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MicroRNAs as biomarkers for early breast cancer diagnosis, prognosis and therapy prediction

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A B S T R A C T

Breast cancer is a major health problem that affects one in eight women worldwide. As such, detecting breast cancer at an early stage anticipates better disease outcome and prolonged patient survival. Extensive research has shown that microRNA (miRNA) are dysregulated at all stages of breast cancer. miRNA are a class of small non-coding RNA molecules that can modulate gene expression and are easily accessible and quantifiable. This review highlights miRNA as diagnostic, prognostic and therapy predictive biomarkers for early breast cancer with an emphasis on the latter. It also examines the challenges that lie ahead in their use as biomarkers. Noteworthy, this review addresses miRNAs reported in patients with early breast cancer prior to chemotherapy, radiotherapy, surgical procedures or distant metastasis (unless indicated otherwise). In this context, miRNA that are mentioned in this review were significantly modulated using more than one statistical test and/or validated by at least two studies. A standardized protocol for miRNA assessment is proposed starting from sample collection to data analysis that ensures comparative analysis of data and reproducibility of results.

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1. Introduction

microRNA (miRNA) are a major class of endogenous, small noncoding RNA molecules approximately 18–25 nucleotides in length. Since the discovery of the first miRNA lin-4 in *C. elegans* in 1993 (Lee et al., 1993), their role has drastically shifted from transcriptional noise to modulators of gene expression and master regulators of multiple pathways.

Abbreviations: 5-FU, 5-fluorouracil; 5NP, five negative; AC, adjuvant chemotherapy; AGO, Argonaute; BC, breast cancer; CA153, cancer antigen 153; CEA, Carcinoembryonic antigen; DCIS, ductal carcinoma in situ; DFS, disease-free survival; DGCR8, DiGeorge Syndrome Critical; EBC, early breast cancer; ER, estrogen receptor; FEC, 5-fluorouracil, epirubicin and cyclophosphamide; FFPE, formalin fixed paraffin embedded; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; LN, lymph node; LNA-ISH, LNA-enhanced probes in situ hybridization; miRNA, microRNA; NAC, neoadjuvant chemotherapy; NAT, normal adjacent tissue; PTX/CBP, paclitaxel plus carboplatin; RECIST, Response Evaluation Criteria in Solid Tumors; RFS, recurrence-free survival; RISC, RNA-induced silencing complex; ROC, receiver operating characteristic; RT-qPCR, reverse transcription quantitative real-time PCR; TAC, doxorubicin, docetaxol and cyclophosphamide; TCH, Taxotere, carboplatin and Herceptin; TN, triple negative; TNBC, triple negative breast cancer; TRBP, transactivation-responsive RNA-binding protein; Xpo5, Exportin-5.

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miRNA encoding genes are either intergenic or intronic (embedded within protein encoding genes) and are mainly transcribed by RNA polymerase II as long primary sequences called pri-miRNA (Fig. 1). The first processing step involves excision of the stem-looped pri-miRNA by a microprocessor complex composed of nuclear DROSHA, a ribonuclease-III protein, and DiGeorge Syndrome Critical (DGCR8), a double-stranded RNA-binding protein. The product is a 70–100 nucleotide hairpin-shaped precursor referred to as pre-miRNA that is exported in a Ran-GTP-dependent manner from the nucleus to the cytoplasm by Exportin-5 (XPO5). The second processing step includes further cleavage into 20–25 nucleotide duplexes by *endo*-ribonuclease DICER and its cofactor transactivation-responsive RNA-binding protein (TRBP) (Bartel, 2004). Alternatively, miRNA can be generated through a non-canonical splicing pathway. This subclass of miRNA, referred to as mirtrons, is encoded by introns and spliced in a DROSHA-independent manner (Havens, Reich, Duelli, & Hastings, 2012).

miRNA duplex unwinds either in a slicer dependent or independent manner. The slicer-dependent manner involves Argonaute (AGO) 2-mediated cleavage of one strand to facilitate its rapid removal, while the slicer-independent manner relies on complementarity mismatch (Kwak & Tomari, 2012; Park & Shin, 2015). One of the strands acts as a mature functional miRNA that is incorporated into a large protein complex called the RNA-induced silencing complex (RISC) with AGO protein as the primary component. The other miRNA strand either undergoes rapid degradation or becomes functional as part of the RISC complex (Guo & Lu, 2010). A seed region between nucleotide positions 2 to 8 from the 5′ region of the miRNA allows either perfect or imperfect base-pairing with the 3′ untranslated region or other sites of the mRNA target. In animals, imperfect pairing mainly occurs and can affect the mRNA by blocking its translation, degrading it via endonucleolytic cleavage or destabilizing it via deadenylation (Wu, Fan, & Belasco, 2006; Gu & Kay, 2010; Park & Shin, 2014). Moreover, miRNA can function in a RISC-independent manner either by directly targeting proteins like ribonucleoproteins to inhibit their activity in a decoy process (Eiring et al., 2010) or by targeting DNA to regulate their transcription through chromatin remodeling or direct binding (Gonzalez, Pisano, & Serrano, 2008; Khraiwesh et al., 2010).

Over 2500 mature miRNAs have been identified in humans according to miRbase, a database of published miRNA sequences and annotations. Their functions, however, are still under investigation. Importantly, miRNA can regulate multiple genes and can therefore control multiple critical pathways that are involved in physiological (such as development) as well as pathological processes including diabetes, neuro-degenerative disorder and cardiac hypertrophy. The first correlation between miRNA and cancer was reported upon the frequent deletions and downregulation of the miRNA-encoding gene clusters of miR-15 and miR-16 in chronic lymphocytic leukemia (Calin et al., 2002). miRNA have been shown to be differentially expressed in different types of cancer. The widespread disruption of miRNA expression in cancer has been explained by various potential mechanisms (Calin & Croce, 2006). First, miRNA genes, such as those of miR-15 and miR-16, are often proximal to common breakpoint regions, minimal regions of loss of heterozygosity, minimal regions of amplification and fragile sites. Such locations increase the propensity of miRNA genes to chromosomal abnormalities (deletion, amplification and translocation). Second, epigenetic control and DNA-binding factors could be another mechanism affecting the promoter regions of miRNA genes. The downregulation of miRNA genes could result from aberrant hyper-methylation, as in the case of miR-9-1 gene in breast cancer (Lehmann et al., 2008), or from histone deacetylation and tri-methylation, as in the case of miR-29 in B-cell lymphomas (Zhang et al., 2012). Histone acetylation could also act as an activator of miRNA genes, such as that of miR-224 in hepatocellular carcinoma (Wang, Toh, et al., 2012). An example of a DNA-binding factor is the tumor suppressor p53 that can activate

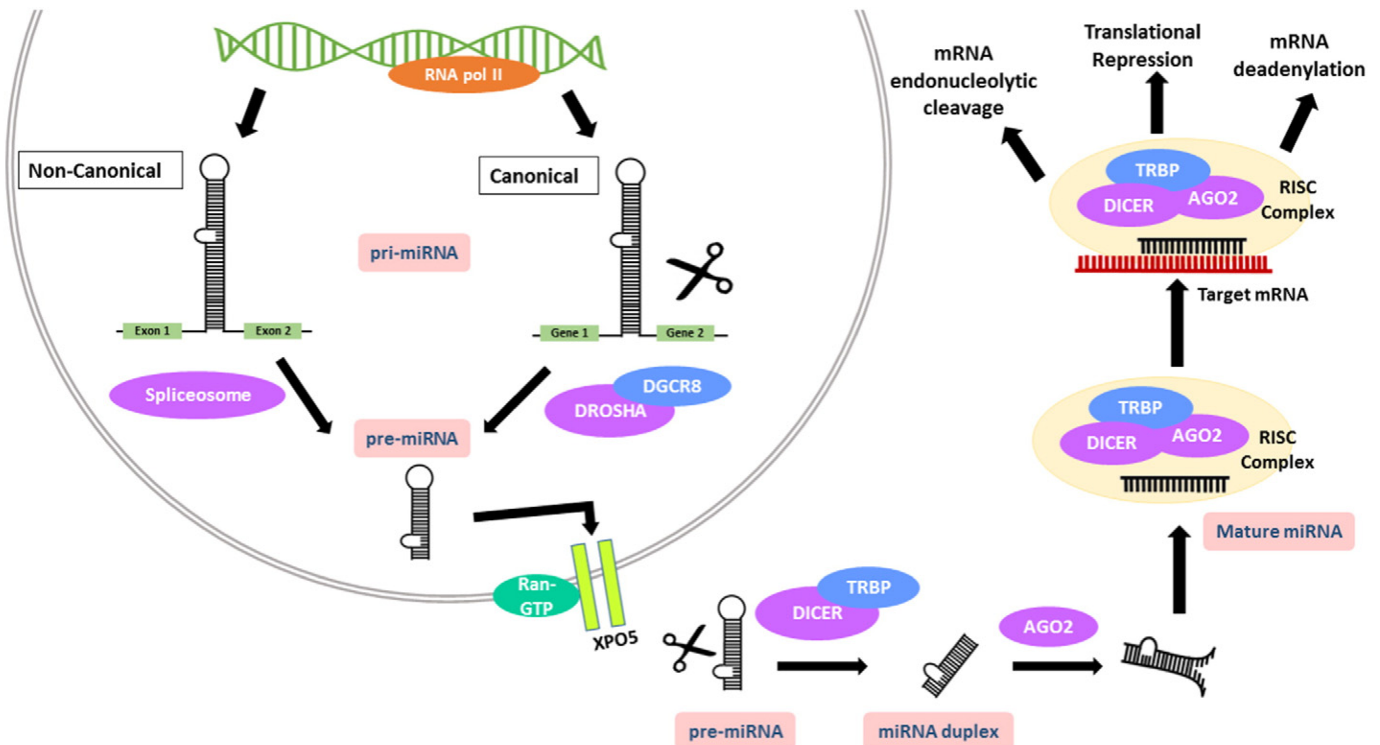

Fig. 1. miRNA biogenesis. miRNA intronic and intergenic genes are transcribed by RNA polymerase II forming the primary miRNA (pri-miRNA). Pri-miRNA is cleaved either by DROSHA in association with DGCR8 (canonical pathway) or by Spliceosome (non-canonical pathway). The resulting precursor miRNA (pre-miRNA) is exported to the cytoplasm via Exportin-5 (XPO5) in a Ran-GTP dependent manner. Pre-miRNA is further processed by DICER in conjunction with TRBP to produce a miRNA duplex that unwinds with the help of Argonaute 2 (AGO2). miRNA mature strand incorporates into the RNA-induced silencing complex (RISC) and then interacts with the target mRNA leading to its translational repression, endonucleolytic cleavage, or deadenylation. Purple colored oval denotes an enzyme protein while blue color denotes a cofactor protein.

