patients (ER ⁺ [63%] vs ER ⁻ [37%]) and 58 healthy controls	Serum	SYBR green qRT-PCR (Qiagen)	Increased circulating levels of miR-182 in BC cases.	139
miR-484	Training set: 39 early-stage BC cases (ER ⁺ [71.8%] vs ER ⁻ [28.2%]; LN ⁺ [48.7%] vs LN ⁻ [51.3%]) and ten healthy controls Validation set: 98 early-stage BC cases (ER ⁺ [44.9%] vs ER ⁻ [55.1%]; LN ⁺ [44.9%] vs LN ⁻ [55.1%]) and 25 healthy controls	Serum	TaqMan qRT-PCR (Thermo Fisher Scientific)	Increased circulating levels of miR-484 in BC cases.	140

Notes: This is a representative sample of 20 recent studies found on a PubMed query (breast cancer blood miRNA miR) that describe individual miRNAs or miRNA signatures having potential application for early disease detection. Studies with fewer than 20 BC cases were excluded. While these signatures mainly reflect higher amounts of circulating miRNAs, some miRNAs are detected at lower levels in blood samples of BC patients. Blood collection was performed before surgery unless otherwise indicated. miRNAs shown in bold indicate a recurrent presence in at least three independent studies.

Abbreviations: BC, breast cancer; DCIS, ductal carcinoma in situ; ER, estrogen receptor; LN, lymph node status; miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 2 miRNA-related risk loci associated with BC

Gene locus	SNP	Comments	Population	Clinical observation	Reference	
MIR27A	rs895919 *C		Asians	No risk association	141	
			Caucasians	Protective		
	Jewish BRCA2 carriers		Increased risk	142		
	rs895819 A/G		pre-miRNA	Caucasian	Decreased risk	143
			pre-miRNA	Asians	No risk association	144
				Caucasians	Decreased risk	
				Chinese (young)	Decreased risk	35
				Chinese	Decreased risk	34
				Asians	No risk association	31
				Caucasians	No risk association	
MIR34B cluster	rs4938723 T/C		African Americans	Increased survival	145	
MIR100	rs1834306 G/A		African Americans	No risk association	33	
			European Americans	Decreased overall risk		
MIR101-2	rs1053872 C/G rs462480 A/C	Primary transcript Primary transcript	Chinese	Increased risk	38	
			Chinese	Increased risk	38	
MIR106B	rs1527423 A/G		African Americans	No risk association	33	
			European Americans	Increased overall risk		
MIR122A	rs17669 A/G		African Americans	Decreased risk of ER ⁺ BC	33	
			European Americans	No risk association		
MIR146A	rs2910164 G/C		Italian	Earlier age of onset	146	
			Caucasians	No risk association	147	
			Chinese	No risk association	83	
			Asians	No risk association	38	
			Caucasians	No risk association		
			Asians	Decreased risk (C allele)	144	
			Asians	No risk association	31	
			Caucasians	No risk association		
			Chinese	No risk association	36	
					Asians	No risk association
MIR149	rs2292832 T/G		Caucasians	No risk association		
			Chinese	No risk association	36	
			Asians	No risk association	31	
			Caucasians	No risk association		
			African Americans	Reduced risk	145	
MIR185	rs2008591 C/T		African Americans	Reduced risk	145	
	rs887205 A/G		African Americans	Reduced risk	145	
MIR196A-2	rs11614913 T/C		Korean	Survival of HER2 ⁺ cases	148	
			Italian and German	No risk association	37	
			Asians	Decreased risk	141	
			Caucasians	No risk association		
			Brazilian	Decreased risk	149	
			Caucasian	Decreased risk	147	
			Chinese and Korean	Decreased risk	32	
			Chinese	Increased risk	36	
			Chinese	Increased risk	83	
			African Americans	No risk association	33	
MIR204	rs7861254 G		European Americans	No risk association		
			African Americans	Decreased risk of ER ⁻ BC	33	
			European Americans	No risk association		
			African Americans	Increased survival	145	
			African Americans	Increased risk of ER ⁻ BC	33	
MIR206	rs6920648 A/G		European Americans	No risk association		
			European Americans	No risk association	33	
MIR219	rs107822 G/A		African Americans	No risk association		
			European Americans	Increased overall risk		
MIR331	rs11107973 A/G		African Americans	No risk association	33	
			European Americans	Increased overall risk		
MIR373	rs12983273 T/C		Asians	No risk association	38	
			Caucasians	No risk association		

(Continued)

Table 2 (Continued)

Gene locus	SNP	Comments	Population	Clinical observation	Reference
MIR423	rs6505162 A/C	pre-miRNA	Caucasian	Reduced risk	150
			Jewish BRCA2 carriers	Later age of onset	142
			Asians	No risk association	38
MIR455	rs2060133 C/G		Caucasians	No risk association	
			African Americans	No risk association	33
MIR487	rs1951032 G/A		European Americans	Decreased risk of ER ⁺ BC	
			African Americans	No risk association	33
MIR495	rs2281611 C/A		European Americans	Increased overall risk	
			African Americans	No risk association	33
MIR499	rs3746444 A/G		European Americans	Increased risk of ER ⁻ BC	
			Chinese	Increased risk	36
			Asians	Increased risk	151
			Caucasians	No risk association	
			Asians	Increased risk	152
			Caucasians	No risk association	
			Chinese	Increased risk	32
			Caucasians	No risk association	147
			Asians	Increased risk	153
			Caucasians	No risk association	
			Asians	Increased risk	31
			Caucasians	No risk association	
			Asians	Decreased risk	141
			Caucasians	No risk association	
			MIR513A-2	rs2018562 A/G	
MIR544	rs10144193 A/T		European Americans	No risk association	
			African Americans	No risk association	33
MIR548A-2	rs878175 A/G		European Americans	Increased overall risk	
			African Americans	No risk association	33
MIR595	rs4909238 A/G		European Americans	Decreased risk of ER ⁺ BC	
			African Americans	Increased risk of ER ⁻ BC	33
MIR605	rs2043556 *A		European Americans	No risk association	
			Asians	Decreased risk	141
MIR606	rs12266981 G/A		Caucasians	No risk association	
			African Americans	Increased risk of ER ⁺	33
MIR608	rs4919510 C/G		European Americans	No risk association	
			African Americans	No risk association	33
MIR628	rs8041885 A/G		European Americans	Decreased risk of ER ⁻ BC	
			African Americans	Decreased risk of ER ⁺ BC	33
MIR659	rs8041044 C/A		European Americans	No risk association	
			African Americans	Decreased risk of ER ⁺	33
			European Americans	No risk association	
MIR758	rs5750504 T/A		African Americans	No risk association	33
			European Americans	Increased overall risk	
			African Americans	Decreased overall risk	33
ATF1 3' UTR	rs11169571 C/T	miR-320 MRE	European Americans	No risk association	
			African Americans	No risk association	
			European Americans	Increased risk of ER ⁻ BC	
BMPRI3 3' UTR	rs1434536 C/T	miR-125b MRE	African Americans	Increased risk	142
			Caucasian	Increased risk	43
BRCA1 3' UTR	rs799917 C/T	miR-638 MRE	Italian	Increased risk	154
HPGD 3' UTR	rs8752 G/A	miR-485-5p MRE	Chinese	Increased risk in ER ⁺	155
IGF-1R 3' UTR	rs28674628 A/G	miR-515-5p MRE	Jewish BRCA1 carriers	Earlier age of onset	156
IL23R 3' UTR	rs10889677 A/C	let-7 MRE	Chinese	Increased risk (A allele)	157
				Earlier age of onset (C allele)	
				Decreased risk	158
IQGAP1 3' UTR	rs1042538 A/T	miR-124 MRE	Chinese	Decreased risk	159
ITGB4 3' UTR	rs743554 A/G	miR-34a MRE ^{predicted}	Swedish	Increased risk	160
				Reduced survival	

