

Circulating microRNAs and their role in the immune response in triple-negative breast cancer (Review)

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Abstract. Breast cancer (BC) is the most common type of cancer in women worldwide, and despite advances in treatments, its incidence and mortality are increasing. Therefore, it is necessary to develop new, non-invasive tests that provide more accurate diagnosis and prognosis in a timely manner. A promising approach is measuring the presence of biomarkers to detect tumors at various stages and determine their specific characteristics, thus allowing for more personalized treatment. MicroRNAs (miRNAs) serve a role in gene expression, primarily by interacting with messenger RNAs, and may be potential biomarkers for detecting cancer. They are detectable in tissues and blood, including plasma and/or serum, are stable and often tumor specific. Also, different miRNAs are associated with specific BC molecular subtypes. Triple-negative BC (TNBC) is a type of BC in which the primary targets for hormonal therapy are absent. It is an aggressive phenotype, which frequently metastasizes and is associated with an unfavorable prognosis. The present review focuses on circulating miRNAs in patients with TNBC, with an emphasis on their interaction with the immune response checkpoint genes PD-1, PD-L1 and CTLA4. Modulation and response of the immune system are of interest in cancer treatment due to the success of immunotherapy in the treatment of various neoplasms. Based on the findings of this literature review and the *in silico* analysis performed as part of this review, it is concluded that circulating hsa-miR-195 and hsa-miR-155 in TNBC interact with checkpoint genes involved in the

immune response. Further analysis of the expression of these circulating miRNAs and their association with prognosis in patients with TNBC treated with immunotherapy should be assessed to evaluate their possible use as non-invasive predictive biomarkers. In addition, functional studies to analyze biologically relevant targets in the development and prognosis of TNBC, which could be therapeutic targets, are also recommended.

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1. Introduction

Breast cancer (BC). BC is a heterogeneous malignant tumor that develops in the mammary gland. It has the highest incidence and mortality rates of all types of cancer in women worldwide, with >2,088,849 cases and 626,679 deaths reported in 2018, making it a primary global public health issue. The incidence of BC varies greatly with geographic region, but the death rates are less variable. For example, New Zealand and Australia reported an estimated age-standardized rate (ASR per 100,000) for incidence of 92.6 and 94.5, respectively, and a death rate (ASR) of 14.2 and 12.3, respectively, whereas for Southern and Central Asia the incidence and death ASRs were 25.9 and 13.6, respectively (1). Nearly 75% of all cases and 60% of deaths occur in high- and upper-middle-income countries. However, mortality rates among women <50 years old are higher in countries with low and lower-middle income compared with countries with high or upper-middle income (2). Regarding 5-year survival rates, there are significant differences between regions; in the United States and Australia the 5-year survival rate is estimated to be 90%, whereas in South Africa it is 40% (3). Not only are there epidemiological differences in BC, there are also differences in tumor biology that affect the prognosis and response to treatment.

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Classification of BC. BC is a highly heterogeneous disease that has been classified according to a variety of characteristics, such as histology, anatomical features (Tumor-Node-Metastasis staging system) (4), tumor grade and molecular traits. BC is classified histologically as non-invasive (*in situ*) or invasive by location. At present, 21 histological subtypes have been described, the most frequently occurring of which are ductal carcinoma (40-75% of cases) and lobular carcinoma (5-15% of cases) (5). BC is not only classified according to its clinical and histopathological characteristics, but also according to its prognosis and molecular traits (4).

In 2000, Perou *et al* (6) proposed the first molecular classification of BC based on global expression profiles. They described the subtypes estrogen receptor (ER)⁺/luminal-like, human epidermal growth factor receptor 2 (HER2) enriched, basal-like and normal-like (Table I). Subsequent studies confirmed the molecular subgroups, and new prognostic tests based on messenger RNA (mRNA) expression profiles in tumor tissue, such as MammaPrint[®], Oncotype Dx[®], PAM50[®], Endopredict[®] and 2-gene Breast Cancer Index, became available. These tests, however, are used for specific cases; for example, MammaPrint[®] is used for women in stage I and II ER⁺ to predict recurrence at 10 years (7). However, the use of expression profile tests has not been applied on a larger scale due to technical and financial limitations. As a consequence, immunohistochemical (IHC) biomarkers are used as an alternative method for classification (8). *Ad hoc* international agreements have developed and ratified guidelines for BC evaluation and classification according to the expression levels of the following biomarkers: Estrogen receptor, progesterone receptor, HER2 and Ki-67 proliferation factor (Table I) (9). IHC biomarkers are essential for classification, prognosis and therapeutic decision-making in BC. Tumors that are positive for hormonal receptors as well as those that express high levels of HER2 are susceptible to specific therapies and represent ~85% of all cases of BC. However, ~15% of BC cases are classified as triple-negative BC (TNBC) as they do not express hormonal receptors or have high expression levels of HER2; thus endocrine therapy and trastuzumab are inefficient treatments for this subgroup (10). In recent years, TNBC has been the subject of numerous clinical and biological studies with the aim of improving understanding of these tumors and improving patient outcomes.