the expression of miR-34 family by binding to conserved p53 consensus sequences in the promoter regions (Tarasov et al., 2007). Third, miRNA dysregulation could be explained by the changes in the miRNA processing machinery. Gene mutations, post-translational modifications and binding to regulatory proteins can affect the miRNA biogenesis proteins: DROSHA, DICER, DGCR8, TRBP, XPO5 and AGO (Hata & Kashima, 2015). Dysregulation of DROSHA and DICER has been reported in various cancer types and has been associated with altered miRNA profile and poor prognosis (Lambertz et al., 2010; Zhu et al., 2012; Diaz-Garcia et al., 2013). Furthermore, miRNA differential expression could be attributed to editing at early stages of biogenesis. For instance, adenosine deaminases mediate the conversion of adenosine to inosine within the hairpin region of pri-miR-142, miR-142 primary precursor (Yang et al., 2006).

Interestingly, miRNA are present in several biological fluids including blood, plasma, serum, saliva, urine, breast milk, seminal plasma, tears, amniotic fluid, cerebrospinal fluid, peritoneal fluid, pleural fluid, bronchial lavage and colostrum (Park et al., 2009; Hanke et al., 2010; Kosaka, Izumi, Sekine, & Ochiya, 2010; Weber et al., 2010). The first extracellular tumor-associated miRNA was detected in sera of B-cell lymphoma patients (Lawrie et al., 2008). It was afterwards validated to be abundant, nuclease-resistant and consistently quantifiable in sera of individuals of the same species (Chen et al., 2008). The fact that circulating miRNA are stable and resistant to degradation under harsh conditions (Mitchell et al., 2008) is due to their presence in microvesicles (exosomes and shedding vesicles), in apoptotic bodies and/or in association with high density lipoprotein particles or with AGO family of proteins (Turchinovich, Weiz, & Burwinkel, 2012). A key advantage of miRNA is the easiness of their detection using microarray, deep sequencing or reverse transcription quantitative real-time PCR (RT-qPCR). Hence, being stable, non-invasive, specific and measurable makes miRNA ideal biomarkers for cancer diagnosis, prognosis and therapy prediction.

2. Breast cancer and miRNA

Breast cancer is the most prevalent cancer in women worldwide (Ferlay et al., 2015). A brief overview of normal breast architecture is required to understand the different classifications of breast cancer. The normal breast is composed of ducts that branch into multiple lobules lined with an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. These structures are separated from the stroma, which consists of fat and connective tissue, by a basement membrane (Russo & Russo, 2014).

Breast cancer is not a single disease but rather a heterogeneous one that could be classified according to histology, immunopathology, mRNA expression profiling, and miRNA expression signature (Bertos & Park, 2011). Ductal carcinoma in situ (DCIS) is the most common histotype of non-invasive breast cancer, while invasive ductal carcinoma (IDC) followed by invasive lobular carcinoma are the most frequent histotypes of invasive carcinomas that breach the basement membrane. Other less common histotypes include medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous, inflammatory, comedo, adenoid cystic and micropapillary types. Immunopathologically, breast cancers are classified according to the receptor profile that includes estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). These receptor-based subtypes could be indicative of the therapy outcome and the level of prognosis as follows: best overall prognosis for indolent ER+ (ER+, HER2−) tumors treated with ER antagonists or aromatase inhibitors (Lumachi, Santeufemia, & Basso, 2015), better outcome for HER2+ (ER−, HER2+) and triple positive (ER+, PR+, HER2+) tumors only when treated with HER2-targeted agents like monoclonal antibody trastuzumab (Ahmed, Sami, & Xiang, 2015), and worst outcome for triple negative (TN) (ER−, PR−, HER2−) tumors, whose targeted treatment regimen is still under thorough investigation (Griffiths & Olin, 2012). Moreover, mRNA expression profiling has revealed the molecular heterogeneity of breast cancer and identified five intrinsic subtypes with distinct clinical outcomes: (1) luminal A subtype (resembles receptor-defined ER+ subtype) and (2) normal-like subtype (resembles normal tissue) that is associated with good prognosis, (3) luminal B (resembles receptor-defined ER+ subtype) that is associated with poor prognosis and higher proliferation compared to luminal A, (4) HER2-enriched subtype that overlaps with receptor-defined HER2+ subtype and (5) basal subtype that overlaps with receptor-defined TN subtype (Sorlie et al., 2001; Liu, Zhang, & Zhang, 2014; Prat et al., 2015). Besides, miRNA signature can sub-classify breast cancer (Blenkiron et al., 2007) and can even determine new subtypes, as recently reported (Bhattacharyya, Nath, & Bandyopadhyay, 2015).

Several tests have been developed for breast cancer detection and prognosis. The primary screening test for breast cancer detection is mammography; however, it is not recommended for women younger than 40 years as they tend to have denser breast tissue (Boyd et al., 2007; Checka, Chun, Schnabel, Lee, & Toth, 2012). Other than pain and discomfort that it might cause (Miller, Livingstone, & Herbison, 2008), mammography is associated with increased rates of false positives ranging from 12% to 65% and is correlated with over-diagnosis that is 31% of all breast cancer diagnosed (Bleyer & Welch, 2012; Kerlikowske et al., 2013). Various multi-gene expression based tests are utilized for breast cancer diagnosis and prognosis such as MammaPrint, MapQuant Dx, Oncotype Dx, PAM50 Breast Cancer Intrinsic Subtype Classifier (Prosigna), and Theros Breast Cancer Index. For instance, MammaPrint measures the mRNA expression levels of 70 genes and categorizes ER+ cancer patients into low-risk or high-risk prognostic groups while it classifies all ER− cancers as high-risk (Tian et al., 2010). Another diagnostic test is Prosigna that quantifies the mRNA expression of 50 genes and predicts the risk of distant recurrence of ER+ breast cancer in postmenopausal women treated with adjuvant endocrine therapy (Wallden et al., 2015). A 21-gene assay called Oncotype DX is also used for computing a recurrence score for ER+ breast cancer (Gyorffy et al., 2015). However, all of these tests mainly require formalin fixed paraffin embedded (FFPE) or fresh frozen invasive tissue biopsies and are only useful for hormone receptor-positive or invasive breast cancer. Moreover, carbohydrate antigens such as carcinoembryonic antigen (CEA) and cancer antigen 153 (CA153), have been identified as prognostic tools for breast cancer since they were reported to be elevated in sera of breast cancer patients, especially those with HER2+ and ER− subtypes respectively. However, the use of CEA and CA153 as prognostic serum markers still remains controversial due to conflicting results of different studies and their low sensitivity (Shao, Sun, He, Liu, & Liu, 2015).

Notably, miRNA is aberrantly expressed in breast cancer, as shown by microarray profiling of tumor and normal breast tissues (Iorio et al., 2005). Several studies have investigated the diverse functions of the dysregulated miRNA in malignant breast transformation whereby they can act as oncogenes (oncomiR) or as tumor suppressors. In breast cancer, miRNA has been shown to regulate cell cycle progression, apoptosis, angiogenesis, epithelial-mesenchymal transition, tumor microenvironment, migration, invasion, metastasis and drug resistance, as well as the differentiation and self-renewal of breast cancer stem cells (Li et al., 2012). As such, this review highlights miRNA as diagnostic, prognostic and therapy predictive biomarkers for early breast cancer (EBC) and discusses the challenges along with optimization steps for their use as biomarkers. All literature included in this review addressed miRNAs that were associated in patients with EBC prior to chemotherapy, radiotherapy, surgical procedures or distant metastasis (unless indicated otherwise) and that were significantly dysregulated using more than one statistical test (Receiver operating characteristic or ROC curve, Cox regression model & Spearman rank correlation) or reported in more than one study.

2.1. Diagnostic role

miRNAs have been proposed as diagnostic biomarkers for EBC detection to distinguish between healthy subjects and breast cancer patients.