(Continued)

Table 2 (Continued)

miRNA locus	SNP	Comments	Population	Clinical observation	Reference
KRAS 3' UTR	rs61764370 T/G	let-7 MRE	Caucasian	Increased frequency in BRCA1 carriers/no risk association	40
				Increased risk of TNBC	39
RAD52 3' UTR	rs7963551 A/C	let-7 MRE	Chinese	Decreased risk	42
RYR3 3' UTR	rs1044129 A/G	miR-367 MRE	Chinese	Increased risk and poor survival	161
SET8 3' UTR	rs16917496 C/T	miR-502 MRE	Asian	Earlier age of onset	162
TGFBR1 3' UTR	rs334348 A/G	miR-628-5p MRE	Italian	Increased risk	154
TGFBI Exonic	rs1982073 C/T	miR-187 MRE	Italian	Increased risk	154
XRCCI Exonic	rs1799782 T/C	miR-138 MRE	Italian	Decreased risk	154
AGO4	rs7354931 C/A	miRNA RISC-loading, miRNA-RISC activity	African Americans	Decreased overall risk	33
	rs16822342 A/G		European Americans	No risk association	
			African Americans	Decreased risk of ER ⁺ BC	33
			European Americans	No risk association	
	rs3820276 G/C		African Americans	Increased risk of ER ⁺ BC	33
			European Americans	No risk association	
DGCR8	rs417309 G/A	Pre-miRNA processing	Chinese	Increased risk	42
	rs9606241 A/G		African Americans	No risk association	33
			European Americans	Increased risk of ER ⁺ BC	
PACT	rs2059691 G/A	miRNA-RISC activity	African Americans	No risk association	33
			European Americans	Increased overall risk	
XPO5	rs11077 A/C	Pre-miRNA nuclear export	African Americans	Decreased risk of ER ⁺ BC	33
			European Americans	No risk association	

Abbreviations: BC, breast cancer; ER, estrogen receptor; HER2, human EGF-like receptor 2; miRNA, microRNA; MRE, microRNA recognition element (ie, binding site); RISC, RNA-induced silencing complex; UTR, untranslated region.

cancer tissues. Typically, these platforms require a large amount of sample, making direct studies of blood or other biological fluids having low miRNA content difficult. Stem-loop primer reverse transcription polymerase chain reaction (RT-PCR) analysis provides an alternative platform that can detect a much lower number of miRNA copies. Such analysis was initially used as an independent validation tool for array-based expression profiling findings and is the current gold standard practice for technical validation of altered miRNA expression. High-throughput RT-PCR multiplexing platforms have enabled characterization of miRNA expression in blood. More recently, NanoString and RNA-Seq analyses have added new high-throughput tools with single molecule detection capabilities. All of these detection methods, each with unique advantages and limitations, have been applied to expression profiling of miRNAs in breast cancer tissues and blood samples from breast cancer patients.^{12–15}

miRNA biomarkers for early disease detection

The prognosis for breast cancer patients is strongly influenced by the stage of the disease. For instance, the 5-year survival rate is 99% for localized disease, 84% for regional disease, and 24% for distant-stage disease.¹⁶ Larger tumor size also correlates with poorer prognosis. Therefore, it is essential that breast cancer lesions are diagnosed at

the earliest stages. Mammography, ultrasound, magnetic resonance, and nuclear medicine are used to identify breast lesions at their earliest stages.¹⁷ Mammography is the current gold standard for breast cancer detection for women over the age of 39 years. However, its limitations include high false-positive rates (12.1%–65.8%)¹⁸ that lead to additional imaging and biopsies,¹⁹ and low success rates in the detection of neoplastic tissue within dense breast tissue. A combination of mammography with magnetic resonance or other imaging platforms can enhance tumor detection, but this additional imaging is costly and is not a routine screening procedure.²⁰ Consequently, more sensitive and more specific detection assays are needed that avoid unnecessary additional imaging and surgery from initial false-positive mammographic results. miRNA analysis of blood or other body fluids offers an inexpensive and noninvasive screening approach to more thoroughly examine high-risk individuals, either those with genetic predispositions or post-treatment patients at risk of recurrence.

miRNA biomarkers in blood

miRNAs are promising blood biomarkers because cell-free miRNA molecules that are circulating unaccompanied, associated with protein complexes, or encapsulated in membrane-bound vesicles (eg, exosome and microvesicles) are very stable in blood.^{21,22} However, circulating miRNAs may emanate from

Table 3 miRNA signatures for prognosis and treatment response in ER⁺ breast cancer subtypes

miRNA(s)	Patient cohort	Sample	Methodology	Clinical observation(s)	Reference
let-7b	2,033 cases (ER ⁺ [84%] vs ER ⁻ [16%])	FFPE tissue cores	In situ hybridization	Higher levels of let-7b correlate with better outcome in ER ⁺ cases.	163
miR-7, miR-128a, miR-210, miR-516-3p	147 early-stage ER ⁺ cases with LN ⁻	FFPE tissue	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter time to distant metastasis.	65
miR-10a, miR-126	Training set: 12 early-stage ER ⁺ cases (LN ⁻ [83.3%] vs LN ⁺ [16.7%]) Validation set: 81 ER ⁺ cases (Stage I-II [77.5%] vs Stage III [23.5%], LN ⁻ [46.9%] vs LN ⁺ [51.8%]) treated with tamoxifen monotherapy	FFPE tissue	TaqMan qRT-PCR (Thermo Fisher Scientific)	Predicts response to tamoxifen and correlates with longer recurrence-free survival.	60
miR-19a, miR-205	68 luminal A ^a cases (Stage II [16.2%] vs Stage III [83.8%]) treated with neoadjuvant epirubicin + paclitaxel	Serum	SYBR-based qRT-PCR (Quantobio Technology)	Predicts response to epirubicin + paclitaxel.	118
miR-30c	246 advanced-stage ER ⁺ cases (local recurrence [13%] vs distant recurrence [87%]) treated with tamoxifen	FFPE tissue	TaqMan qRT-PCR (Thermo Fisher Scientific)	Predicts response to tamoxifen and correlates with longer progression-free survival.	61
miR-210	89 early-stage ER ⁺ cases (LN ⁻ [56%] vs LN ⁺ [38%]) treated with adjuvant tamoxifen monotherapy	FFPE tissue	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter recurrence-free survival.	62
miR-519a	50 ER ⁺ cases	FFPE tissue	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter recurrence-free survival.	63

Notes: ^aLuminal A subtype was defined by expression of ER and/or PR, absence of HER2 expression, and less than 14% of cells positive for Ki-67.

