

Review

miRNA Biomarkers in Breast Cancer Detection and Management

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Abstract

Breast cancer is considered as a heterogeneous disease comprising various types of neoplasms, which involves different profile changes in both mRNA and micro-RNA (miRNA) expression. Extensive studies on mRNA expression in breast tumor have yielded some very interesting findings, some of which have been validated and used in clinic. Recent miRNA research advances showed great potential for the development of novel biomarkers and therapeutic targets. miRNAs are a new class of small non-coding regulatory RNAs that are involved in regulating gene expression at the posttranscriptional level. It has been demonstrated that miRNA expression is frequently deregulated in breast cancer, which warrants further in-depth investigation to decipher their precise regulatory role in tumorigenesis. We address briefly the regulatory mechanism of miRNA, the expression of miRNAs in tumorigenesis, and their potential use as breast cancer biomarkers for early disease diagnosis and prognosis. In addition, we discuss the use of the Formalin-Fixed, Paraffin-Embedded (FFPE) tissue as an invaluable source for breast cancer biomarker discovery and validation, and the potential use of circulating miRNAs in blood for early breast cancer detection. We envision the potential use of miRNAs in breast cancer management in the near future, particularly in improving the early diagnosis, prognosis and treatment.

Key words: breast cancer, miRNA, microarray, FFPE, biomarker

Regulatory mechanisms of miRNAs

MicroRNAs (miRNAs) are a new class of small non-coding regulatory RNAs that are involved in regulating gene expression at the posttranscriptional level. These small (18–24 nucleotides in length) RNA molecules regulate numerous biological processes. The base-pairing interactions between miRNAs and their target mRNAs, often within the 3' untranslated regions (UTR) of target genes, results in degradation and/or translation inhibition of the target genes [1] [2]. Although the complementary binding to the 3' UTR is not necessarily perfect, it results in transla-

tional repression. In contrast, the complementary binding within the open reading frame (ORF) needs almost perfect match in order for Ago2 to cleave or degrade target mRNAs [3] [4]. miRNAs, generally transcribed by RNA polymerase II, are initially made as large RNA precursors, called pri-miRNAs [5]. Transcription of miRNA genes are regulated through the modulation of various transcription factors as that of protein-coding genes [6]. About 1000 miRNA genes are thought to be encoded in the human genome [7] [8]. A total of 1048 human miRNAs have been identi-

fied according to miRBase Release 16 (September 2010) (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa) [9] [10]. miRNAs and their targets seem to form a complex regulatory network. It is believed that a single miRNA can regulate multiple mRNAs and a single mRNA can be targeted by a number of different miRNAs. There are about one third of all human protein-coding genes that are regulated by miRNA based on computational predictions [11].

miRNA expression in human cancer.

The role of miRNAs in cancer was initially suspected based on the observation that miRNA controlled aspects of cell proliferation and apoptosis in *C. elegans* and *Drosophila* [12] [13]. miRNA genes were located at fragile sites in the genome, which are frequently amplified or deleted in human cancers [14] and other genetic diseases, such as fragile X syndrome. Generally, genes encoding miRNAs located in chromosomal regions that are amplified in cancers function as oncogenes while those deleted in cancers may act as tumor suppressors. Expression of various miRNAs has been reported to be differentially altered across a variety of tumor types, suggesting their direct involvement in oncogenesis [3] [15] [16]. Deregulated miRNA expression profiles were identified in many human cancers using different miRNA profiling

techniques. miRNAs have been associated with embryogenesis and stem cell maintenance [17], hematopoietic stem cell differentiation [18], and cancers [19] [20] [21]. Many studies show that miRNA expression appeared to be deregulated in cancer versus normal tissue [22] [14, 23] [24]. Since those initial studies, examples of miRNA deregulation have been shown in chronic lymphocytic leukemia [25], B-cell lymphoma [26] [27] and breast cancer [19] [28] [29] [30, 31]. However, it is not clear whether the changes in miRNA expression are a cause or effect of the disease for many miRNA species.

Role of miRNA in breast cancer

Although extensive research on molecular mechanisms involved in breast cancer has been done over the decades, challenges still prevail in the early diagnosis and management of breast cancer patients, such as unpredictable response and development of resistance to adjuvant therapies. miRNAs, as mRNA regulators, could serve as novel diagnostic and prognostic candidates, and potential therapeutic targets. Since the miRNA deregulation in breast cancer was first reported in 2005 [28], there have been many studies on the expression of various miRNAs and their roles in breast cancer as summarized (Table 1).