However, this review discusses miRNAs that were confirmed in more than one study. miR-21, one of the most studied oncomiRs in cancer, has been shown in multiple studies to serve as a diagnostic biomarker for EBC. It was significantly over-expressed in plasma/serum and frozen/FFPE tumor (T) tissues of breast cancer patients compared to normal controls (NC) (Asaga et al., 2011; Wu et al., 2011; Gao, Zhang, Xu, Guo, & Li, 2013; Ng et al., 2013; Si et al., 2013; Matamala et al., 2015; Chen et al., 2016). miR-21 studies were performed on different ethnic groups (American, Chinese and European) and using distinct experimental methodology. Most of the studies detected miR-21 in breast tissues using different platforms (microarray/sequencing/RT-qPCR) followed by validation in serum and plasma (Wu et al., 2011; Si et al., 2013; Matamala et al., 2015). Others performed array panels on plasma samples followed by verification in plasma using RT-qPCR (Ng et al., 2013) or started with RT-qPCR on tissues then direct serum RT-qPCR (Asaga et al., 2011). Notably, serum miR-21 exhibited higher sensitivity in EBC diagnosis than other conventional cancer markers, such as CA153 and CEA (Gao et al., 2013).

Another extensively studied oncomiR for EBC detection is miR-155. miR-155 was shown to be upregulated in sera and tissues of breast cancer patients of diverse ethnicities (Chinese, Mexican and European) using RT-qPCR and was normalized to dissimilar endogenous controls (Sun et al., 2012; Mar-Aguilar et al., 2013; Sochor et al., 2014; Shaker, Maher, Nassar, Morcos, & Gad, 2015). An additional well-described diagnostic miRNA is miR-18a, which was over-expressed in sera of ethnically-varied patients mainly of ER+ receptor profile, as shown using microarray of a large cohort (205 T & 205 NC) and RT-qPCR of more than one sample sets (Godfrey et al., 2013; Kodahl et al., 2014). Furthermore, the decreased expression of another miRNA, miR-181a, in serum and whole blood was confirmed by two studies as a tool for breast cancer screening, particularly for luminal A subtype using RT-qPCR and normalized to miR-16 (Guo & Zhang, 2012; McDermott et al., 2014). In contrast, a study reported the upregulation of miR-181a in serum using miRNA microarray analysis, and the results were validated in a small sample using RT-qPCR with a different endogenous control, miR-1825, which might explain the discrepancy of the results (Godfrey et al., 2013). miR-145 was also proposed as a diagnostic biomarker in plasma/serum and tissues of breast cancer patients, particularly those with ER+ receptor profile. However, studies disagreed about its mode of dysregulation (Ng et al., 2013; Kodahl et al., 2014), which could be attributed to the variability in the cohort ethnicities or the endogenous controls used for normalization. Similarly, each of miR-195, miR-199a, miR-133a and miR-29a were dysregulated inconsistently in two separate studies. Thus, these miRNAs might not be suitable as indicators for EBC (Heneghan, Miller, Kelly, Newell, & Kerin, 2010; Wu et al., 2011; Chan et al., 2013; Kodahl et al., 2014; McDermott et al., 2014; Zhao et al., 2014; Shin, Siu, Cheuk, Ng, & Kwong, 2015; Zhang et al., 2015). Despite that miR-451 and miR-92a were identified as potential circulating diagnostic biomarkers in more than one study, these miRNA are also not suitable for this purpose since they are highly expressed by red blood cells and their levels are increased upon hemolysis (Pritchard et al., 2012). Other potential diagnostic miRNA that were detected in one study and require further investigation are briefly elaborated on in Table 1 (miR-505-5p, 125b, 96, 152, 16, 29b-2, 197, 205, 19a, 181b, 24, 493, 30e, 27a, 382, 15a,107, 425, 139-5p, 143, 365, 222, 652, 29c, 424, 1, 133b, 48a, 27a, 30b, 148b, 376c, 409-3p, 801, 484, 182, 202, 30a, 3613-3p, 4668-5p, 3656, 5704, 3676-3p, 3196, 3941, 585, 1264, 200a-3p, 1273g-3p, 5581-3p, 877-5p, 744-3p, 2276, 342-5p, 760, 203, 185-5p, 20b-5p, 4521, 4692, miRPlus-A1086).

2.2. Prognostic role

miRNA could serve as a prognostic tool in EBC through estimating the patients' overall survival (OS), anticipating disease outcome and predicting its recurrence. Several studies reported particular miRNA as potential prognostic biomarkers that are worth pointing out. The most studied was miR-21, whose upregulation was associated with reduced disease-free survival (DFS) and OS as well as with lymph node (LN) metastasis and advanced stage in patients of various ethnicities (Yan et al., 2008; Lee, Lee, Lee, Kim, & Bae, 2011; Markou, Yousef, Stathopoulos, Georgoulias, & Lianidou, 2014). Similar trend was noted in two studies using RT-qPCR and in situ hybridization (ISH) for miR-205, especially within ductal tumor tissues (Quesne et al., 2012; Markou et al., 2014). miR-210 was also over-expressed in breast cancer tissues taken from two separate populations, especially in TN patients, and was associated with higher risk of recurrence, poor DFS and OS (Rothe et al., 2011; Toyama et al., 2012). On the other hand, upregulated miR-9 in breast cancer tissues has been associated with poor OS and local recurrence, especially in ER+ patients, as shown using microarray and sequencing data that was validated using RT-qPCR (Zhou et al., 2012; Zhou et al., 2014). Moreover, downregulation of miR-30a and let-7b in breast cancer tissues was correlated with reduced recurrence-free survival (RFS) and OS, advanced stage or LN metastasis (Cheng et al., 2012; Ma, Li, Wu, & Meng, 2014; Quesne et al., 2012; Zhang, Wang, Huo, et al., 2014). Nevertheless, these associations concluded for each miRNA were established using techniques of different sensitivities, whereby RT-qPCR and array used for miR-30a are more sensitive than ISH used for let-7b analysis (Li & Ruan, 2009).

Most studies reporting miRNA as prognostic biomarkers were performed on breast tissues; however, other studies addressed miRNA in serum/plasma, such as miR-106b that was associated with high recurrence risk and shorter OS in plasma and tissues of EBC patients (Zheng et al., 2015) and miR-122 that predicted metastasis and was upregulated in serum of relapsing group. Another example in serum was a miRNA signature (miR-18b, miR-103, miR-107 and miR-652) that was associated with tumor recurrence and reduced OS in TN breast cancer patients as it was exclusively upregulated in relapsing TN breast cancer compared to non-relapsing TN breast cancer, healthy subjects or ER+ patients (Kleivi Sahlberg et al., 2015). In addition, a panel of 4 miRNAs (miR-155, miR-493, miR-30e and miR-27a) was found to act as both diagnostic and prognostic tool through sub-classifying TN breast cancer into basal-like or Core Basal (CB) and five negative (5NP). 5NP refers to the absence of all five markers: ER, PR, HER2, EGFR (epidermal growth factor receptor) and CK5/6 (cytokeratin 5/6), while CB refers to the presence of EGFR and/or CK5/6. This classification using miRNA could help in predicting the OS and the outcome of TN breast cancer since CB subdivision tends to have poor prognosis compared to 5NP (Gasparini et al., 2014). Other miRNA suggested as prognostic biomarkers and reported to date in a single study are listed in Table 2 (miR-155, 19a, 181b, 24, 27a, 27b-3p, 23a, 324-5p, 711, 122, 375, 126, 10a).

2.3. Therapy predictive role

miRNA could have a therapy predictive function in breast cancer, whereby their dysregulation could be indicative of the patient's response to treatment manifested as either resistance or sensitivity to therapy. Breast cancer treatment depends on the tumor stage and subtype and could include radiotherapy, chemotherapy, targeted therapy and/or hormonal therapy. Most of therapy predictive miRNA are initially studied or validated in an in vitro setting (Kutanzi, Yurchenko, Beland, Checkhun, & Pogribny, 2011; Robertson & Yigit, 2014). This is the case for radiotherapy where therapy predictive miRNA were exclusively studied in breast cancer cell lines. Several dysregulated miRNA such as the upregulation of miR-21, miR-144 and miR-27a as well as the downregulation of miR-205, miR-200c and miR-302 were correlated with radioresistance (Anastasov et al., 2012; Liang, Ahn, Guo, Votaw, & Shim, 2013; Ren, Fu, & Han, 2015; Sun et al., 2015; Yu et al., 2015; Zhang, Wang, Rodriguez-Aguayo, et al., 2014).

This review will focus on studies performed on breast cancer patients that require long follow-ups to examine their chemo- or targeted

Table 1
List of diagnostic miRNA in early breast cancer. miRNA are placed in chronological order of appearance in the article. Bold miRNA are reported by more than one study.