Abbreviations: ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; LN, lymph node status; miRNA, microRNA; PR, progesterone receptor; HER2, human EGF-like receptor 2; qRT-PCR, quantitative real-time polymerase chain reaction.

different cell types in the primary tumor lesion or systemically, and reflect: 1) the number of lysed cancer cells or other cells in the tumor microenvironment, 2) the number of cells expressing and secreting those particular miRNAs, and/or 3) the number of cells mounting an inflammatory or other physiological response against diseased tissue. Ideally for analysis, circulating miRNAs would reflect the number of cancer cells or other cell types specific to breast cancer in the primary tumor. Many studies have compared changes in miRNA levels in blood between breast cancer cases and age-matched healthy

controls in order to identify miRNA biomarkers (Table 1). Unfortunately, there is significant variability among studies in the patient characteristics, experimental design, sample preparation, and detection methodology that complicates the interpretation of these studies:

- Patient characteristics: Clinical and pathological characteristics of patients and their tumor tissues differ broadly. Age, ethnicity, stage, histology, molecular subtype, and treatment history are variables that can affect miRNA expression.²³

Table 4 miRNA signatures for prognosis and treatment response in HER⁺ breast cancer subtypes

miRNA(s)	Patient cohort	Sample	Methodology	Clinical observation(s)	Reference
miR-21	32 Stage III HER2 ⁺ cases (ER ⁺ [56.2%] vs ER ⁻ [43.8%])	Frozen tissues (pre- and post-neoadjuvant treatment)	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher levels correlate with poor treatment response.	79
miR-21, miR-210, miR-373	127 HER2 ⁺ cases (ER ⁺ [56%] vs ER ⁻ [44%]; LN ⁻ [40%] vs LN ⁺ [60%]; M0 [84%] vs M1 [16%]) with neoadjuvant treatment (trastuzumab [50%] vs lapatinib [50%])	Serum (pre- and post-neoadjuvant treatment)	TaqMan qRT-PCR (Thermo Fisher Scientific)	No correlation with pathologic complete response. High levels of miR-21 correlate with overall survival.	120
miR-210	29 HER2 ⁺ cases (ER ⁺ [44.8%] vs ER ⁻ [55.2%]; LN ⁻ [34.4%] vs LN ⁺ [65.6%]; with neoadjuvant treatment (trastuzumab + chemotherapy)	Plasma (pre- and post-neoadjuvant treatment)	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher circulating levels correlate with pathologic complete response, tumor presence, and LN ⁺ status.	119

Abbreviations: ER, estrogen receptor; HER2, human EGF-like receptor 2; miRNA, microRNA; LN, lymph node status; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 5 miRNA signatures for prognosis and treatment response in TNBC subtype

miRNA(s)	Patient cohort	Sample	Methodology	Clinical observation(s)	Reference
miR-10b, miR-21 , miR-122a, miR-145, miR-205, miR-210	49 TNBC cases	FFPE tissues	SYBR green qRT-PCR (Qiagen NV)	Correlates with shorter disease-free and overall survival.	164
miR-10b-5p, miR-21-3p , miR-31-5p, miR-125b-5p, miR-130a-3p, miR-155-5p , miR-181a-5p, miR-181b-5p, miR-183-5p, miR-195-5p, miR-451a	15 TNBC cases	Fresh tissues	SYBR green qRT-PCR (Takara Bio Inc.)	Separates TNBC tissues from normal breast tissue. Signature enriched for miRNAs involved in chemoresistance.	86
miR-16, miR-125b, miR-155 , miR-374a	173 TNBC cases (LN ⁻ [35.8%] vs LN ⁺ [64.2%])	FFPE tissues	NanoString nCounter	Correlates with shorter overall survival.	165
miR-21	72 TNBC cases (Stage I–II [45.8%] vs Stage III–IV [54.2%]; LN ⁻ [51.3%] vs LN ⁺ [48.6%])	Frozen tissues	SYBR green qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter recurrence-free survival.	166
	105 early-stage TNBC cases (Stage I [48.5%] vs Stage II [51.5%]; LN ⁻ [67.6%] vs LN ⁺ [32.4%])	FFPE tissue cores	In situ hybridization	High levels in stroma compartment correlate with shorter recurrence-free and breast cancer-specific survival.	97
miR-27a, miR-30e, miR-155 , miR-493	173 TNBC cases (LN ⁻ [35.8%] vs LN ⁺ [64.2%])	FFPE tissues	NanoString nCounter	Divides cases into risk subgroups.	84
miR-27b, miR-150, miR-342	37 TNBC cases	Frozen tissues	Illumina miRNA arrays	Correlates with shorter recurrence-free survival.	67
miR-190a, miR-200b-3p, miR-512-5p	Eleven TNBC cases (Stage I–II [36.3%] vs Stage III–IV [63.7%]; LN ⁻ [27.2%] vs LN ⁺ [72.8%]) treated with different neoadjuvant chemotherapy regimens	Tissue core biopsies	SYBR green qRT-PCR (Exiqon)	Predicts response to treatment.	85
miR-34b	39 TNBC cases (Stage I–II [80%] vs Stage III–IV [20%]; LN ⁻ [44%] vs LN ⁺ [56%])	FFPE tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter disease-free and overall survival.	167
miR-200c, miR-205	32 TNBC cases (LN ⁻ [50%] vs LN ⁺ [50%])	FFPE tissues	SYBR green qRT-PCR (Qiagen NV)	Lower levels correlate with LN ⁺ status.	168
miR-210	114 early-stage ER ⁻ cases with LN ⁻ status	FFPE tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter time to distant metastasis.	65
	58 TNBC cases (LN ⁻ [68.9%] vs LN ⁺ [29.3%])	Frozen tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter disease-free and overall survival.	66
miR-376b, miR-381, miR-409-5p, miR-410, miR-766	114 TNBC cases	Frozen tissues	miRNA arrays (Agilent Technologies)	Correlates with shorter distant metastasis-free and breast cancer-specific survival.	169

Note: microRNAs in bold show a recurrent presence in at least three independent studies.

Abbreviations: FFPE, formalin-fixed paraffin-embedded; LN, lymph node status; TNBC, triple-negative breast cancer; miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction.