Table 1 miRNAs function as tumor suppressors or oncogenes in breast cancer.

miRNAs	Targets	Functional pathways	References
Tumor suppressor miRNAs			
miR-206	ESR1	ER signaling	[28, 32]
miR-17-5p	AIB1,CCND1,E2F1	Proliferation	[33, 34]
miR-125a,b	HER2,HER3	Anchorage-dependent growth	[35, 36]
miR-200c	BMI1,ZEB1,ZEB2	TGF-beta signaling	[37-39]
let-7	H-RAS, HMGA2, LIN28,PEBP1	Proliferation,differetiation	[40-42]
miR-34a	CCND1,CDK6,E2F3,MYC	DNA damage, proliferation	[43-45]
miR-31	FZD3,ITGA5,M-RIP,MMP16,RDX,RHOA	Metastasis	[46]
miR-335	SOX4,PTPRN2,MERTK,TNC	Metastasis	[47]
miR-27b	CYP1B1	Modulation of the response of tumor to anti-cancer drugs	[48, 49]
miR-126	IRS-1	Cell cycle progression from G1/G0 to S	[50]
miR-101	EZH2	oncogenic and metastatic activity	[51]
miR-145	miR-145 in p53-mediated repression of c-Myc.	Suppresses Cell Invasion and Metastasis	[52]
miR-146a/b	NF-κB	negatively regulate factor-κB, and impaired invasion and migration capacity	[53]
miR-205	ErbB3 and VEGF-A expression	inhibits tumor cell growth and cell invasion,	[54]
Oncogenic miRNAs			
miR-21	BCL-2,TPM1,PCCD4,PTEN,MASPIN	Apoptosis	[55-58]
miR-155	RHOA	TGF-beta signaling	[59]
miR-10b	HOXD10	Metastasis	[60]
miR-373/520c	CD44	Metastasis	[61]
miR-27a	Zinc finger ZBTB10, Myt-1	Cell cycle progression G2- M checkpoint regulation	[62]
miR221/222	p27 ^{Kip1}	Tamoxifen resistance	[63]

miRNA profiling studies have led to the identification of miRNAs that are aberrantly expressed in human breast cancer, with miR-10b, miR-125b and miR-145 being down-regulated and miR-21, and miR-155 being up-regulated. More recent studies have not only identified miRNA downstream targets but also associated particular miRNA expression with prognostic information. The miRNAs have either been shown to be consistently up-regulated or down-regulated. Tumor formation may arise from down-regulation of a tumor suppressor miRNA and / or overexpression of an oncogenic miRNA. miRNA expression studies in breast cancer also revealed the importance and potential use in tumor classification and better prognosis. [28] Twenty-nine differentially expressed miRNAs were identified when comparing breast cancer tissue with normal, and a subset of 15 miRNAs could be used to discriminate tumor from normal. In addition, miRNA expression correlated with clinical-pathological features such as ER and PR expression (*miR-30*) and tumor stage (*miR-213* and *miR-203*). The differential expression of several *let-7* isoforms was associated with clinical-pathologic features including PR status (*let-7c*), lymph node metastasis (*let-7f-1*, *let-7a-3*, *let-7a-2*), or high proliferation index (*let-7c*, *let-7d*) in tumor samples. Interestingly, unique sets of miRNAs were identified to be associated with breast cancers currently defined by their HER2/*neu* or ER/PR status [35]. Significantly, there was overlap between the miRNAs identified in both studies.

In recent years, the functional role of miRNAs in tumor initiation and progression has been the focal point since the discovery of the role of miR-10b in breast cancer invasion and metastasis [60]. The study indicates that miR-10b is up-regulated in promoting invasion and metastasis, whereas down-regulated in most breast cancer in comparison with the normal control [28]. Interestingly, miR-10b might not have an effect on proliferation due to the fact that almost 50% overexpression in metastatic breast cancers compared with the low-expression in most breast cancer. Rather it is mainly involved in breast cancer invasion and metastasis, which is validated by migration and invasion assay [60]. These demonstrate that miR-10b may confer specific invasive properties only in metastatic cells since it is ubiquitously down-regulated in primary breast cancer cell. It has been reported that MDA-MB-231 cells transfected with lentivirus-miR-106b have a higher potential of invasion and migration, suggesting that miR-106b may play a role in breast cancer metastasis [64]. In addition, miR-9, upregulated in breast cancer cells, directly targets *CDH1*, the E-cadherin-encoding mRNA, leading to

increased cell motility and invasiveness [65]. Interestingly, miR-378(*) was identified as a molecular switch in cancer cell bioenergetics pathway, known as the Warburg effect, by regulating ERBB2 expression [66]. On the other hand, enhancing the expression of some tumor suppressor miRNAs can prevent progression of breast tumors [67]. For example, the expression of tumor suppressor miR-127 can down-regulate the expression of proto-oncogene *BCL6*, a potential target of miR-127 [68].