Diagnostic miRNA	Biological sample	RNA extraction method	Cohort ethnicity	Case cohort	Detection method	Validation cohort	validation method	Endogenous control	Mode of dysregulation	Reference
miR-21	Serum	A1	Chinese	89 BC patients & 55 NC	B2	NA	NA	miR-16	Up in T vs N	Gao et al. (2013)
miR-21	Serum	Serum: A3, tissue: NR	American	Tissues: 4 of stage I, 1 of stage II, 5 of stage III & 4 of stage IV BC patients, serum: 40 BC patients (10 patients for each stage I–IV) & 10 NC	B2, B3	102 BC patients & 20 NC	B3	Plasma: miR-16 & tissue: RNU6B	Up in T vs N (serum and tissues), higher in BC patients with AJCC stage IV than other stage	Asaga et al. (2011)
miR-21, 505-5p, 125b, 96	FFPE tissues & plasma	A4 & A6	White Spaniard	tissues: 122 BC patients & 11 NC	B7	Plasma: 114 BC patients & 116 NC	B9	miR-103a	Up in T vs N (plasma)	Matamala et al. (2015)
miR-21, **29a**	Frozen tissues & serum	A5	Chinese	T & NAT from one BC patient	B8	Serum: 20 BC patients & 20 NC	B2	RNU6B	Up in T vs N (tissue and serum)	Wu et al. (2011)
miR-21, 152	Plasma	A4	American	53 sporadic BC patients & 40 patients with benign T & 49 NC	B1	NA	NA	cel-miR-39	miR-21: up in T vs N, miR-152: up in T vs N & benign vs N	Chen et al. (2016)
miR-21, 92a	Tissues & serum	A4, A5	Chinese	Tissues: 48 BC patients (48 T and 48 NAT)	B2	Serum: 100 BC patients & 20 NC	B2	miR-16	Down: miR-92a & up: miR-21 in tissues & serum, miR-92a correlates with tumor size and LN metastases	Si et al. (2013)
miR-21, 16, 145, 451	Tissues & plasma	Tissue: A1, serum: A2 & A4	Chinese	Plasma: 5 BC patients & 5 NC, 5 BC patients: 5 T & 5 NAT	B11	170 BC patients & 100 NC (60 female & 40 male) & 95 other types of cancers, Blind validation: 70 BC patients & 50 NC	B2	RNU6B	Up: miR-16, 21 & 451, down: miR-145 in plasma & tissues	Ng et al. (2013)
miR-155	Serum	A5	Chinese	103 BC patients & 55 NC	B1	NA	NA	cel-miR-39	Up in T vs N	Sun et al. (2012)
miR-155, 29b-2, 197, 205	Serum	A4	Egyptian	80 EBC patients & 30 NC	B2	NA	NA	SNORD	Up in T vs N	Shaker et al. (2015)
miR-155, 19a, 181b, 24	Tissue & serum	A4	Caucasian (Czech)	Serum: 63 EBC patients & 21 NC	B1	Tissues: 10 BC patients = 10 T & 10 NAT	B1	let-7a	Up in T vs N (tissue and serum)	Sochor et al. (2014)
miR-155, 493, 30e, 27a	FFPE tissues	A7	Caucasian	173 TNBC BC patients	B10	NR	B12, B1	RNU6B	Differentiate between CB and 5NP	Gasparini et al. (2014)
miR-155, 145, 382	Serum	A4	Mexican	61 BC patients & 10 NC	B1	NA	NA	18S RNA	Up in T vs N	Mar-Aguilar et al. (2013)
miR-15a, **18a**, 107, 425, 133a, 139-5p, 143, 145, 365	Serum	A4	Caucasian (Denmark)	48 ER+ EBC patients (24 with and 24 without LN metastasis) & 24 NC	B9	60 ER+ ECB patients & 51 NC	B9	miR-10b & miR-30a	Up in T vs N: miR-15a, 18a, 107, & 425 & down in T vs N: miR-133a, 139-5p, 143, 145, & 365	Kodahl et al. (2014)
miR-18a, **181a**, 222	Serum	A8	American & Puerto Rican	205 BC patients & 205 NC	B6	5 BC patients & 5 NC	B1	miR-1825	Up in T vs N	Godfrey et al. (2013)
miR-181a	Serum	A2	Chinese	10 EBC patients & 10 NC	B1	152 BC patients & 75 NC	B2	miR-16	Down in T vs N	Guo and Zhang (2012)

miR-29a, **181a**, 652	Blood & tissues	A3	Ireland	Blood: 10 luminal A-like BC patients & 10 NC	B11	Blood: 44 Luminal A BC patients & 46 NC, Tissue: 11 BC patients = 11 BC & 10 NAT	B1	miR-16	miR-181a & 652: up in array, down in blood & tissue of luminal A patients, miR-29a: down in blood & array of luminal A patients but not tissues, luminal A-like BC biomarkers	McDermott et al. (2014)
miR-195	Blood	A3	Ireland	83 BC patients, 80 patients of other cancer types & 63 NC	B1	NA	NA	miR-16	Up in T vs N, BC specific	Heneghan et al. (2010)
miR-195	Serum	A2	Chinese	20 EBC patients & 20 NC & 100 patients with other cancer types	B2	210 BC patients & 102 NC	B2	miR-16	Down in T vs N	Zhao et al. (2014)
miR-199a	Plasma & FFPE tissues	A1, A2, A4	Chinese	5 TNBC & 5 nonTNBC patients & 5 NC	B7	Small cohort: 30 BC patients & 30 NC, tissues: 90 patients (90 T vs 90 NAT), large cohort: 67 TNBC patients, 95 non-TNBC patients & 90 NC	B2	miR-484	Down: TNBC vs non-TNBC & NC sera, T vs NAT, associated with tumor stage and subtype	Shin et al. (2015)
miR-199a, 29c, 424	Serum	NA	Chinese	25 EBC patients & 20 NC	B3	76 BC patients & 52 NC	B3	miR-103a & miR-132	Up in T vs N	Zhang et al. (2015)
25 miRNA set[a]	FFPE tissues	A4 &A6	White Spaniard	Tissues: 122 BC patients & 11 NC	B7	Tissues: 44 BC patients from the case set & 12 NC	B9	miR-103a	Smallest set of miRNA discriminating T vs N	Matamala et al. (2015)
miR-1, 92a, **133a**, 133b	Frozen tissues & serum	Tissue:A5, serum: A4	Chinese	32 BC patients: 32 T & 32 NAT, serum: 32 BC patients & 22 NC	B5	132 BC patients & 101 NC	B9	miR-103 & miR-191	Up in T vs N serum only	Chan et al. (2013)
miR-148a, 451, 27a, 30b	Serum	A5	Chinese	60 BC patients, 20 patients with benign T & 29 NC	B2	NA	NA	cel-miR-356	miR-451,27a & 30b: down in benign and T vs N, miR-148a: down in T vs N	Luo et al. (2014)
miR-148b, 376c, 409-3p, 801	Plasma & frozen tissues	A2, A4	Caucasian	10 EBC patients & 10 NC	B11	Plasma: 127 sporadic BC patients & 80 NC, tissues: 24 BC tissues & 8 benign breast biopsies	B1	Plasma: cel-miR-39, tissue: RNU6B	Plasma: all up in T vs N; tissues: down miR-148b, 376c & 409-3p in T vs benign, but no change in miR-801	Cuk et al. (2013)
miR-484	Serum	A5	Australian	39 BC patients & 10 NC	B11	98 BC patients & 25 NC	B1	miR-16	Up in T vs N	Zearo et al. (2014)
miR-182	Tissue & serum	Serum: A5, tissues: A1	Chinese	Tissues: 3 BC patients (3 T vs 3 NAT)	B2	Serum: 46 BC patients & 58 NC	B2	Spiked in human 5S rRNA	Up in T vs N (tissues and serum esp. ER− patients)	Wang et al. (2013)
miR-202	Blood	A4	Caucasian (Germany)	48 EBC patients & 57 NC	B4	24 EBC patients & 24 NC	B1	miR-16	Up in T vs N	Schrauder et al. (2012)
miR-30a	Plasma	A1	Chinese	100 BC patients & 64 NC	B2	NA	NA	miR-16	Down in T vs N	Zeng et al. (2013)

A1: TRizol Reagent (Ambion), A2: TRizol LS Reagent (Ambion), A3: TRI Reagent (Sigma), A4: miRNeasy Mini Kit (Qiagen), A5: mirVana PARIS Kit (Invitrogen), A6: miRNeasy FFPE Kit (Qiagen), A7: Recover All kit (Ambion), A8: Norgen total RNA purification kit.

B1: TaqMan RT-qPCR, B2: SYBR-Green RT-qPCR, B3: Direct Serum SYBR-Green RT-qPCR, B4: Geniom® Biochip, B5: Agilent Human miRNA Microarray, B6:GeneChip miRNA 2.0 array (Affymetrix), B7: LNA-based miRNA microarray (Exiqon), B8: SOLiD sequencing, B9: miRCURY LNA Universal RT-qPCR (Exiqon), B10: NanoString nCounter Microarray, B11: TaqMan human miRNA arrays, B12: sequencing data from TCGA and GEO: GSE28884.

[a] 25 miRNA set: miR-125b, 3613-3p, 4668-5p, 3656, 5704, 3676-3p, 3196, 3941, 585, 1264, 200a-3p, 1273g-3p, 5581-3p, 877-5p, 96-5p, 744-3p, 2276, 342-5p, 760, 203, 185-5p, 20b-5p, 4521, 4692, miRPlus-A1086.

Table 2
List of prognostic miRNA in early breast cancer. miRNA are placed in chronological order of appearance in the article. Bold miRNA are reported by more than one study.