Clinical and molecular characteristics of TNBC. TNBC is a subgroup of heterogeneous tumors that primarily affects younger patients of African and Hispanic ethnicity, although it has also been reported in patients of Asian origin (11). These tumors are generally larger than non-TNBC types, and patients who present with affected lymph nodes at diagnosis have a high risk of metastasis and mortality. The histological traits of these tumors include high grade and a high mitotic index, although low grade triple-negative neoplasms have been reported (12). Several studies have shown lower 5-year survival rates in patients with TNBC compared with patients with other molecular subtypes of BC (75 vs. 93%). Hispanic patients with TNBC have lower survival rates compared with those with other BC subtypes (62 vs. 80%) (13). It has been reported that 28-36% of patients with TNBC benefit from neoadjuvant chemotherapy based on a combination of anthracyclines, alkylating agents

and microtubule inhibitors (14). Gass *et al* (15) observed higher rates of complete pathological response (cPR) to taxane-platinum combinations (50%), compared with anthracycline-taxane combinations (41.8%) in patients with TNBC. The cPR rate in BRCA1/2-negative patients with TNBC to neoadjuvant therapy based on carboplatin is higher than that in HER2-positive patients with BC (16). Recurrence occurs, on average, after 1.6 years, with a recurrence rate of 25% in patients with TNBC, 74% of which is systemic recurrence (17). Among recurrent cases, 51% have distant metastases in multiple sites, most frequently in the lung (56%), bone (44%), liver (30%) and brain (22%) (17). This highlights the importance of identifying biomarkers to predict recurrence and metastasis as this may improve treatments and prognoses, thus increasing survival rates and times. There is a need for molecular analysis that recognizes alterations that may impact prognosis and prediction, and enables improvements and/or modifications in therapy to be made in a timely manner.

The first biological characteristic described for TNBC was its association with mutations in the BRCA genes, particularly BRCA1 (15). Subsequently, various clinical studies assessed the benefits of immunotherapy, particularly the use of programmed cell death 1 ligand 1 (PD-L1)/programmed cell death 1 (PD-1) inhibitors, in patients with TNBC (18). Although the rate of response to treatment in patients selected for PD-L1/PD-1 expression was greater (20-30%) than that for non-selected patients (10%), the cPR results for monotherapy were improved when immunotherapy was used in combination with other therapeutic agents (18). Improved clinical, pathological and molecular characterization of TNBC is essential to identify the cases in which specific systemic and/or targeted treatments may potentially benefit the patient.

To determine the molecular characteristics of TNBC, Lehmann *et al* (19) analyzed the gene expression profiles of 3,247 cases of TNBC. They identified six subgroups, which comprised two basal-like subgroups, in addition to immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like and luminal androgen receptor (LAR) subgroups. In further analyses of inflammatory infiltrate and stromal cells, TNBC tumors were stratified into four categories: Basal 1, basal 2, M and LAR (Table II) (20). These molecular subgroups exhibit differences in cPR to neoadjuvant anthracycline-taxane chemotherapy. In particular, significant differences were identified in the cPR for basal type 1 tumors compared with the other subgroups (41 vs. 31%; Table II). Similarly, Burstein *et al* (21) identified four molecular subtypes according to expression profiles and copy-number variations, which they termed LAR, M, basal-like immunosuppressor (BLIS) and basal-like immunoactivator (BLIA). Additionally, they found that the BLIA subtype was associated with improved overall survival compared with the other subtypes, whereas BLIS was associated with the worst overall survival. Furthermore, they identified potential therapeutic targets, which included androgen receptor, mucin 1, platelet-derived growth factor receptor and PD-1. The clinical, pathological and molecular heterogeneity of TNBC requires comprehensive analysis to achieve a consensus regarding its classification in order to determine suitable therapies and improve its prognosis.

Table I. Characteristics of molecular subtypes of breast cancer.