Molecular Signatures in Breast Cancer

Recent advances in phenotyping and expression profiling of human cancers have greatly enhanced the diagnosis and biological classification of several tumors, in particular breast cancers. Prior to this a very limited number of prognostic markers were available in clinic beyond those of histopathological analysis. The microarray-based gene expression signatures were identified to classify breast cancers into distinct subtypes largely based on their ER, progesterone (PR), and HER2 receptor status [69] [70] [71]. Subtypes were designated *Luminal A*, which strongly expressed ER and/or PR, but not HER2; *Luminal B*, which were ER, PR and HER2 positive; *Basal* tumors which were ER, PR, and HER negative, preferentially affecting young women and women of African origin, usually of high histological grade and more aggressive clinical behavior [72]. Survival analyses showed significantly different outcome for patients based on their tumor subtype, suggesting the clinical relevance of molecular profiling. This method of disease classification based on the molecular profiling heralds the promise of personalized medicine [73]. Great scientific endeavors in the field of microarray-based gene expression profiling help translate such technological advances to clinical practice in developing new tools for accurate molecular diagnosis of breast cancer [74]. One such application has been the development of a multi-gene assay to predict the recurrence of tamoxifen-treated, node-negative breast cancer (Oncotype DX) [75]. This and other similarly novel genomic tests, such as MammaPrint, Theros and MapQuant Dx, prove the feasibility of accelerating the transition from traditional to molecular medicine. With more studies, miRNA signatures, which are currently showing capability of accurately classifying tumors according to currently available prognostic variables, would serve as novel biomarkers for diagnosis and treatment. An interrogation of miRNA expression profiling in breast cancer classification could further characterize the molecular basis and define more precise subsets of breast cancer for the identification of novel targets that can be exploited for targeted therapy.

miRNA expression levels should represent the functional activity of the gene more accurately compared with the those of mRNAs, which have to be translated to proteins to attain their biological effects [76]. Thus, miRNAs can be novel targets to further our understanding in breast cancer diagnosis at the molecular level. At the same time, miRNAs can be used as cancer therapeutic agents when breast cancers can be classified based on their miRNA signatures. Furthermore, in each particular cancer or disease developmental stage, specific miRNAs with their implicated signaling pathways need to be elucidated. Of interest, the identity and functions of each cell are reflected by a unique miRNA expression signature [22, 77]. Since a single miRNA can target multiple genes of a particular signaling pathway, it is therefore more feasible to target miRNAs involved in a particular cellular process, rather than targeting one mRNA [22]. For example, introduction of anti-miR-21 to MCF-7 breast cancer cells and a xenograft mouse model considerably suppressed miR-21 expression, which resulted in decreased cell growth via increased apoptosis and reduced cell proliferation [78]. Topotecan can increase the tumor growth reduction capacity of anti-miR-21, indicating that suppression of the oncogenic miR-21 can sensitize cancer cells to chemotherapy, which is helpful for patients who show resistance to chemotherapeutic agents [78]. Recent report showed that miR-125b expression causes a marked inhibition of paclitaxel-induced cytotoxicity and apoptosis by targeting is the pro-apoptotic Bak1, which could help explain the paclitaxel-resistant in breast cancer [79].

miRNA expression profiling from FFPE tissues.

FFPE (Formalin-Fixed, Paraffin-Embedded) tissues are an invaluable resource for biomarker discovery and validation. The formalin fixation process allows for permanent preservation of the architecture of tissues in optimal histological condition and easy long-term storage. However, this process compromises the yield, quality and integrity of mRNA through degradation, cross-linking with proteins and various chemical modifications [80] [81]. miRNAs, due to their small size, are potentially more robust to degradation than mRNAs during the process. Therefore miRNA expression profiling could be more reliable using FFPE tissues. Recent report indicated that correlations of expression profiles between fixed and frozen mouse liver tissues are higher with miRNAs than with mRNAs [82]. miRNAs can efficiently be detected by qRT-PCR in archived colorectal cancer specimens up to 10 years and in liver specimens up to

30 years [83], suggesting that miRNAs are better alternative for expression analyses in archived samples, such as FFPE samples. In addition, it has been demonstrated in multiple studies that miRNAs are minimally affected by FFPE treatment, and miRNAs extracted from FFPE samples provide reliable expression levels by direct comparison with frozen samples [82] [84] [85] [86]. Furthermore, we have recently isolated miRNAs from microdissected breast tissues successfully using the *RecoverAll Total Nucleic Acid Isolation Kit* from Ambion. The isolated miRNAs were used in both miRNA microarray expression profiling study as well as real-time QPCR validation (unpublished data). Taken together, these demonstrated that the FFPE tissue would be an invaluable source for miRNA expression study and marker validation.