Prognostic miRNA	Biological sample	RNA extraction method	Cohort ethnicity	Case cohort	Detection method	Validation cohort	Validation method	Endogenous control	Mode of dysregulation	Reference
miR-21	FFPE tissues	A3	Korean	109 IDC BC	B1	NA	NA	RNU6	Up in BC, associated with poor prognosis, poor survival & aggressive tumor	Lee et al. (2011)
miR-21	Frozen tissues	A1 & A9	Chinese	8 BC patients: 8 T & 8NAT	B7	113 BC patients: 113 T & 40 NAT	B15	U6	Up correlated with advanced clinical stage, LN metastasis & poor prognosis, independent risk factor	Yan et al. (2008)
miR-21, **205**	FFPE tissues	A2	Greek	84 EBC patients & 13 NC (mammoplasties)	B1	NA	NA	miR-191	Up miR-21 associated with shorter DFS & down miR-205 associated with shorter DFS & OS	Markou et al. (2014)
miR-205	FFPE tissues	A6	British	1304 ductal BC patients	B4	26 ductal BC patients	B1	RNU44	Up: predicted survival within ductal T	Quesne et al. (2012)
miR-210	FFPE & frozen tissues	A1	Japanese	58 TNBC vs 103 ER+/HER2−	B1	NA	NA	RNU6B	Up in TNBC vs ER+/HER2−, independent factor associated with poor prognosis, OS & DFS.	Toyama et al. (2012)
miR-210	Frozen tissues	A1	British	56 BC patients	B16	73 BC patients	B1	RNU44 & RNU48	Up associated with higher risk of recurrence	Rothe et al. (2011)
miR-9	FFPE tissues	A7	American	16 BC patients (8 with & 8 without local recurrence)	B11	68 BC patients (23 with & 45 without local recurrence)	B1	U6	Up in BC patients with local recurrence & with ER+	Zhou et al. (2012)
miR-135b, 187, 18a, 210, 224, 3200, 452, 455, 505, 584, **9-1**, 9-2, 190b, 375	Tissues	NR	American	596 TCGA dataset BC patients (456 ER+ & 140 ER−)	B8	319 TCGA dataset BC patients (251 ER+ & 68 ER−)	B8	NA	Up: 12 miRNAs (miR-135b, 187, 18a, 210, 224, 3200, 452, 455, 505, 584, 9-1 & 9-2) & down: (miR-190b, 375) in ER− vs ER+ patients, associate with poor OS	Zhou et al. (2014)
let7b	FFPE tissues	A6	British	1342 luminal BC patients	B4	29 luminal BC patients	B1	RNU44	Up: associate with less aggressive tumors & survival within luminal T, not validated in B1	Quesne et al. (2012)
let-7b	FFPE tissues	NA	Chinese	80 BC patients & 22 patients with benign T	B4	NA	NA	NA	Down in BC vs benign tissues, inversely associated with LN metastasis & with better OS and RFS	Ma et al. (2014)
miR-30a	Frozen tissues	A5	Taiwanese	9 stage I/II BC patients & 5 stage III/IV BC patients	B11	221 BC IDC patients	B13 & B1	RNU6B	Down associated with unfavorable outcome, advanced stage, & decreased RFS and OS	Cheng et al. (2012)
miR-30a	Tissues	NR	Chinese	28 BC patients (T and NAT)	B2	96 BC patients	B2	U6	Down associated with LN metastasis & with lung metastasis	Zhang et al., (2014a)
miR-27a	FFPE tissues	NA	Chinese	102 BC patients	B4	NA	NA	NA	UP in invasive BC with distant metastasis vs non-metastatic cancers, associated with shorter DFS & OS	Tang et al. (2012)
miR-155, 493, 30e, **27a**	FFPE tissues	A7	Caucasian	173 TNBC BC patients	B10	NR	B12	RNU6B	Up: miR-155 & miR-493 correlated with better outcome, down: miR-30e & miR-27a correlated with worse outcome	Gasparini et al. (2014)
miR-155, 19a, 181b, 24	Serum	A4	Caucasian (Czech)	63 EBC patients & 21 NC: 1 day pre-operation, 14–28 days after surgery, & 14–28 days after first treatment: chemo/radiotherapy	B1	NA	NA	let-7a	miR-155, 181b & 24 down upon surgery and therapy with delay in high risk EBC patients, miR-19a down upon therapy	Sochor et al. (2014)
miR-106b	Plasma & tissue	A1	Chinese	Tissues: 187 EBC patients & 50 fibroadenoma patients	B4 & B14	Plasma: 187 EBC patients & 20 NC	B14	Plasma: miR-16 & tissue: RNU6B	Up in BC tissues vs benign tissues & T vs N in plasma, up correlated with shorter DFS and OS, a higher T grade, more aggressive disease & poor prognosis, independent prognostic factor, associated with a high risk of BC recurrence	Zheng et al. (2015)

miR-122	Serum	A2	American	42 stage II–III locally advanced & inflammatory BC patients	B3	26 stage II–III BC patients (8 with metastatic recurrence)	B2	miR-16	Up in relapse group, predicts metastasis	Wu et al. (2012)
miR-18b, 103, 107, 652	Serum	A8	Caucasian	20 ductal TNBC patients (10 relapsing & 10 non-relapsing) & 10 NC	B9	40 ductal TNBC patients (20 relapsing & 20 nonrelapsing) & 33 ER+ BC patients & 30 NC, 70 TNBC patients	B1	U6	Up in relapsing vs nonrelapsing TNBC, NC & ER+ group, associated with tumor recurrence & reduced OS	Kleivi Sahlberg et al. (2015)
miR-27b-3p	FFPE tissues	A7	Chinese	58 IDC TNBC patients (31 developed distant metastasis vs 27 disease-free patients)	B2	41 TNBC patients	B2	U6	Up in metastatic disease group vs disease-free patients, associated with increased risk of distant metastasis	Shen et al. (2014)
miR-23a	Frozen tissues	A4	Egyptian	16 BC patients, 8 with benign T & 8 NC	B2	76 BC patients, 36 with benign T & 36 NC (breast reduction surgery)	B2	SNORD 68	Up in BC vs benign tumor & NC, correlate with decrease in RFS and increase in cumulative hazards	Eissa, Matboli, & Shehata (2015)
miR-324-5p	Tissues	NR	Norway	MicMa BC patient cohort (101 T)	B5	METABRIC cohort (1302 T)	B17	NA	Up associated with longer OS	Leivonen et al. (2014)
miR-711	FFPE tissues	A9	Chinese	30 BC patients & 30 NC	B1	161 BC patients (underwent radical mastectomy)	B1	U6	Up in BC, independent prognostic factor associated with poor OS and DFS	Hu, Yi, et al. (2016)
miR-375	FFPE tissues	A6	Caucasian (Austria)	32 BC patients (16 with local relapse vs 16 with no relapse)	B5	115 BC patients (30 with local relapse vs 85 without relapse)	B2	SNORD61	Up in the relapse group especially in ER+ subgroup	Zehentmayr et al. (2016)
miR-126, 10a	FFPE Tissues	A7	Caucasian (Germany)	Postmenopausal ER+ BC patients (6 with & 6 without recurrence following tamoxifen)	B6	Postmenopausal ER+ BC patients (34 with & 47 without recurrence following tamoxifen)	B1	NA	Independent predictors of recurrence, low miR-126 & 10a correlate with higher risk for recurrence in tamoxifen treated patients	Hoppe et al. (2013)

A1: TRizol Reagent (Ambion), A2: TRizol LS Reagent (Ambion), A3: TRI Reagent (Sigma), A4: miRNeasy Mini Kit (Qiagen), A5: mirVana PARIS Kit (Invitrogen), A6: miRNeasy FFPE Kit (Qiagen), A7: Recover All kit (Ambion), A8: miRCURY RNA Isolation Kit (Exiqon), A9: phenol/chloroform extraction, A10: miRNA Isolation Kit (Ambion).

B1: TaqMan RT-qPCR, B2: SYBR-Green RT-qPCR, B3: Solexa deep sequencing (Illumina), B4: LNA-in situ hybridization, B5: Agilent Human miRNA Microarray, B6: GeneChip miRNA 2.0 array (Affymetrix), B7: CapitalBio Microarray, B8: IlluminaHiSeq_miRNASeq, B9: miRCURY LNA Universal RT-qPCR (Exiqon), B10: NanoString nCounter Microarray, B11: TaqMan human miRNA arrays, B12: sequencing data from TCGA and GEO: GSE28884, B13: laser confocal microscopy dissection, B14: molecular beacon based RT-qPCR, B15: unspecified RT-qPCR, B16: mirVana miRNA Bioarrays, B17: data from European Genome–Phenome Archive (EGAS00000000122).

therapy efficacy (Table 3). Each of the studies has utilized a different approach to identify the therapy predictive miRNA biomarker starting at the level of patients and then validating the results in drug resistant breast cancer cell lines or vice versa. Interestingly, despite the limited number of studies, various methods were utilized to stratify the patients according to the treatment response.