- Experimental design: Sample size and the inclusion of training and validation sets vary. Some studies analyzed changes in miRNA levels between fewer than 30 breast cancer and 30 control samples in a single patient cohort, whereas others analyzed these changes in much larger patient cohorts and validated miRNA signatures using independent cohorts. Such differences affect the statistical power of analysis. The miRNA field must be aware of the pitfalls associated with small sample sizes, poor experimental design, and statistical choices.²⁴
- Sample preparation: Whole blood, serum, and plasma have been used as sample material for miRNA detection. Whole blood contains various cell types (white cells, red cells, and platelets) that contribute their miRNA content to the sample being analyzed, confounding interpretation of results. For this reason, serum or plasma are preferred sources of circulating miRNAs. Serum is obtained after blood coagulation and contains the liquid portion of blood with its proteins and other soluble molecules, but without cells or clotting factors. Plasma is obtained from

Table 6 miRNA signatures for detection, monitoring, and characterization of MBC

microRNA(s)	Patient cohort	Sample	Methodology	Clinical observation	Reference
miR-10b	23 cases (M0 [21.7%] vs MI [78.3%])	FFPE tissues	SYBR green qRT-PCR (Thermo Fisher Scientific)	Higher levels in MBC cases.	100
	101 cases (ER ⁺ [62.4%] vs ER ⁻ cases [37.6%]; LN ⁻ [33.7%] vs LN ⁺ [66.3%]; Stage I-II [59.4%] vs Stage III-IV [40.6%])	FFPE tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher levels in MBC cases; higher levels correlate with shorter progression-free and overall survival in metastasis-free cases.	102
	84 early-stage cases (ER ⁺ [53.6%] vs ER ⁻ cases [41.1%]; LN ⁻ [24.1%] vs LN ⁺ [75.9%])	FFPE tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	No correlation with disease progression, metastasis, or clinical outcome.	106
	219 cases (LN ⁻ [58%] vs LN ⁺ [42%])	Frozen tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	No correlation with formation of distant metastasis or clinical outcome.	107
	122 cases (M0 [82%] vs MI [18%]) and 59 age-matched healthy controls	Serum	SYBR green qRT-PCR (Thermo Fisher Scientific)	Higher levels in MBC cases with bone metastases.	104
	152 cases (M0 [78.9%] vs MI [21.1%]) and 40 healthy controls	Serum (post-surgery for M0 cases)	TaqMan qRT-PCR (Thermo Fisher Scientific)	No change in levels change between non-MBC and MBC cases.	170
miR-10b, miR-373	60 cases (ER ⁺ [60%] vs ER ⁻ cases [40%]; LN ⁻ [41.7%] vs LN ⁺ [58.3%]; Stage I-II [%])	Plasma	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher levels in cases with LN ⁺ .	103
miR-17, miR-155, miR-19b	152 cases (M0 [78.9%] vs MI [21.1%]) and 40 healthy controls	Serum	TaqMan qRT-PCR (Thermo Fisher Scientific)	Levels change between non-MBC and MBC cases.	170
	113 cases (HER2 ⁻ [42.4%] vs HER2 ⁺ [57.5%]; M0 [31%] vs MI [69%]) and 30 age-matched healthy controls	Serum	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with longer overall survival in HER2 ⁺ MBC cases with inflammatory disease.	105
miR-21, miR-205	84 early-stage cases (ER ⁺ [53.6%] vs ER ⁻ cases [41.1%]; LN ⁻ [24.1%] vs LN ⁺ [75.9%])	FFPE tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Correlates with shorter recurrence-free survival. Only lower levels of miR-205 correlate with shorter overall survival.	106
miR-210	219 cases (LN ⁻ [58%] vs LN ⁺ [42%])	Frozen tissues	TaqMan qRT-PCR (Thermo Fisher Scientific)	Higher levels correlate with shorter recurrence-free survival.	107
miR-1258	166 BC cases (M0 [48.7%] vs MI [51.3%]), 62 cases with benign breast disease and 54 healthy controls	Serum (post-surgery but before treatment)	SYBR green qRT-PCR (Shanghai Novland Co. Ltd)	Lower circulating levels in BMC cases compared to non-BMC cases and healthy controls. Higher circulating levels correlate with good clinical outcome.	171

Note: microRNAs in bold show a recurrent presence in at least three independent studies.

Abbreviations: BC, breast cancer; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; LN, lymph node status; MBC, metastatic breast cancer; miRNA, microRNA; HER2, human EGF-like receptor 2; qRT-PCR, quantitative real-time polymerase chain reaction.

uncoagulated blood; it contains the liquid portion of blood with clotting factors, proteins, and molecules not present in serum, but it also retains some cells. Additionally, different anticoagulants can be used to prepare plasma (eg, heparin and ethylenediaminetetraacetic acid [EDTA]), and these can have different effects on plasma composition and downstream molecular assays. The lysis of red blood cells or other cell types (hemolysis) during blood separation procedures can contaminate the miRNA content in serum and plasma preparations. Several miRNAs are known to be expressed at high levels in specific blood cell types, and these miRNAs are typically excluded from analysis to avoid confusion.

Moreover, it appears that miRNA concentration in serum is higher than in plasma, hindering direct comparison of studies using these different starting materials.²⁵

- **Detection methodology:** The miRCURY LNA Universal RT miRNA and PCR assay, and the TaqMan Low Density Array RT-PCR assay are among the most frequently used high-throughput RT-PCR platforms for miRNA detection. Each uses a different strategy to reverse transcribe mature miRNA molecules and to PCR-amplify the cDNA, which results in different detection biases.
- **Data analysis:** One of the biggest challenges to date is the normalization of circulating miRNA levels. Since

there is not a unique cellular source or mechanism by which miRNAs reach circulation, choosing a reference miRNA (eg, miR-16, miR-26a) or other non-coding RNA (eg, U6 snRNA, snoRNA RNU43) is not straightforward. Spiking samples with RNA controls and/or normalization of miRNA levels to volume are some of the strategies used to standardize analysis. In addition, various studies apply different statistical methods and criteria for normalization, background or control reference subtraction, and significance cutoff values.¹²

Due to this variability in assay methods and analysis, it is not surprising that the reported signatures present little overlap. If one focuses on common trends, there are some miRNAs that might be useful for early detection of all types of breast cancer, whereas others might be useful for specific subtypes, histologies, or disease stages (Table 1). We briefly describe recent studies that used previous works to inform their experimental approach and analysis. Leidner et al drew and harmonized miRNA data from 15 previous studies and compared circulating miRNA signatures.²⁶ They found very few miRNAs whose changes in circulating levels between breast cancer and control samples were consistent even when using similar detection methods (mainly quantitative real-time polymerase chain reaction [qRT-PCR] assays). There was no consistency at all between circulating miRNA signatures generated using different genome-wide detection platforms after filtering out contaminating miRNAs from cellular sources in the blood. The authors then performed their own study that included plasma samples from 20 breast cancer patients before surgery, 20 age- and race-matched healthy controls, an independent set of 20 breast cancer patients after surgery, and ten patients with lung or colorectal cancer. Forty-six circulating miRNAs showed significant changes between pre-surgery breast cancer patients and healthy controls. Using other reference groups in the study, the authors could assign miRNA changes to different categories. The change in the circulating amount of 13 of these miRNAs was similar between post-surgery breast cancer cases and healthy controls, suggesting that the changes in these miRNAs in pre-surgery patients reflected the presence of a primary breast cancer tumor.²⁶ However, ten of the 13 miRNAs also showed altered plasma levels in patients with other cancer types, suggesting that they may more generally reflect a tumor presence or tumor burden. After these analyses, only three miRNAs (miR-92b*, miR-568, and miR-708*) were identified as breast cancer-specific circulating miRNAs. These miRNAs had not been identified in previous studies.