Circulating miRNAs as early diagnostic biomarkers

miRNAs in the circulation system might function as feasible biomarkers in early stage breast cancer detection. Since miRNAs are exceptionally stable in visceral tissue, it is plausible if those serum preserved miRNAs are detectable, and quantifiable in the circulation system. Those promising ideas are generating great excitement in both clinic and scientific fields. After the serum preserved miRNAs were first documented within the patients with diffuse large B-cell lymphoma [87], subsequent studies has continually reported the presence of miRNAs in circulation system and body fluid, and hypotheses that their potential for use as novel biomarkers for disease and physiological states including invasive cancers, diabetes mellitus and even pregnancy [88-91]. This concept still needs further extensive investigation to validate, however, due to the limited methodologies, small numbers and great deficiency interrogations have been made in this area, especially in breast cancer. If the detection of invasive metastasizing cancer cells and their miRNAs migration to the lymph node is feasible, their potential use as minimally invasive biomarker would be an incredible breakthrough in disease monitoring, and in particularly, serves as significant control to study the comparatively poorly invasive early stage cancer cells.

Limitations of current methodologies of discovering molecular biomarkers for breast cancer

Microdissection of the archived FFPE tumor tissue provides great potential, as one can compare the expression profiling of the different regions of the tumor representing specific histological types within

the same genetic background. Nevertheless, based on the current multistep carcinogenesis model, which predicts tumor cells might consecutively acquire alternations in genomics and epigenomics before they escalate toward more aggressive phenotype. It's very likely that those small metastasizing cells, which may have acquired additional mutations that are not present in the primary tumor [92-96], could escape the detection by current expression profiling techniques, unless the tumor cells can be sub-grouped and analyzed separately, which is not an easy task. The fate of the tumor is determined from the initialization events and the following genomic and / or epigenomic mutations. It might also depend on the origin of the cells [97]. Microarray profiling is suitable to detect both cellular origins and subsequent mutations. However this does not exclude the possibility that the metastatic phenotype is inherited by an accident mutation from a single metastatic cell in the primary tumor or a non-invasive cell acquires the de nova invasive potential characteristic. On the other hand, even though microarray technology is relatively mature, numbers of expression alterations could still be attributed to differences in the technology rather than biology, which is why it requires careful and rigorous validations using a large number of samples.

Apparently, the miRNA microarray has the potential to alleviate the dilemma and provides more robust data as miRNAs are so small in size and proportionally have less possibility of alternative splicing and less vulnerability to intrinsic platforms. However, since the miRNA expression is tissue specific, and the degradation of mRNA endogenous control in long time FFFPE archived tissue is commonly severe, which would certainly challenge the availability and reliability of the endogenous controls [98] [99]. Instead, we have to identify more new reliable endogenous miRNA markers to serve as controls. In addition, sample collection is of concern because most of the breast cancer patients have received adjuvant chemotherapy. It is hard to determine whether a tumor without metastasis is due to chemotherapy.

Conclusions

The biological function of miRNAs in breast cancer is diverse, comprehensive and remains to be fully understood. The involvement of miRNAs in the initiation and progression of breast cancer holds great potential for new development in current diagnostic and therapeutic strategies in breast cancer management. Each year more than 1.3 million women will be diagnosed with breast cancer globally and about 465,000 will die from the disease despite the fact that breast cancer is still highly curable if only diagnosed

and treated appropriately at the early stage. At the cellular level, not all early stage tumor cells will eventually progress into notorious invasive carcinoma, otherwise need aggressive chemotherapy which will generate intense physical and financial trauma to the patient. So it is invaluable to establish a reliable and confident detecting and scoring system to guide the clinic to avoid over-treatment to the patients who do not benefit from aggressive standard treatment.

miRNAs would provide easily accessible, sensitive, reliable data resources to shed light on the research of early stage breast cancer development and progression. Combined with other existing technologies, miRNA microarrays and / or miRNA deep sequencing would identify novel miRNA biomarkers that could be used for more accurate early detection, prognosis and targeted therapy. Of note, scientific endeavors on the functional investigation of miRNAs and their targets will elucidate the intrinsic molecular mechanisms of breast cancer development and progression.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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