2.3.1. miRNA and response to chemotherapy

Chemotherapy is the most studied treatment option that could be administered either before surgical operation to decrease tumor size and to increase surgical options called neoadjuvant chemotherapy (NAC) or after surgery to prevent recurrence referred to as adjuvant chemotherapy (AC). Most common chemotherapy drugs include anthracyclines (e.g. doxorubicin and epirubicin), taxanes (e.g. paclitaxel and docetaxel), cyclophosphamide and 5-fluorouracil (5-FU). let-7a expression was examined by ISH on pre-treated tissue biopsies of 39 IDC patients who later received epirubicin-based NAC and it was also validated using RT-qPCR on pre-treated tissue biopsies of 31 additional patients. Patients were divided according to Response Evaluation Criteria in Solid Tumors (RECIST) which are radiological guidelines based on tumor size and define cancer patients who have complete response (with the disappearance of target lesion), partial response (with 30% tumor size reduction), stable disease (neither sufficient shrinkage nor increase to qualify) or progressive disease (with 20% tumor size increase or new lesions) upon treatment. Responders to treatment were those with complete and partial response. Reduced level of let-7a was associated with epirubicin chemoresistance in non-responder tissues (Wu et al., 2015). This was followed by in vitro experiments using two breast cancer cell lines (SKBR3 cell line and epirubicin-resistant SK-3rd sphere cell line that was generated from the former cell line) that were injected in the mammary fat pad of mice with 10–12 weeks continuous epirubicin treatment until the xenografts reached ~2 cm diameter (Yu et al., 2007). This epirubicin- resistant breast tumor-initiating cell line (SK-3rd sphere cells) restored their sensitivity to epirubicin and showed increased apoptosis after in vitro transfection with let-7a mimics. Similar association was reported with miR-204 and miR-205 in tissues taken before administering NAC with combination treatment of 5-fluorouracil (5-FU), epirubicin and cyclophosphamide (FEC) and combination treatment of doxorubicin, docetaxol and cyclophosphamide (TAC) respectively. For both miRNA studies, patients were also categorized as therapy responders based on RECIST. Low miR-204 expression in FFPE tissues was correlated with chemoresistance seen in 53 non-responsive breast cancer patients as compared to higher expression in 76 responsive patients (Li, Jin, et al., 2014). As for miR-205, better chemotherapeutic response rate was correlated with high levels of miR-205 in 30 patients. Then the association between miR-205 and taxol and doxorubicin resistance was validated in vitro in multi-drug resistant MCF-7/A02 and CALDOX that are derived from MCF-7 and Cal51 breast cancer cell lines. It was also confirmed in vivo upon subcutaneously injecting these drug-resistant breast cancer cells stably expressing miR-205 or vector control into the fat pad of nude mice (Hu, Qiu, et al., 2016; Hu, Yi, et al., 2016). Another miRNA that correlated with taxane and anthracycline chemoresistance was miR-221 which was upregulated in plasma of non-responder patients grouped according to standard RECIST criteria. miR-221 study was based on upregulation of this miRNA in adriamycin resistant MCF-7 compared to MCF-7 (Zhao et al., 2011).

Furthermore, miR-125b was correlated with chemoresistance in 56 IDC patients who received NAC with combination treatment of 5-FU and FEC and were stratified according to RECIST, PCNA immunostaining for cell proliferation, and TUNEL assay for apoptotic index in surgical biopsies after chemotherapy. This miRNA was investigated after identifying that it was significantly overexpressed in the pre-treated sera of 21 stage III IDC patients as compared to that of 35 stage II patients. miR-125b was found to be upregulated in sera of non-responsive IDC patients and was correlated with cell proliferation as well as inversely associated with apoptosis (Wang, Tan, et al., 2012). This was also confirmed in primary breast cancer cells isolated from biopsies of 11 breast cancer patients before chemotherapy, whereby knockdown of miR-125b would sensitize the non-responder cells to 5-FU. It was further validated in breast cancer cell lines (T47D, BT20, MDA-MB 231, MCF-7 and 5-FU resistant MCF-7), whereby miR-125b overexpression in T47D, BT20, MDA-MB 231 and MCF-7 cell lines would confer chemoresistance to 5-FU and miR-125b knockdown in 5-FU resistant MCF-7 and MDA-MB 231 (with high endogenous miR-125b) would increase 5-FU sensitization.

Other miRNA studies have utilized a different approach other than RECIST radiological assessment for patient stratification. For example, a study divided patients based on Miller and Payne histopathology scoring system that ignores tumor size and nodal status and focuses on tumor cellularity by evaluating pathological response as grade 1: no reduction; grade 2: minor loss (≤30%); grade 3: some loss (30%–90%); grade 4: marked loss (>90%) and grade 5: no residual invasive cancer (Ogston et al., 2003). The sensitive group was considered with a grade >2 and the resistant group with a grade ≤2. TaqMan Real-time PCR miRNA Array was performed on 6 pooled sera from the luminal A group sensitive to NAC with epirubicin plus paclitaxel and on 6 from the resistant luminal A group. As per array results and RT-qPCR validation in sera of 56 luminal A patients (32 sensitive and 24 resistant), miR-19a and miR-205 were found to be over-expressed in non-responsive patients prior taking NAC with epirubicin plus paclitaxel regimen; thus, these two miRNAs were found to predict chemo-sensitivity (NAC with epirubicin plus paclitaxel) in luminal A patients (Li, Liu, et al., 2014).

Another method for patient grouping is Symmans Residual Cancer Burden (RCB) score that is derived from the primary tumor size, tumor cellularity and axillary nodal burden. If patients had an RCB score of 0, they were defined as pathologic complete response, whereas those with RCB score of ≥1 were defined as pathologic non-complete response (Symmans et al., 2007). After dividing patients according to RCB, chemoresistance was associated with the downregulation of miR-375 and upregulation of miR-122 in sera of patients before taking NAC, especially in HER2+ patients (Wu et al., 2012). These miRNAs were chosen upon deep sequencing of sera from 42 stage II–III locally advanced and inflammatory patients and this was followed by validation on another 26 patient set using RT-qPCR.

Elevated levels of miR-621 in tissues was associated with better therapeutic response to paclitaxel/carboplatin (PTX/CBP) NAC regimen after analyzing miR-621 expression in 50 patients divided according to pathologic complete response that is defined as absence of invasive tumor cells in breast and axillary lymph nodes. This correlation of miR-621 with PTX/CBP was validated by overexpressing of miR-621 in MCF-7 (with low endogenous miR-621) that sensitizes the cells to PTX/CBP in vitro and when orthotopically injected into the mammary gland fat pads of BALB/c nude mice as stably overexpressing miR-621 (Xue et al., 2016). Serum levels of miR-155 and plasma levels of miR-21 and miR-505-5p were also found to be low following chemotherapy, but these miRNAs require further investigation before being identified as indicators for treatment response since they were examined on a small sample size (Sun et al., 2012; Matamala et al., 2015).

2.3.2. miRNA and response to hormonal and targeted therapy

Moreover, other miRNA have been associated with resistance to hormonal therapy that includes ER blockers (tamoxifen and Fulvestrant) and estrogen production inhibitors (aromatase inhibitors) as well as to targeted therapy (trastuzumab/Herceptin and Everolimus). Upregulated miR-210 in tissues has been associated with poor outcome and higher risk of recurrence in ER+ tamoxifen-treated breast cancer patients (Rothe et al., 2011). Eighty-nine ER+ tamoxifen-treated patients were divided according to miR-210 expression as per RT-qPCR results and their clinical outcomes were monitored over 10 years.

Table 3
List of therapy predictive miRNA in early breast cancer. miRNA are placed in chronological order of appearance in the article.

Therapy predictive miRNA	Biological sample	RNA extraction method	Cohort ethnicity	Case cohort	Detection method	Validation cohort	Validation method	Endogenous control	Division of patient response	Mode of dysregulation	Reference
let-7a	FFPE & frozen tissues	A1 & A3	Chinese	39 IDC BC patients before epirubicin based NAC	B4	31 IDC BC patients before epirubicin based NAC	B1	U6	RECIST	Down associated with lower chemosensitivity to epirubicin	Wu et al. (2015)
mR-204	FFPE tissues	A1, A4, A7	Chinese	129 BC patients after NAC with 5-FU & FEC	B1	NA	NA	U6	RECIST	Down in non-responsive patients, correlate with chemoresistance	Li, Jin, et al. (2014)
miR-205	Frozen tissues	A9	Chinese	30 BC patients prior to TAC	B2	NA	NA	U6	RECIST	Up with better chemotherapeutic response	Hu, Qiu, et al. (2016); Hu, Yi, et al. (2016)
miR-221	Plasma	No RNA extraction placed directly in cDNA kit	Chinese	93 non-metastatic stage I–III BC patients before taxane- & anthracycline-based NAC & 32 NC	B8	NA	NA	No control	RECIST	Up associated with chemoresistance, a predictor for NAC response	Zhao et al. (2011)
miR-125b	Serum	A4	Chinese	56 IDC patients before and after NAC with FEC & 10 NC	B1	NA	NA	miR-16	RECIST, PCNA immunostaining & TUNEL assay for apoptotic index	Up in non-responsive patients, correlated with chemotherapeutic resistance	Wang et al., (2012a)
miR-19a, 205	Serum	A2	Chinese	Stage IIa–IIIc luminal A BC patients before received NAC with epirubicin plus paclitaxel (pooled 6 from sensitive group & pooled 6 from resistant group)	B6	56 stage IIa–IIIc luminal A BC patients before received NAC with epirubicin plus paclitaxel (24 resistant vs 32 sensitive)	B2	miR-484, external controls Quanto EC1 and Quanto EC2	Miller and Payne histopathology scoring system	Up in luminal A subtype resistant to epirubicin plus paclitaxel regimen	Li, Liu, et al. (2014)
miR-375, 122	Serum	A2	American	42 stage II–III locally advanced and inflammatory BC patients before treatment with NAC & surgery	B2	32 of the case cohort	B2	miR-16	RCB score	Down miR-375 & up miR-122 correlate with resistance to NAC in HER2+ patients	Wu et al. (2012)
miR-621	Frozen tissues	A1	Chinese	50 BC patients before received PTX/CBP	B8	NA	NA	U6	NR	Up with better chemotherapeutic response	Xue et al. (2016)
miR-155	Serum	A4	Chinese	29 nonmetastatic BC patients before & after surgery & AC & 55 NC	B1	NA	NA	cel-miR-39	NA	Down upon treatment post-operative	Sun et al., 2012
miR-21, 505-5p	FFPE tissues & plasma	A3 & A5	White Spaniard	Tissues: 122 BC patient pre-therapy & 11 NC	B7	Plasma: 83 BC patients (36 before and 47 after treatment) & 26 NC	B5	miR-103a	NA	Down after surgery and treatment	Matamala et al. (2015)
miR-210	Frozen tissues	A1	British	56 BC patients (pre-therapy)	B9	89 ER-positive BC patients after treatment with tamoxifen only (61 high genomic grade vs 28 low genomic grade; genomic grade reflect tumor proliferation and differentiation)	B1	RNU44 & RNU48	miR-210 expression	Associated with poor clinical outcome in ER+, tamoxifen-treated BC patients	Rothe et al. (2011)
miR-210	Plasma	A6	Case: American, validation: Korean	29 BC patients before a& after 24 weeks of treatment with NAC (paclitaxel + FEC) & trastuzumab & 28 NC	B1	43 BC patients (39 preoperative and 30 postoperative) did not receive NAC or AC	B1	U6	MD Anderson guidelines	Associated with trastuzumab resistance & tumor presence, up before surgery and in BC patients with LN metastasis	Jung et al. (2012)
miR-21	FFPE & frozen tissues	NR	Chinese	32 BC patients before & after pre-operative trastuzumab (treated with NAC: TCH)	B4	32 BC patients before & after pre-operative trastuzumab	B1	U6	RECIST	Up in resistant tumors than the sensitive ones, up associated with poor trastuzumab response	Gong et al. (2011)