More recently, Shen et al found 43 miRNAs that were detected at significantly different levels in plasma samples from a training set of 52 patients with invasive breast cancer, 35 with noninvasive ductal carcinoma in situ (DCIS), and 35 healthy controls;²⁷ all study subjects were Caucasian. miR-33a, miR-136, and miR-199-a5-p were among those with the highest fold change between invasive carcinoma cases and healthy controls or DCIS cases. These changes in circulating miRNA levels may reflect advanced malignancy events. Twenty-three miRNAs exhibited consistent changes between invasive carcinoma and DCIS cases relative to healthy controls, which may reflect early malignancy changes. Interestingly, only three of these 43 miRNAs overlapped with miRNAs in previously reported signatures. These three, miR-133a, miR-148b, and miR-409-3p, were all part of the early malignancy signature and their fold changes were relatively modest, less than four-fold. Nonetheless, the authors validated the changes of miR-133a and miR-148b in plasma samples from an independent cohort of 50 patients with stage I and II breast cancer and 50 healthy controls. Moreover, miR-133a and miR-148b were detected in culture media of MCF-7 and MDA-MB-231 cells, suggesting that they are secreted by the cancer cells. The authors did not investigate the mechanism of miRNA secretion.

Some studies have also compared changes in the amount of circulating miRNAs in blood samples obtained before or after surgery (Table 1). A four-miRNA signature (miR-107, miR-148a, miR-223, and miR-338-3p) was identified in a patient cohort of 24 ER⁺ breast cancers.²⁸ Circulating serum levels of miR-148a, miR-223, and miR-338-3p decreased, while that of miR-107 increased after surgery.²⁸ Normalization of circulating miRNA levels after surgery could be useful in detecting disease recurrence if the changes are also observed in blood samples collected during follow-up visits. In another study, circulating levels of miR-19a, miR-24, miR-155, and miR-181b were monitored longitudinally in serum samples from a cohort of 63 breast cancer patients collected 1 day before surgery, 2–4 weeks after surgery, and 2–4 weeks after the first cycle of adjuvant treatment.²⁹ Levels of miR-24, miR-155, and miR-181b decreased after surgery, while the level of miR-19a only significantly decreased after adjuvant treatment.²⁹ The authors noted that three patients relapsed during the study follow-up. This limited number did not allow the authors to determine whether the altered levels of these miRNAs could be useful for detecting disease recurrence.²⁹

The lack of consensus about circulating miRNA signatures for early detection of primary or recurrent breast tumor requires

careful and thoughtful examination. Does this mainly indicate technical difficulties in preanalytic sample preparation, miRNA detection, and/or statistical analysis? Or does it more deeply question the validity of miRNAs as biomarkers for detecting a wide array of heterogeneous presentations of breast cancer? Longitudinal studies that collect blood from breast cancer patients, ideally before diagnosis (healthy baseline), at diagnosis, before surgery, and after surgery, that also consistently process and analyze miRNA changes should be considered to address these questions. High-risk individuals, such as *BRCA* gene mutation carriers, those with other genetic predispositions to breast cancer, or breast cancer survivors at high risk of recurrence, could provide cohorts of appropriate size for such longitudinal studies.

Finally, detection of miRNAs within isolated exosomes or microvesicles is a potential new biomarker assay to consider.^{21,22} Enrichment of miRNAs in these membrane-bound particles may more directly reflect the secretory phenotype of cancer cells or other cells in the tumor microenvironment, than circulating miRNAs in whole blood samples. Such miRNAs may be less subject to noise and inter-patient variability, and thus may be a more appropriate material for analysis in longitudinal studies.

Risk alleles of miRNA or target genes associated with breast cancer

By mining the genome for allele variants of miRNA genes or their known target genes, miRNA research has shown some promise in helping identify individuals at risk of developing breast cancer. Single nucleotide polymorphisms (SNPs) in the miRNA precursor hairpin can affect its stability, miRNA processing, and/or altered miRNA–target mRNA binding interactions if the SNPs are within the functional sequence of mature miRNAs. Similarly, SNPs in the 3′-UTR of mRNAs can decrease or increase binding interactions with miRNA, altering protein expression. In addition, SNPs in coding sequences of proteins involved in miRNA processing (eg, DROSHA), export (eg, XPO5), and maturation (eg, Dicer) can also affect the expression levels and activity of miRNAs (Table 2). Depending on the tumor suppressive or oncogenic functions of a protein, disruption of miRNA-mediated regulation can increase or decrease cancer risk. According to the miRdSNP database, there are currently 14 unique genes experimentally confirmed as miRNA targets with breast cancer-associated SNPs in their 3′-UTRs (*APC*, *BMP1B*, *BRCA1*, *CCND1*, *CXCL12*, *CYP1B1*, *ESR1*, *IGF1*, *IGF1R*, *IRS2*, *PTGS2*, *SLC4A7*, *TGFBR1*, and *VEGFA*).³⁰ Table 2 provides a comprehensive

summary of miRNA-related SNPs linked to breast cancer; some well-studied SNPs are highlighted below.

SNPs in the precursors of five miRNAs (miR-27a, miR-146a, miR-149, miR-196, and miR-499) have been associated with increased risk of developing certain types of cancer, including breast cancer.³¹ Race, ethnicity, and molecular subtype can influence the relative risk associated with SNPs.^{32,33} The rare [G] allele of rs895819 is located in the loop of pre-miR-27; it interferes with miR-27 processing and is associated with a lower risk of developing familial breast cancer.³⁴ The same allele was associated with lower risk of sporadic breast cancer in a patient cohort of young Chinese women,³⁵ but the allele had no prognostic value in individuals with breast cancer in this cohort.³⁵ The [C] allele of rs11614913 in the pre-miR-196 and [G] allele of rs3746444 in the pre-miR-499 were associated with increased risk of developing breast cancer in a case–control study of Chinese women (1,009 breast cancer patients and 1,093 healthy controls).³⁶ In contrast, the same variant alleles were not associated with increased breast cancer risk in a case–control study of Italian and German women (1,894 breast cancer cases and 2,760 healthy controls).³⁷ The [C] allele of rs462480 and [G] allele of rs1053872, within 61 bp and 10 kb of pre-miR-101, were associated with increased breast cancer risk in a case–control study of Chinese women (1,064 breast cancer cases and 1,073 healthy controls).³⁸ The authors suggest that these SNPs may interfere with stability or processing of primary miRNA transcripts.³⁸