Molecular subtype	Immunohistochemical classification
Luminal A	ER ⁺ and/or PR ⁺ , HER2 ⁻ , Ki67<14%
Luminal B	HER2 ⁻ , ER ⁺ and/or PR ⁺ , Ki67≥14%; or HER2 ⁺ , ER ⁺ and/or PR ⁺ , any Ki67 ⁺
HER2	ER ⁻ , PR ⁻ , HER2 ⁺
Triple-negative	ER ⁻ , PR ⁻ , HER2 ⁻
Basal-like	ER ⁻ , PR ⁻ , HER2 ⁻

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Taken and modified from St. Gallen Consensus 2011 (9).

Another factor that has been the subject of various studies of BC is non-coding RNA, particularly microRNAs (miRNAs). However, non-coding RNAs are not considered in current molecular classification systems of TNBC despite their regulatory importance; nevertheless, numerous studies have identified miRNA signatures that may differentiate molecular subtypes (22-24).

2. miRNAs as biomarkers in BC

miRNAs dysregulation has been shown in the majority of different types of cancer, including BC (25). Some miRNAs are upregulated, whereas others are downregulated in BC tissues compared with healthy tissues, which highlights their potential as biomarkers.

PubMed (<https://ncbi.nlm.nih.gov/pubmed/>; accessed January 10, 2020) includes 5,351 reports regarding miRNAs in BC (search term, 'microRNA AND breast cancer'); 537 reports for 'microRNA AND triple negative breast cancer'; 207 reports for 'circulating microRNA AND breast cancer'; and 21 reports for 'circulating microRNA AND triple negative breast cancer'. The present review highlights the findings of various studies on miRNAs and BC, with a focus on circulating miRNAs and TNBC.

miRNAs. miRNAs are small RNAs that are primarily considered to function as regulators of mRNAs. miRNAs interact with complementary sequences in the 3' or 5' untranslated regions (UTRs) of mRNAs, thus blocking translation or promoting the breakdown of that mRNA (26). A single miRNA can interact with and regulate a wide range of mRNAs (27). Other noncanonical functions of miRNAs include increasing the translation of target mRNAs by recruiting protein complexes and increasing ribosome biogenesis (28). Since the discovery of miRNAs (29), our understanding of the regulation of gene expression and its role in a wide variety of biological functions has improved greatly.

The first miRNA repository (version 1.0) was published in December 2002 and included 218 precursor and mature miRNAs from five different species (<http://www.mirbase.org/>). The most recent database (version 22) contains

271 organisms with 48,860 mature miRNAs. For humans, 1,917 precursor sequences and 2,654 mature sequences have been reported (30).

Structural and functional characterization of miRNAs has prompted investigation of their roles in the development and progression of various types of cancer. Several studies have identified expression profiles of miRNAs in cancer and healthy tissues that suggest they function as oncogenes (onco-miRNAs) or tumor suppressor genes (25,31). It is estimated that >50% of miRNAs are contained within fragile areas or genomic regions associated with cancer (32), and ~60% of coding mRNAs are regulated by miRNAs (33).

miRNAs in TNBC. Interest in the molecular characterization of TNBC has more recently included miRNAs. The first report to indirectly include profiles of miRNA expression in BC, including TNBC, was published in 2008 (34). hsa-miR-210 was determined to be associated with a poor prognosis (34). A search of PubMed (accessed February 24, 2019; search term 'breast AND cancer AND triple negative AND miRNA') found 548 reports on this topic. The most notable of these are included in Tables III (23,35-62) and IV (36,37,46,63-65), where there is a brief description of some of the most representative meta-analyses.

Li *et al* (36) analyzed 1,299 BC tumours from the METABRIC database (https://www.cbioportal.org/study/summary?id=brca_metabric) and compared TNBC (n=204) with non-TNBC (n=1,095) samples to identify miRNAs associated with survival. They identified 376 differentially expressed miRNAs, of which only 10 showed a statistically significant association with poor survival in patients with TNBC: hsa-miR-301b, hsa-miR-181a-2-3p, hsa-miR-105-5p and hsa-miR-93-3p were upregulated; whereas hsa-miR-7-1-3p, hsa-miR-135a, hsa-miR-628-5p, hsa-miR-638, hsa-miR-3173 and hsa-miR-4245 were downregulated in TNBC. The authors suggested the use of these miRNAs as survival biomarkers exclusive to TNBC, as no association was found for these miRNAs in non-TNBC samples. To validate this hypothesis, an analysis was conducted using a different dataset (Gene Expression Omnibus; ID, GSE40267), confirming the upregulation of four miRNAs: hsa-miR-301b, hsa-miR-181a-2-3p, hsa-miR-105-5p and hsa-miR-93-3p, but the downregulation of only one miRNA, hsa-miR-628-5p. Functional analysis confirmed the roles of hsa-miR-93-3p and hsa-miR-105 in chemoresistance to cisplatin. It was suggested that this resistance is caused by activation of the Wnt/β-catenin pathway, via regulation of secreted frizzled-related protein 1 (36).