A1: TRizol Reagent (Ambion), A2: TRizol LS Reagent (Ambion), A3: miRNeasy Mini Kit (Qiagen), A4: mirVana PARIS Kit (Invitrogen), A5: miRNeasy FFPE Kit (Qiagen), A6: Norgen's RNA purification kit, A7: PureLink™ FFPE RNA Isolation Kit, A8: RNABee, A9: miRCURY RNA Isolation Kit (Exiqon).
B1: TaqMan RT-qPCR, B2: SYBR-Green RT-qPCR, B3: Solexa deep sequencing (Illumina), B4: LNA-in situ hybridization, B5: miRCURY LNA Universal RT-qPCR (Exiqon), B6: TaqMan human miRNA arrays, B7: LNA-based miRNA microarrays, B8: unspecified RT-qPCR, B9: mirVana miRNA Bioarrays.

Increased levels of miR-210 in the plasma of HER2+ breast cancer patients were also correlated with trastuzumab resistance and tumor presence (Jung et al., 2012). In this study, miR-210 was assessed in the plasma of the trastuzumab treatment group (n = 29) and the healthy control group (n = 28) and then in plasma of 29 patients before and after 24 weeks of trastuzumab treatment. Patients were classified with pathological complete response if they have no residual invasive cancer in breast and axilla and all other cases were considered non-responders (18 with complete response and 11 with residual disease) (Buzdar et al., 2005). RT-qPCR results showed that miR-210 was upregulated in breast cancer patients compared to healthy subjects and was significantly higher in patients with residual disease as compared to those with complete response. miR-210 upregulation was validated in trastuzumab resistant BT474 clone and its correlation with tumor burden was shown in the plasma of 43 Korean patients (39 preoperative and 30 postoperative) who did not receive either neoadjuvant or adjuvant chemotherapy.

Similarly, miR-21 was associated with poor trastuzumab response but rather in patient tissue biopsies and using a more comprehensive approach (Gong et al., 2011). HER2+ breast cancer cell lines, SKBR3, MDA-MB-453, and BT474, which acquired resistance to trastuzumab by continuous low-dose (5 μg/ml) exposure for 6 months, were first used to prove the upregulation of miR-21 in trastuzumab resistant cells. miR-21 expression level was then modulated in those cells either blocked by miRNA inhibitor or overexpressed by miRNA mimic to demonstrate their sensitization and resistance to trastuzumab respectively. miR-21 inhibition was also performed in vivo after injecting the mammary fat pads of mice with trastuzumab resistant BT474 in growth factor-reduced Matrigel and it caused sensitization of the resistant cells. This was followed by miR-21 examination in biopsies from 32 patients receiving pre- and post-operative neoadjuvant trastuzumab therapy and that were divided according to RECIST. ISH and RT-qPCR results have shown that miR-21 is highly expressed in trastuzumab-resistant tumors as compared to the sensitive ones.

Therefore, various methods are present to categorize patients according to treatment response. An evaluation study performed on 151 locally advanced breast cancer patients to assess three of the methodologies (RECIST, RCB and Miller and Payne) has shown that RCB index and Miller and Payne classification are highly in agreement with each other but differ from RECIST criteria (Romero et al., 2013). Treatment outcome predicted by RCB and Miller and Payne classification correlated with better OS and RFS and was superior to RECIST. The group with an RCB of score 0 was the best in estimating the patients with better OS. This could be explained by the fact that RCB takes more than one variable into consideration which are the tumor volume, number and size of axillary lymph node metastases and the cellularity of the residual tumor, while the Miller and Payne classification only considers the decrease of cancer cellularity and RECIST are only based on radiological assessment of tumor dimensions. Moreover, prospective studies with long patient follow up are needed instead of retrospective studies that could be affected by selection bias. Hence, identifying a therapy predictive miRNA biomarker requires choosing a method for assessing tumor response and a comprehensive approach that evaluates the biomarker at the level of patients in vivo as well as in vitro.

3. miRNA as biomarkers: challenges to overcome

miRNAs are qualified to be therapy predictive biomarkers for ECB since they are characterized by being stable, easily detected and acquired in a minimally invasive manner. Studies have identified several challenges and suggested the need to develop and implement an optimal strategy for miRNA biomarker detection. This strategy focuses on limiting the variability in patients' characteristics, experimental design, isolation and detection methodologies of miRNA and data analysis as discussed below.

3.1. Patient characteristics

Breast cancer is a heterogeneous disease whereby each subtype and histotype is characterized by a specific miRNA profile. Therefore, when studying miRNA as biomarkers, the selected cohort should have the least variability in subtype, histotype, ethnicity and age at diagnosis (Fig. 2A). One study has shown that miRNA associated with cancer risk, particularly breast cancer, are affected by population-specific genetic variations, as shown after examining the whole genome sequencing of 69 unrelated individuals from 14 global populations, including European, Asian and African populations (Rawlings-Goss, Campbell, & Tishkoff, 2014). In addition, differences in dysregulated miRNA have been noted in our laboratory upon comparative miRNA profile analysis between breast cancer tissues taken from American and Lebanese patients. This was attributed to patient's age at diagnosis, ethnic variation in miRNA epigenetic regulation or sequence variation of precursor miRNA (data not published). Another study has identified 145 Single Nucleotide Polymorphisms in 6 miRNA processing genes and in 78 miRNA genes that differed between 906 African American and 653 European American women (Yao et al., 2013). Interestingly, a pilot study has shown that dysregulated plasma miRNA levels in 10 Caucasians and 10 African-Americans differ as compared to their ethnically matched controls, with an overlap of only two miRNAs suggesting that circulating miRNA profile could depend on ethnicity to some degree (Zhao et al., 2010). Age could be another factor that affects miRNA expression in breast cancer, knowing that younger patients tend to have more aggressive disease with higher mortality and to differ in their response to therapy (McGuire, Brown, Malone, McLaughlin, & Kerin, 2015). A recent study has shown a distinct miRNA profile in FFPE breast cancer tissues obtained from patients younger than 35 years as compared to older patients (Pena-Chilet et al., 2014). In addition, differential miRNA expression was identified in the serum/plasma of breast cancer patients from different age groups (Hatse et al., 2014). Even control samples should be age- and ethnicity-matched to tumor samples. An important variable that should be taken into consideration when studying miRNA for therapy prediction is to limit the patients to those having similar treatment regimen.

3.2. Experimental design

Many studies have poor experimental design either having no validation cohort or having both case and validation cohorts but of small sample size (Fig. 2B). The sample size is a critical variable as it determines the reliability of the study and the statistical power of the analysis. Other important factors include sample collection, handling and processing, especially when dealing with blood specimens (Cheng et al., 2013). Fresh and FFPE breast cancer tissues showed no significant difference in miRNA expression using RT-qPCR and sequencing (Yan et al., 2008; Meng et al., 2013). As for blood sampling, serum and plasma specimens are preferred over whole blood, since the latter contains multiple cell types that might release miRNA. Both serum and plasma face a dilemma of residual or hemolyzed blood cells that can contribute to circulating miRNA levels. Interestingly, measurements of serum miRNA are also affected by possible release of miRNA from cells (red blood cells and platelets) into the serum during the coagulation process; hence suggesting a difference between plasma and serum (Wang, Yuan, et al., 2012). As such, it is not recommended to compare studies of circulating miRNA from different sample types (plasma, serum and whole blood) and not to pool different sample types but rather use either plasma or serum. Besides excluding miRNA that are highly expressed by the blood cells from the analysis, a study has reported a quality control system as well as a sensitive hemolysis indicator to assess samples (Blondal et al., 2013; Shah, Soon, & Marsh, 2016). The quality control system includes comparing miRNA profiles from serum and plasma with a normal reference range values of 119 miRNAs that are most commonly present in serum and plasma. The hemolysis indicator assesses the