The [G] allele of rs61764370 in the 3′-UTR of *KRAS*, which disrupts a binding site for let-7 family members, is associated with an increased risk of developing certain types of cancer, including breast cancer. The [G] allele of rs61764370 was associated with the TNBC subtype in younger women in case–control studies from Connecticut, US cohort with 415 breast cancer cases and 475 healthy controls, as well as from an Irish cohort with 690 breast cancer cases and 360 healthy controls.³⁹ This allele was also associated with familial *BRCA1* breast cancer in a case–control study with 268 mutated *BRCA1* families, 89 mutated *BRCA2* families, 685 non-mutated *BRCA1/2* families, and 797 geographically matched healthy controls.⁴⁰ However, there was no association between ER status and this allele in this study cohort.⁴⁰ No association between this allele and the TNBC subtype or *BRCA1* mutation status was found in an independent case–control study with 530 sporadic postmenopausal breast cancer cases, 165 familial breast cancer cases (regardless of *BRCA* status), and 270 postmenopausal healthy controls.⁴¹

Interestingly, the [C] allele of rs7963551 in the 3'-UTR of RAD52 also disrupts a binding site for let-7. This allele is associated with decreased breast cancer risk in two independent case-control studies of Chinese women with 878 and 914 breast cancer cases and 900 and 967 healthy controls, respectively.⁴² The authors suggest that relief of let-7-mediated regulation may contribute to higher baseline levels of this DNA repair protein, which could be protective against cancer development. The [T] allele of rs1434536 in the 3'-UTR of the bone morphogenic receptor type 1B (BMPRI1B) disrupts a binding site for miR-125b.⁴³ This variant allele was associated with increased breast cancer risk in a case-control study with 428 breast cancer cases and 1,064 healthy controls.⁴³

Prognostic and predictive miRNA biomarkers in breast cancer subtypes with targeted therapies

ER⁺ breast cancers account for 70% of all cases and have the best clinical outcome. For ER⁺ cancers, several targeted therapies exist to block hormone signaling, including tamoxifen, aromatase inhibitors, and fulvestrant. However, as many as half of these patients are resistant to endocrine therapy intrinsically (de novo) or will develop resistance over time (acquired).⁴⁴ Thus, there is a clinical need for prognostic and predictive biomarkers that can indicate which ER⁺ patients can be effectively treated with hormone therapies alone and which tumors have innate (or will develop) resistance to hormone therapy, thereby requiring more aggressive treatment. For HER2⁺ breast cancers, treatment with the targeted inhibitor trastuzumab is the standard course.^{45,46} Even though trastuzumab is effective, almost half of the breast cancer patients that overexpress HER2 are either nonresponsive to trastuzumab or develop resistance.⁴⁷⁻⁴⁹ There have been numerous mechanisms identified for trastuzumab resistance, yet there is no clinical assay available to determine which patients will respond to trastuzumab.

Profiling of miRNA expression in clinical tissue specimens and/or in breast cancer cell line models of drug resistance has linked individual miRNAs or miRNA signatures to drug resistance and disease outcome (Tables 3 and 4). Functional characterization of some of the highlighted miRNAs in cell line models has provided mechanistic insights on their role in resistance.^{50,51} Some miRNAs can directly control expression levels of ER and HER2 via interaction with complementary binding sites on the 3'-UTRs of mRNAs.^{50,51} Other miRNAs can affect output of ER and HER2 signaling

by controlling expression levels of downstream effectors and signaling factors.^{50,51}

miRNAs in ER signaling and endocrine resistance

miR-22, miR-27a, miR-206, miR-221/222, and miR-302c have been shown to regulate ER expression in breast cancer cell line models and, in some instances, miRNA overexpression is sufficient to promote resistance to endocrine therapies.⁵²⁻⁵⁵ In some studies (but not others), these miRNAs have been detected at lower levels in ER⁺ tumor tissues relative to ER⁻ tumor tissues.^{55,56} Expression of the miR-191~miR-425 gene cluster and of miR-342 is driven by ER signaling in breast cancer cell lines and their expression correlates with ER status in breast tumor tissues.⁵⁶⁻⁵⁹ Several clinical studies have identified individual miRNAs or miRNA signatures that correlate with response to adjuvant tamoxifen treatment.⁶⁰⁻⁶⁴ These signatures do not include any of the above-mentioned miRNAs that have a mechanistic link to ER regulation or signaling. A ten-miRNA signature (miR-139-3p, miR-190b, miR-204, miR-339-5p, miR-363, miR-365, miR-502-5p, miR-520c-3p, miR-520g/h, and miRPlus-E1130) was associated with clinical outcome in a patient cohort of 52 ER⁺ cases treated with tamoxifen, but this signature could not be validated in two independent patient cohorts.⁶⁴ Individual expression changes in miR-30c, miR-210, and miR-519 correlated with clinical outcome in independent patient cohorts treated with tamoxifen.⁶⁰⁻⁶³ High miR-210 correlated with shorter recurrence-free survival in a cohort of 89 patients with early-stage ER⁺ breast tumors.⁶² The prognostic performance of miR-210 was comparable to that of mRNA signatures, including the 21-mRNA recurrence score from which US Food and Drug Administration (FDA)-cleared Oncotype Dx is derived. High miR-210 expression was also associated with poor outcome in other patient cohorts of either all comers or ER⁻ cases.⁶⁵⁻⁶⁹ The expression of miR-210 was also upregulated under hypoxic conditions.⁷⁰ Thus, miR-210-based prognostic information may not be specific or limited to ER signaling or ER⁺ breast tumors.

miRNAs in HER signaling and trastuzumab resistance

miR-125b, miR-134, miR-193a-5p, miR-199b-5p, miR-331-3p, miR-342-5p, and miR-744* have been shown to regulate expression of HER2 via binding to sites on the 3'-UTR of its mRNA in HER2⁺ breast cancer cell lines (eg, BT-474, MDA-MB-453, and SK-BR-3).⁷¹⁻⁷³ miR-125b and miR-205 also indirectly affect HER2 signaling

via inhibition of HER3 in SK-BR-3 and MCF-7 cells.^{71,74} Expression of other miRNAs, including miR-26, miR-30b, and miR-194, is upregulated upon trastuzumab treatment in BT-474 and SK-BR-3 cells.^{75,76} Altered expression of these miRNAs has been associated with breast cancer, but for most of them, there is not a clear, exclusive link to the HER2⁺ tumor subtype. miR-21, miR-302f, miR-337, miR-376b, miR-520d, and miR-4728 have been reported by some studies (but not others) to be overexpressed in HER2⁺ breast cancer tissues.^{56,77,78} Indeed, miR-4728 is cotranscribed with the HER2 primary transcript and is processed out from an intronic sequence.⁷⁸ High levels of miR-21 interfere with trastuzumab treatment in BT-474, MDA-MB-453, and SK-BR-3 cells via inhibition of PTEN (phosphatase and tensin homolog).⁷⁹ High levels of miR-21 in HER2⁺ tumor tissues before and after neoadjuvant treatment with trastuzumab are associated with poor response to treatment.⁷⁹ miR-221 can also confer resistance to trastuzumab treatment via PTEN in SK-BR-3 cells.⁸⁰ High levels of miR-221 correlate with lymph node involvement and distant metastasis as well as HER2 overexpression,⁸¹ though other studies observed lower levels of miR-221 in HER2⁺ cases.⁸²