Naorem *et al* (65) identified 255 differentially expressed miRNAs between TNBC and non-TNBC tumors, of which 125 were upregulated and 130 were downregulated in TNBC compared with in non-TNBC. The miRNAs with the highest levels of expression were hsa-miR-135b-5p, hsa-miR-18a-5p, hsa-miR-9-5p and hsa-miR-522-3p. By contrast, hsa-miR-190b and hsa-miR-449a were the most significantly downregulated miRNAs. hsa-miR-522-3p had the highest number of predicted target genes with 1,618, and hsa-miR-449a had the least with 500. In a pathway enrichment analysis, the Wnt and RAS/MAPK signaling pathways were the most frequently identified pathways. Activation of the Wnt/β-catenin pathway is associated with poor prognosis in BC and this activation may be independent of CTNNB1 mutations (66). Activation

Table II. Molecular classification of triple-negative breast cancer.

Type	Characteristics	Distribution (%)	cPR (%)
Basal 1	High grade, early stages	35	41
Basal 2	Growth factors, myoepithelial markers, metaplastic carcinomas	22	18
Luminal androgen receptor	Older women, positive lymph nodes, bone metastasis, infiltrating lobular carcinomas	16	29
Mesenchymal	Pulmonary metastasis, metaplastic carcinomas	25	38
Unclassified	NA	2	NA

Characteristics are reflective of the molecular type; they are those most commonly presented for the specified type, but not exclusive for it. Modified from Lehmann *et al* (20). NA, not applicable; cPR, complete pathological response in cohort treated with neoadjuvant chemotherapy.

of the RAS/MAPK pathway has been associated with reduced lymphocyte infiltration in TNBC, and also predicts the response to immune checkpoint inhibitors (67).

Studies based on information available in public databases have focused on the interactions and competition networks of various types of RNA. Koduru *et al* (63) analyzed different small non-coding RNAs and long non-coding RNA (lncRNAs) using next generation sequencing data from TNBC and healthy tumor adjacent tissues. They identified 55 differentially expressed miRNAs, of which hsa-miR-301b, hsa-miR-183, hsa-miR-18a, hsa-miR-3074 and hsa-miR-96 were upregulated in TNBC compared with the adjacent non-tumor tissue. By contrast, hsa-miR-122, hsa-miR-204, hsa-miR-135a-2, hsa-miR-139 and hsa-miR-215 were downregulated in TNBC compared with the adjacent non-tumor tissue. Changes in the levels of expression associated with clinical stage were also assessed, and six miRNAs (hsa-miR-135a-2, hsa-miR-16-1, hsa-miR-215, hsa-miR-301b, hsa-miR-486 and hsa-miR-517b) were found to be associated with stages I-III. The number of dysregulated miRNAs was greater in the earlier stages of cancer, dysregulated small non-coding RNAs were enriched in chromosomes 14, 11 and 1, and the most commonly affected pathway was the TGF- β signaling pathway (63).

Yuan *et al* (37) also identified RNAs that were differentially expressed between TNBC and the healthy adjacent tissue, and found that hsa-miR-105-1, hsa-miR-519a-1, hsa-miR-105-2, hsa-miR-767 and hsa-miR-516a-1 were upregulated, while hsa-miR-486-1, hsa-miR-486-2, hsa-miR-4732, hsa-miR-139 and hsa-miR-451a were downregulated. They later correlated five modules with a weighted gene co-expression network analysis, including 22 hub genes, and identified 11 miRNAs associated with those hub genes. Of these miRNAs, the following were found to form an important part of a competing endogenous RNA network: hsa-miR-183, hsa-miR-9 and hsa-miR-145. The authors also identified seven upregulated RNAs, namely AKAP12, FOS, EMX2OS, MYCNOS, RP11-542B15.1, hsa-miR-9 and hsa-miR-183 that were associated with a poor prognosis, whereas another four upregulated mRNAs, namely hsa-miR-145, LINC00461, RP11-576D8.4 and RP11-496D24.2, were associated with a favorable prognosis. It is important to note that clinically relevant miRNAs were not among the top unregulated miRNAs.