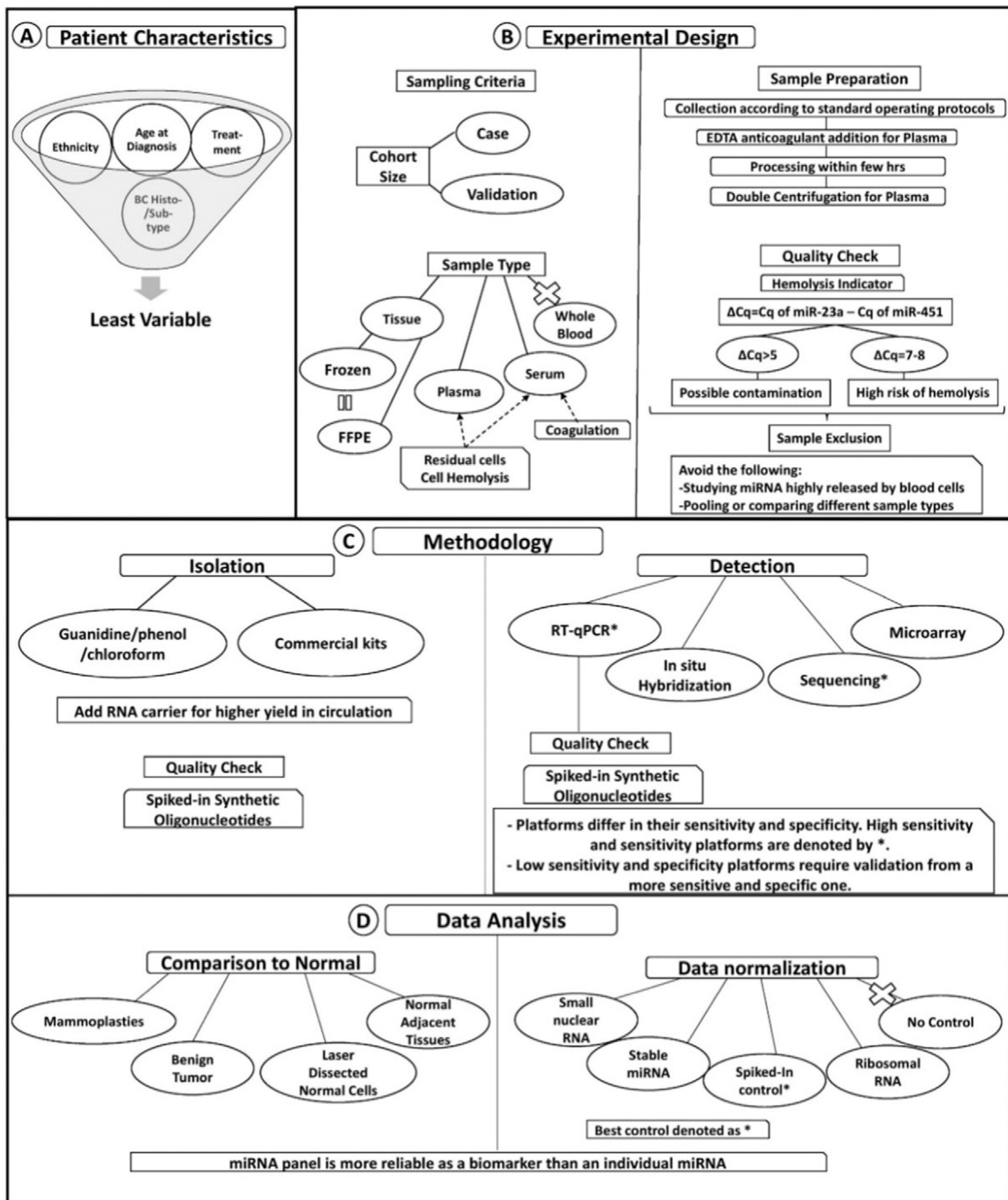

Fig. 2. Summarized challenges and optimal strategy for miRNA detection. Ways to control the variability in patients' characteristics, experimental design, isolation and detection methodologies, and data analysis are suggested in this figure. X denotes that the selected is not recommended. = denotes equivalence. Dotted lines means "affects".

relative expression of the erythrocyte-specific miR-451 and the stable miR-23a (unaffected by hemolysis) or delta Cq (Cq of miR-23a − Cq of miR-451). Possible red blood cell miRNA contamination is indicated if delta Cq is greater than five and a high risk of hemolysis with sample exclusion if delta Cq is 7–8. To ensure universal sample collection, serum and plasma samples should be collected according to the published standard operating protocols (The National Cancer Institute Early Detection Research Network, 2016). Blood processing should be performed within a few hours from collection, and double centrifugation is recommended for plasma. EDTA is the recommended anticoagulant used upon plasma preparation rather than heparin or citrate to avoid interference with downstream applications (Kroh, Parkin, Mitchell, & Tewari, 2010) or hemolysis (Cui, Ma, Wang, & Biswal, 2011).

Considering the overall experimental design used in the literature, some studies investigated miRNA in EBC at the level of breast tissues and then validated their results in circulation or vice versa. However, miRNA mode of dysregulation in tissues might not be similar to that in circulation. This has been explained by the selective release of miRNA from tumor cells into circulation (Pigati et al., 2010). Circulating miRNA could be also secreted from cells either by passive leakage or by active secretion through microvesicles or RNA-binding protein-

dependent pathway (Chen, Liang, Zhang, Zen, & Zhang, 2012). Many studies directly profiled miRNA in circulation and attributed their dysregulation to breast cancer disregarding the fact that it could be due to many other diseases, excretions from normal blood cells, chronological age or even dietary and lifestyle patterns (Hatse et al., 2014; Rome, 2015). Therefore, it is better to study miRNA initially in breast tissues and then validate the obtained data in circulation to ensure that the significantly deregulated miRNA in circulation of breast cancer patient are breast cancer-related.

3.3. Isolation and detection methodologies

The isolation and detection methodologies of miRNA differ from one study to another making it hard to compare results (Fig. 2C). miRNAs are usually isolated as total RNA using guanidine/phenol/chloroform-based protocols and commercial kits. Since the RNA yield in serum and plasma is usually low, RNA carrier could be added during RNA extraction or plasma and serum samples could be directly used for cDNA synthesis without extraction (Asaga et al., 2011; Zhang et al., 2015). In order to eliminate miRNA variation caused by inhibitors in serum and plasma, a quality check at different steps (miRNA extraction, cDNA synthesis, real time PCR) could be performed through adding spike-in synthetic oligonucleotides such as *C. elegans* synthetic miRNA that is used as a control for sample-to-sample normalization of RNA recovery and reverse transcriptase efficiency (Blondal et al., 2013). Different platforms are utilized for miRNA detection, such as microarray, sequencing, ISH and RT-qPCR. Intra-platform variability was noted for miRNA microarray profiling that might be due to different reliable detection stringency utilized by each manufacturer or due to different normalization strategies used in the analysis (Sato, Tsuchiya, Terasawa, & Tsujimoto, 2009; Kolbert et al., 2013). Utilizing microarray and ISH is not enough to assign miRNA as a biomarkers for EBC since these platforms have low sensitivity (van Schooneveld et al., 2015). In addition, sequencing is still an expensive assay to be routinely used for miRNA detection. Thus, its use might be limited to preliminary screening that characterizes an ethnicity's miRNA profile and that identifies new miRNA and mutations affecting miRNA genes. Hence, the most commonly used platform for validation is the sensitive and specific RT-qPCR assay, which is combined with ROC curve, a statistical test that defines the accuracy of the results, as done in most of the studies selected for this review.

3.4. Data analysis

As for data analysis, studies use various strategies to detect miRNA expression (Fig. 2D). Data normalization of samples is still a challenge for miRNA in circulation and in tissues. Each study uses a different endogenous control (small nuclear RNA, stable miRNA, ribosomal RNA, spiked-in control or no control). Small nuclear RNA RNU6B and U6 are the most commonly used for tissue specimens. Nonetheless, the use of RNU6B and U6 is not credible in circulation as they are not detectable or have a very high Ct in plasma/serum (Cheng et al., 2013). miR-16 is frequently utilized for plasma/serum samples even though it is affected by hemolysis and produced by red blood cells (Kirschner et al., 2011; Pritchard et al., 2012). Thus, the preferential endogenous control in circulation is spiked-in control miRNA (Kroh et al., 2010). Others use a global measure of miRNA without normalization such as median or mean, but this is prone to experimental errors. Another challenge in the analysis is the comparison of miRNA expression in tumor tissues to different NCs, including mammoplasties, cancer-free tissues, breast benign tumors (e.g. fibroadenoma), normal adjacent tissues or normal cells dissected by laser dissection microscopy. Finally, choosing one miRNA as a biomarker is not sufficient but a panel of miRNA would be more reliable (Gasparini et al., 2014; Zhang et al., 2015).

4. Conclusion

miRNA are promising biomarkers for EBC diagnosis, prognosis and therapy prediction. However, miRNA have not yet been clinically utilized as reproducible, disease-specific markers, due to the need for an optimized detection strategy. Studies on EBC diagnosis should focus more on finding miRNA in circulation rather than in tissues, while the prognostic and the therapy predictive miRNA biomarkers could be assessed in tissues and/or in circulation. Furthermore, research on therapy predictive miRNA is still not well-established due to the scarcity of studies performed on patients, the variability in methods for assessing patients' treatment response and/or failure to limit the investigation to patients having the same therapy regimen with long follow up. A critical point to consider in studying miRNA as EBC biomarkers is to identify a panel of dysregulated miRNA that can increase sensitivity and specificity of the biomarker rather than identifying individual miRNA. Enhancing existing miRNA databases in breast cancer to include sample sources, patterns of miRNA expression, methods of their detection and their potential use as diagnostic, prognostic and therapy predictive markers would facilitate the rapid progress in this promising field. Finally, even though some miRNA were validated as biomarkers in more than one study, there is little agreement on the detected miRNA among the different studies. This is due to the variability in patients' characteristics, experimental design, isolation and detection methodologies and data analysis. Hence, a standardized protocol, as proposed in this review, starting from sample collection to data analysis must be adapted in future miRNA studies to ensure comparative analysis of data and reproducibility of results.

Conflict of interest

The authors disclose no potential conflicts of interest.

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