While these mechanistic interactions are sound and there are supportive data with clinical specimens, the prognostic value and potential clinical applications of these miRNAs are not clear. Future studies should investigate whether any of these miRNAs can inform disease outcome or treatment response within a more homogenous cohort of HER2⁺ cases.

miRNA biomarkers and therapeutic opportunities in TNBC without targeted therapies

TNBC is a highly heterogeneous disease whose clinical features include a peak risk of recurrence within the first 3 years, a peak of cancer-related deaths in the first 5 years, and a weak relationship between tumor size and lymph node metastasis.⁴ At the molecular level, TNBC has significant overlap with the basal-like subtype, with approximately 80% of TNBCs being classified as basal-like.³ A comprehensive gene expression analysis (mRNA signatures) of 587 TNBC cases revealed extensive molecular heterogeneity within TNBC as well as six distinct molecular TNBC subtypes.⁸³ The molecular heterogeneity increases the difficulty of developing targeted therapeutics that will be effective in unstratified TNBC patients. It would be highly beneficial to be able to identify these molecular subtypes with simplified biomarkers or signatures.

miRNA expression profiling on frozen and fixed tissues using various detection methods have identified miRNA signatures or individual miRNA changes that correlate with clinical outcome in TNBC cases (Table 5). A four-miRNA signature (miR-16, miR-125b, miR-155, and miR-374a) correlated with shorter overall survival in a patient cohort of 173 TNBC cases. Reanalysis of this cohort by dividing cases into core basal (basal CK5/6- and/or epidermal growth factor receptor [EGFR]-positive) and 5NP (negative for all five markers) subgroups identified a different four-miRNA signature (miR-27a, miR-30e, miR-155, and miR-493) that correlated with the subgroup classification based on ER/PR/HER2/basal cytokeratins/EGFR status.⁸⁴ Accordingly, this four-miRNA signature can separate low- and high-risk cases – in some instances, even more accurately than core basal and 5NP subgroup stratification.⁸⁴ Other miRNA signatures could be useful to inform treatment response to specific chemotherapy regimens (Table 5). A three-miRNA signature (miR-190a, miR-200b-3p, and miR-512-5p) obtained from tissue core biopsies before treatment correlated with complete pathological response in a limited patient cohort of eleven TNBC cases treated with different chemotherapy regimens.⁸⁵ An eleven-miRNA signature (miR-10b, miR-21, miR-31, miR-125b, miR-130a-3p, miR-155, miR-181a, miR-181b, miR-183, miR-195, and miR-451a) separated TNBC tumors from normal breast tissue.⁸⁶ The authors noted that several of these miRNAs are linked to pathways involved in chemoresistance.⁸⁶

Categorizing TNBC subgroups by gene expression (mRNA) signatures indicates the influence and contribution of stromal elements in driving and defining specific subgroups.⁸³ Immunomodulatory, mesenchymal-like, and mesenchymal stem-like subtypes are characterized by signaling pathways typically carried out, respectively, by immune cells and stromal cells, including tumor-associated fibroblasts. miR-10b, miR-21, and miR-155 are among the few miRNAs that are represented in multiple signatures found to be associated with poor outcome in TNBC. These miRNAs are known to be expressed in cell types other than breast cancer cells,^{87–91} and thus, their altered expression may reflect aberrant processes in the tumor microenvironment.⁹²

In situ hybridization (ISH) assays are a powerful tool to determine altered miRNA expression at single-cell resolution and to assess the contribution of reactive stroma and immune response.^{13,93} In breast phyllodes tumors,⁹⁴ as well as in colorectal⁹⁵ and pancreatic cancer,⁹⁶ upregulation of miR-21 expression promotes myofibrogenesis and regulates antimetastatic and proapoptotic target genes, including

RECK (reversion-inducing cysteine-rich protein with kazal motifs), *SPRY1/2* (Sprouty homolog 1/2 of *Drosophila* gene), *PDCD-4* (programed cell death 4), and *PTEN*. We have recently shown that high levels of miR-21 expression in the stromal compartment in a cohort of 105 early-stage TNBC cases correlated with shorter recurrence-free and breast cancer-specific survival.⁹⁷ While ISH-based miRNA detection is not as sensitive as that of a qRT-PCR assay, it provides an independent validation tool to determine the predominant cell type(s) that express miRNAs associated with TNBC or other breast cancer subtypes.

miRNA biomarkers for monitoring and characterization of metastatic disease

Although significant progress has been made in detecting and treating primary breast cancer, advances in the treatment of MBC have been marginal. Does molecular analysis of the primary tumor tissues reflect the evolution of metastatic lesions? Are we treating the wrong disease(s)? In the clinic, computed tomography (CT), positron emission tomography (PET)/CT, and magnetic resonance imaging (MRI) are conventional methods for monitoring MBC patients and evaluating therapeutic efficacy. However, these technologies are limited in their ability to detect microscopic lesions and immediate changes in disease progression. Because it is not currently standard practice to biopsy metastatic lesions to inform new treatment plans at distant sites, circulating tumor cells (CTCs) have been effectively used to evaluate disease progression and treatment response. CTCs represent the molecular composition of the disease and can be used as prognostic or predictive biomarkers to guide treatment options. Further advances have been made in evaluating tumor progression and response using circulating RNA and DNA in blood samples. miRNAs are promising markers that can be identified in primary and metastatic tumor lesions, as well as in CTCs and patient blood samples.

Several miRNAs, differentially expressed in primary tumor tissues, have been mechanistically linked to metastatic processes in cell line and mouse models.^{22,98} Most of these miRNAs are thought to exert their regulatory roles within the epithelial cell compartment (eg, miR-10b, miR-31, miR-141, miR-200b, miR-205, and miR-335), but others can predominantly act in other compartments of the tumor microenvironment, including tumor-associated fibroblasts (eg, miR-21 and miR-26b) and the tumor-associated vasculature (eg, miR-126). miR-10b has been more extensively studied than other miRNAs in the context of MBC (Table 6).

We briefly describe below some of the studies that have analyzed miR-10b in primary tumor tissues, as well as in blood from breast cancer cases with concurrent metastatic disease, either regional (lymph node involvement) or distant (brain, bone, lung).

miR-10b promotes invasion and metastatic programs in human breast cancer cell lines and mouse models via HoxD10 inhibition, which derepresses expression of the prometastatic gene *RhoC*.^{99,100} In the original study, higher levels of miR-10b in primary tumor tissues correlated with concurrent metastasis in a patient cohort of five breast cancer cases without metastasis and 18 MBC cases.¹⁰⁰ Higher levels of miR-10b in the primary tumors correlated with concurrent brain metastasis in a cohort of 20 MBC cases with brain metastasis and ten breast cancer cases without brain metastasis.¹⁰¹ In another study, miR-10b levels were higher in the primary tumors of MBC cases.¹⁰² Higher amounts of circulating miR-10b were also associated with cases having concurrent regional lymph node metastasis.^{103–105} However, another study on primary tumor tissues did not find an association between miR-10b levels and disease progression or clinical outcome in a cohort of 84 early-stage breast cancer patients¹⁰⁶ or in another cohort of 219 breast cancer patients,¹⁰⁷ both with long-term (>10 years) clinical follow-up information.