Yang *et al* (46) constructed and analyzed an interaction network comprising miRNAs, lncRNAs and mRNAs for

TNBC using RNA-Seq data for tumors and healthy tissue from The Cancer Genome Atlas (<https://cancer.gov/tcga>). They identified 114 differentially expressed miRNAs. The five most significantly upregulated miRNAs were hsa-miR-105-2, hsa-miR-105-1, hsa-miR-1269a, hsa-miR-767 and hsa-miR-1269b; whereas hsa-miR-133a-2, hsa-miR-133a-1, hsa-miR-1-1, hsa-miR-1-2 and hsa-miR-133b were the most significantly downregulated genes. In addition, the upregulation of hsa-miR-135b and hsa-miR-216a expression was positively correlated with overall survival, whereas the upregulation of hsa-miR-3610 and hsa-miR-7706 expression was negatively correlated. As part of the competition analysis for endogenous RNAs (RNAs-lncRNAs, mRNA), the authors defined a panel of four lncRNAs (LINC00452, ST7-AS2, LINC00461 and LINC00517), three miRNAs (hsa-miR-145, hsa-miR-144 and hsa-miR-137), four mRNAs (EZH2, FSCN1, SOX11 and KLF4), which were validated by reverse transcription-quantitative PCR in independent samples. The expression of LINC00461 or LINC00517 was found to negatively correlate with the expression of hsa-miR-144 or hsa-miR-137, suggesting regulation by competition and a possible role of these transcripts in the development of TNBC.

A recent review of 212 reports by Piasecka *et al* (68) reported that several miRNAs that are dysregulated in TNBC are associated with epithelial-mesenchymal transition (EMT); some of these, hsa-miR-10b, hsa-miR-21, hsa-miR-29, hsa-miR-9, hsa-miR-221/222 and hsa-miR-373 are upregulated, whereas others, including hsa-miR-145, hsa-miR-199a-5p, hsa-miR-200 family, hsa-miR-203 and miR-205 are downregulated in TNBC. In addition, Tang *et al* (64) identified 48 upregulated miRNAs and 74 downregulated miRNAs in TNBC, based on data from the database of differentially expressed miRNAs in human cancers (<https://www.picb.ac.cn/dbDEMCI/>).

Numerous studies have highlighted the importance of detecting circulating miRNAs that are unregulated in TNBC tissues, as they could be used as non-invasive biomarkers. These studies are presented in the next section.

3. Circulating miRNAs in BC

Diagnostic or follow-up tests in BC require biopsies to be obtained; however, the acquisition of samples may involve overcoming certain technical difficulties, particularly regarding small lesions, such as the challenge of obtaining a sample that

Table III. Clinical and functional characteristics of microRNAs in TNBC.

Authors, year	MicroRNA expression level ^a		Outcome, functions and/or targets	Refs.
	High hsa-miR-	Low hsa-miR-		
Bar <i>et al.</i> , 2017	210		Expressed in tumor epithelial and CD45 ⁺ LCA ⁺ cells	(35)
Li <i>et al.</i> , 2017	93-3p, 105		Associated with cisplatin chemoresistance and metastasis promotion. Wnt/ β -catenin signaling activation	(36)
Yuan <i>et al.</i> , 2017	9, 183	145	Poor survival	(37)
Yang <i>et al.</i> , 2018	195, 204, 488, 497	93, 105, 340	Good prognosis. 195 and 497 target CD274 (PD-L1)	(38)
Wu <i>et al.</i> , 2018		199b-5p	Inhibition of proliferation, migration and invasion. Targets DDR1	(39)
Mokhlis <i>et al.</i> , 2019		873	Poor survival. Targets KRAS	(40)
Meng <i>et al.</i> , 2018		645	Decreased invasive growth. Targets uPA	(41)
Hajalirezay Yazdi <i>et al.</i> , 2018		34a	Targets MET and AXL	(42)
Bayraktar <i>et al.</i> , 2018		34a	Poor prognosis. Targets eEF2K and FOXM1	(43)
Guo <i>et al.</i> , 2018		508-3p	Local and distant metastasis. Targets ZEB1 and suppresses the EMT process	(44)
Jinesh <i>et al.</i> , 2018	Chromosome 19 miRNA cluster (C19MC)		Associated with TNBC phenotype	(45)
Yang <i>et al.</i> , 2018	3610, 7706	135b, 216a	Poor survival	(46)
Song <i>et al.</i> , 2018	301b		Promotes proliferation and apoptosis resistance. Targets CYLD	(47)
Romero <i>et al.</i> , 2018		342-3p	Metabolic reprogramming. Targets MCT1	(48)
Razaviyan <i>et al.</i> , 2018		96, 3182, 557	Targets mTOR and S6K1	(49)
García-Vázquez <i>et al.</i> , 2019		143	cPR in neoadjuvant therapy	(50)
Schwarzenbacher <i>et al.</i> , 2019		1287-5p	Poor prognosis. Targets PIK3CB.	(51)
Wang <i>et al.</i> , 2019		136, 4324, 125b, 381, 145	Poorest prognosis in patients with loss of PTEN	(52)
Cantini <i>et al.</i> , 2019		199a/214	Related to EMT-like phenotype	(53)
Wong <i>et al.</i> , 2019		4417	Poor survival.	(54)
Zhao <i>et al.</i> , 2019		4306	Poor survival, metastasis. Targets SIX1, Cdc42 and VEGFA	(55)
Kolesnikov <i>et al.</i> , 2019	20a, 221		Biomarkers of TNBC	(23)
Wang <i>et al.</i> , 2019		890	Inhibition of proliferation and invasion. Targets CD147	(56)
Liang <i>et al.</i> , 2019		5590-3p	Inhibition of proliferation, migration and EMT. Targets YY1.	(57)
Wang <i>et al.</i> , 2019	377-3p		Longer OS. Targets HOXC6	(58)

Table III. Continued.

Authors, year	MicroRNA expression level ^a		Outcome, functions and/or targets	Refs.
	High hsa-miR-	Low hsa-miR-		
Son <i>et al.</i> , 2019	374a-5p		Tumor progression. Targets ARRB1	(59)
Tormo <i>et al.</i> , 2019	449a		Better survival, increased sensitivity to doxorubicin	(60)
Shi <i>et al.</i> , 2019		124	Suppresses proliferation and invasion. Targets STAT3.	(61)
Liu <i>et al.</i> , 2019		4458	Suppresses cell proliferation and promotes cell apoptosis. Targets SOCS1.	(62)

^aLevel of expression found in TNBC relative to other molecular subtypes or adjacent non-tumoral tissues. The terms high and low refer to genes that are upregulated or downregulated, respectively, as compared with the control group in each study. PD-L1, programmed cell death 1 ligand 1; EMT, epithelial-mesenchymal transition; cPR, complete pathological response; TNBC, triple-negative breast cancer; OS, overall survival.

represents the entire heterogeneity of the tumor, image-guided biopsies, patient discomfort, infrastructure requirements and cost. The assessment of non-invasive biomarkers, such as those present in the circulation, would reduce these inconveniences to the patient and limitations in obtaining the samples, and may even be used preventively before clinical manifestations of the disease are observed. Another advantage of circulating biomarkers is that it would be relatively easy to obtain multiple samples over a certain time period to monitor various parameters of progression or regression of the disease following therapeutic intervention. Additionally, as miRNAs can be identified in the urine, blood, plasma, serum, saliva and feces, they are readily accessible non-invasive biomarkers for the diagnosis and prognosis of cancer and its follow-up (69).

The first study to describe the expression of circulating miRNAs in BC was published in 2008 (34). In that year, other studies assessed the presence of circulating miRNAs in patients with various types of cancer (70,71). Several mechanisms for the release of circulating miRNAs have been proposed, which involve microvesicles, exosomes, circulating tumor cells and release from apoptotic bodies (72). Circulating miRNAs are stable, which makes them suitable as potential biomarkers in biological fluids. Their stability in these fluids results from protection against RNase-induced degradation by lipoproteins, argonaute protein 2 and microvesicles. They are also resistant to changes in pH and temperature, long storage periods and multiple freeze/thaw cycles. Additionally, as circulating miRNAs have been shown to be stable post sample collection, they can consequently be identified and quantified without the time interval between sampling and testing biasing the results (73,74). One of the most relevant applications for the detection of circulating miRNAs is as an early diagnostic tool, as well as a predictive biomarker of recurrence and metastasis.

A search of PubMed found 306 publications on circulating miRNAs in PubMed, although only 25 of those were studies specific to TNBC (accessed August 5, 2019; search term, 'circulating microRNAs AND triple negative breast cancer').

Circulating miRNAs in TNBC. A summary of circulating miRNAs in TNBC is presented in Table III, and a description of the most relevant studies is presented hereinafter.

One of the first studies to analyze the expression of a circulating miRNA in patients with TNBC was published by Sahlberg *et al.* (75). The authors first analyzed a small group of TNBC samples (10 recurrent and 10 non-recurrent cases) and their association with recurrence using the Exiqon platform, and then validated the findings in an independent cohort of 70 cases. A total of nine differentially expressed miRNAs were initially identified in the serum of patients with recurrent TNBC compared with non-recurrent TNBC (hsa-miR-18b-5p, hsa-miR-20a-5p, hsa-miR-30d-5p, hsa-miR-32-5p, hsa-miR-101-3p, hsa-miR-103a-3p, hsa-miR-107, hsa-miR-223-3p and hsa-miR-652-3p). Of these miRNAs, the upregulation of hsa-miR-18b, hsa-miR-103, hsa-miR-107 and hsa-miR-652 was found to be associated with reduced recurrence-free survival and low overall survival rates, and thus these may be specific biomarkers for predicting the outcomes of patients with TNBC (75).