We are not aware of any study that has compared miRNA expression between matched primary and metastatic tissues in a large cohort. This could provide information about cancer cell evolution, as well as the tumor microenvironment niche at distant sites. With smaller cohorts, higher levels of miR-9, miR-200 family members (miR-141, miR-200a, miR-200b, miR-200c), and miR-219-5p have been detected in distant metastatic lesions compared with matched primary tumors by RT-PCR and ISH assays.¹⁰⁸

A recent ISH-based study in a limited number of breast cancer cases reported that expression of miR-708 was markedly downregulated in regional lymph node and distant lung metastases.¹⁰⁹ miR-708 modulates intracellular calcium levels via inhibition of neuronatin.¹⁰⁹ miR-708 expression is transcriptionally repressed epigenetically by polycomb repressor complex 2 in metastatic lesions, which leads to higher calcium bioavailability for activation of extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK), and cell migration.¹⁰⁹ Recent mechanistic studies have revealed antimetastatic functions of miR-7,¹¹⁰ miR-18a,¹¹¹ and miR-29b,¹¹² as well as conflicting antimetastatic functions of miR-23b¹¹³ and prometastatic functions of the miR-23 cluster (miR-23, miR-24, and miR-27b)¹¹⁴ in

breast cancer. The prognostic value of these miRNAs needs to be investigated.

miRNA expression profiling in CTCs could be useful for assigning CTC status and for interrogating molecular aberrations in individual CTCs during the course of MBC.¹¹⁵ However, only one study has analyzed miRNA expression in CTC-enriched blood samples after positive selection of epithelial cells with anti-EpCAM antibody binding.¹¹⁶ The authors used a cutoff of 5 CTCs per 7.5 mL of blood to consider a sample positive for CTCs, which is within the range of previous clinical studies. A ten-miRNA signature (miR-31, miR-183, miR-184, miR-200c, miR-205, miR-210, miR-379, miR-424, miR-452, and miR-565) can separate CTC-positive samples of MBC cases from healthy control samples after epithelial cell enrichment.¹¹⁶ However, only miR-183 is detected in statistically significantly different amounts between CTC-positive and CTC-negative samples of MBC cases.¹¹⁶ Another study took a different approach and correlated changes in circulating miRNAs with the presence or absence of CTCs in MBC cases. Higher circulating amounts of seven miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, and miR-375) and lower amounts of miR-768-3p were detected in plasma samples from CTC-positive MBC cases.¹¹⁷ miR-210 was the only overlapping miRNA between these two studies; epithelial cell-expressed miRNAs (miR-141, miR-200a, miR-200b, and miR-200c) did not reach statistical significance in the other study.

Changes in amounts of circulating miRNAs have been reported in various studies of blood samples collected before and after neoadjuvant treatment. Such changes could be useful in monitoring treatment response at an earlier time than current imaging technologies allow. However, there is no evidence at this time that circulating miRNA signatures would contain enough information to dissect molecular aberrations in individual metastatic lesions, which could be many and heterogeneous within the same patient. The amount of circulating miR-19a and miR-205 in serum before treatment correlated with response to neoadjuvant epirubicin + paclitaxel chemotherapy regimen in Stage II and III patients with luminal A breast tumors.¹¹⁸ Relatively lower levels of circulating miR-210 in plasma samples before treatment correlated with complete pathologic response to neoadjuvant trastuzumab treatment in patients with HER2⁺ breast tumors.¹¹⁹ At 24 weeks after surgery, the miR-210 in plasma samples of patients with residual disease (as assessed by pathological response) was reduced to the level of patients with complete pathological response.¹¹⁹ While circulating levels of miR-21, miR-29a, and miR-126 were relatively higher in

plasma samples from breast cancer patients relative to those of healthy controls, there were no significant changes of these miRNAs between pre-surgery and post-surgery plasma samples.¹¹⁹ Another study found no correlation between the circulating amount of miR-21, miR-210, or miR-373 in serum samples before treatment and the response to neoadjuvant trastuzumab (or lapatinib) treatment in patients with HER2⁺ breast tumors.¹²⁰ In this study, however, relatively higher levels of circulating miR-21 in pre-surgery or post-surgery serum samples correlated with shorter overall survival.¹²⁰ More studies are needed that carefully address the technical and biological reproducibility, as we discussed above for miRNA-based early-disease detection assays.

Conclusion

Breast cancer has been widely studied and characterized at the molecular level. Various molecular tools have already been incorporated into the clinic for diagnostic and prognostic applications based on gene (mRNA) and protein expression, but there are still unmet clinical needs for novel biomarkers that can improve diagnosis, management, and treatment. In this review, we provided a general look at the state of miRNA research on breast cancer. We limited our discussion to studies that associated miRNA changes with one of these focused challenges: early disease detection (Tables 1 and 2), management of a specific breast cancer subtype (Tables 3–5), or new opportunities to monitor and characterize MBC (Table 6). There are more studies that have linked altered expression of specific miRNAs with clinical outcome, but we did not review those that did not analyze their findings within the context of specific subtypes based on ER/PR/HER2 status.

The promise of miRNA biomarkers generates great enthusiasm. Their chemical stability in tissues, blood, and other body fluids, as well as their regulatory capacity to modulate target networks, are technically and biologically appealing. miRNA-based diagnostics have already reached the clinic in laboratory-developed tests that use qRT-PCR-based detection of miRNAs for differential diagnosis of pancreatic cancer, subtyping of lung and kidney cancers, and identification of the cell of origin for cancers having an unknown primary.^{121,122} For breast cancer applications, there is little agreement on the reported individual miRNAs and miRNA signatures among studies from either tissues or blood samples. We considered in detail parameters that may contribute to these discrepancies in blood samples. Most of these concerns also apply to tissue studies, namely, patient characteristics, experimental design, sample size, methodology, and analysis tools. Another limitation of most expression-profiling studies in whole-tissue

specimens is that they measure collective levels of RNA from a mixture of different cell types. Intratumoral and intertumoral heterogeneity at the cellular and molecular levels are confounding factors in interpreting altered miRNA expression. This may explain in part the low overlap of reported miRNA signatures in tissues. We discussed the influence of altered miRNA expression in the stroma in the context of TNBC. Stromal features are known to influence cancer cell characteristics.^{123,124} Therefore, it is likely that miRNA-mediated regulation in other cellular compartments of the tumor microenvironment also influences cancer cells. Detection methods that incorporate the context of altered expression, such as multiplex ISH/immunohistochemistry assays, may provide additional validation tools for altered miRNA expression.^{13,93}

In conclusion, it is premature to make specific recommendations for clinical implementation of miRNA biomarkers in managing breast cancer. More research is needed that includes multi-institutional participation and longitudinal studies of large patient cohorts, with well-annotated pathologic and clinical characteristics to validate the clinical value of miRNAs in breast cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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