Kahraman *et al.* (76) analyzed the expression profiles of miRNAs in the blood cells of patients with basal-like TNBC

Table IV. Differentially expressed microRNAs in TNBC tissues.

Authors, year	Samples	MicroRNAs differentially expressed in tumor vs. non-tumor			Comment	Refs.
		Upregulated hsa-miR-	Downregulated hsa-miR-			
Koduru <i>et al.</i> , 2017	24 TNBC/14 adjacent normal tissues	18a, 96, 183, 301b, 3074	122, 135a-2, 139, 204, 215		55 microRNAs aberrantly expressed (P<0.005) in TNBC; 8 microRNAs upregulated and 47 downregulated	(63)
Yuan <i>et al.</i> , 2017	116 TNBC/11 adjacent normal tissues	105-1, 105-2, 516a-1, 767, 519a-1	139, 451a, 486-1, 486-2, 4732		miRNA-Seq data from TCGA. 67 DE microRNAs by limma analysis: 46 upregulated and 21 down regulated	(37)
Yang <i>et al.</i> , 2018	111 TNBC/104 non-cancerous tissues	105-1, 105-2, 767, 1269a, 1269b	133a-1, 133a-2, 1-1, 1-2, 133b		miRNA-Seq data from TCGA. 114 DE microRNAs in TNBC; 91 upregulated and 23 downregulated	(46)
Tang <i>et al.</i> , 2019	209 datasets from GEO and TCGA	7, 9, 15a, 15b, 16, 17, 18a, 18b, 19a, 24, 25, 29b, 32, 93, 96, 98, 105, 106b, 107, 125a-5p, 128, 130b, 141, 142-3p, 142-5p, 146a, 155, 181b, 182, 183, 200a, 200b, 200c, 210, 219-5p, 301a, 301b, 324-5p, 331-3p, 340, 429, 454, 484, 590-5p, 629, let-7d	10b, 99a, 100, 133b, 134, 139-3p, 139-5p, 140-3p, 143, 145, 188-5p, 193a-5p, 195, 198, 202, 204, 299-3p, 335, 370, 376a, 376c, 378, 381, 451, 452, 483-3p, 483-5p, 486-5p, 497, 498, 513a-5p, 513b, 513c, 516a-5p, 520b, 520e, 542-5p, 551b, 557, 564, 572, 601, 617, 623, 636, 638, 654-5p, 659, 662, 665, 671-5p, 708, 877, 887, 936, 939, 1225-3p, 1225-5p		dbDEMC data for DE microRNAs in TNBC, 48 upregulated and 74 downregulated	(64)
Li <i>et al.</i> , 2017	204 TNBC/1,095 non-TNBC	93-3p, 105-5p, 181a-2-3p, 301b,	7-1-3p, 135a, 628-5p, 638, 3173, 4245		METABRIC data: 376 of 616 microRNAs were significantly DE in TNBC; 151 upregulated and 225 downregulated	(36)
Naorem <i>et al.</i> , 2019	TNBC data from PubMed and Scopus, GEO database, miRBase v21	9-5p, 18a-5p, 135b-5p, 522-3p	190b, 449a method.		Meta-analysis performed using a novel RRA	(65)

MicroRNAs differentially expressed in TNBC vs. non-TNBC. In the non-TNBC tissues, estrogen receptor, progesterone receptor and/or human epidermal growth factor receptor 2 were positive. DE, differentially expressed; TNBC, triple-negative breast cancer; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; dbDEMC: Database of differentially expressed miRNAs in human cancer; RRR, robust rank aggregation.

tumors (cytokeratin 5+) prior to and following neoadjuvant chemotherapy with carboplatin and paclitaxel to identify miRNAs that may predict cPR. The analysis demonstrated that although the expression levels of hsa-miR-34a-5p and hsa-miR-34b-5p in patients with TNBC prior to chemotherapy were comparable with those in healthy controls, their expression increased following neoadjuvant chemotherapy. By contrast, the expression of hsa-miR-664b-3p was reduced following therapy. Notably, the expression levels of hsa-miR-144-3p, hsa-miR-144-5p, hsa-miR-126-5p and hsa-let-7d-5p were higher in the patients with TNBC compared with the controls prior to chemotherapy, but were similar to those of the controls following therapy. Despite the differences observed in the expression levels of various miRNAs prior to and following chemotherapy, none of the miRNAs were identified to be predictive biomarkers of cPR. Nevertheless, an impact of these miRNAs on other outcomes, such as metastasis and overall survival, was observed.

Braicu *et al* (77) studied TNBC tumors and BC tumors positive for hormonal receptors and HER2 based on data available in The Cancer Genome Atlas, and validated their findings in plasma samples. The authors identified differentially expressed miRNAs between tumors and normal tissues, and analyzed the overlap of these differentially expressed miRNAs with those in plasma. Their discoveries included the upregulation of hsa-miR-200b, hsa-miR-200c and hsa-miR-210 in plasma and tumors, as well as the downregulation of hsa-miR-29c in plasma in patients with TNBC compared with BC positive to hormonal receptors. The hsa-miR-29c family has a role in EMT regulation and is considered an early biomarker for the prediction of metastasis (78).

According to the descriptions listed in Tables IV (36,37,46, 63-65) and V (36,75-92), few miRNAs have been identified consistently in different studies.

4. Checkpoints and miRNAs in TNBC

Immune response in TNBC. Immune responses in cancer have been studied for a number of decades. The first studies on this were published in the 1950s (93-95). Several studies in the 1970s described tumor-infiltrating lymphocytes (TILs) and their possible involvement in the development of neoplasms (96-98). The number of publications on cancer and immune response significantly has gradually grown over time, generating a vast field of knowledge and information on the relationship between immunity and cancer.

A seminal report highlighting the role of the immune system in cancer is that of Hanahan and Weinberg (99), in which they established evasion of immune response as one of the hallmarks of cancer.

Currently, modulation of the immune response is a major focal point in cancer treatment due to the success of immunotherapy in the treatment of malignancies, such as skin and lung cancer, which has led to the use of immunotherapy for other neoplasms, including BC (100). Similarly, the Immunoscore, which was first developed for colon cancer, has been adopted for use in other types of cancer and its inclusion in the traditional classification of cancer has been proposed for prognostic and predictive purposes (101). The Immunoscore is based on quantification of lymphocyte populations (CD3/CD45RO,

CD3/CD8 or CD8/CD45RO) in the marginal and central regions of tumors (92).

Several studies have described the predictive value of inflammatory infiltrates in BC, particularly for TNBC and HER2 positive BC. Consequently, recommendations have been made for the systematic assessment of BC with TILs (102). Early studies assessed Immunoscore in BC, in addition to its contribution to pro- or antitumor activity (103). The evaluation of lymphocyte infiltrates using hematoxylin and eosin staining has been shown to have predictive and prognostic value in TNBC. It has been estimated that tumors with >50% TILs may benefit from neoadjuvant chemotherapy, as evaluated by cPR, even when tissues from core needle biopsy specimens are used for TIL quantification (103-106). In this regard, the use of platinum-based agents improves cPR (107). The cPR has been reported to increase from 37 to 52% when platinum-based agents are used in the treatment of TNBC, with the exclusion of TNBC with mutations; however, hematological toxicity is increased (108). The impact of TILs has only been observed in TNBC and HER2-positive tumors, whereas in other molecular subtypes and low-grade tumors, the impact is small or non-existent (109).

The aforementioned molecular classification of TNBC performed by Lehmann *et al* (19) includes an IM subgroup, characterized by enrichment with genes associated with immune processes, including cytotoxic T-lymphocyte associated protein 4 (CTLA4), interleukin (IL)-12, natural killer cell (NK) cell and IL-7 pathways, antigen processing and presentation, NF- κ B and TNF pathways, T-cell transduction and NK-mediated cytotoxicity. A study notably observed that the overlap of genes from this subgroup with the molecular signature for medullary cancer is associated with a favorable prognosis regardless of advanced histological grade (110). However, in a later molecular classification, the number of TNBC subgroups was reduced from six to four, with removal of the IM subgroup as it resulted from enrichment with infiltrating lymphocytes (20). This posed the question of whether this molecular subtype was the expression profile of TILs or tumor cells. The authors found that TILs were more abundant in the IM subgroup than in the other subgroups, and that there was a correlation between the number of lymphocytes and the IM component of each tumor. Immune checkpoint regulators, such as CTLA4, CD274 and PDCD1, were observed amongst the genes identified in the IM subtype, and the IM subtype was associated with improved overall survival and recurrence-free survival in comparison with all other TNBC subtypes (20).

These findings have contributed to interest in the development of a mode of immunotherapy for TNBC. The first studies of immunotherapy for TNBC showed a response rate to pembrolizumab of 18%, which was very similar to the initial percentage of patients classed as possessing the IM subtype (20%) (18,103). Clinical trials using immunotherapy for the treatment of TNBC have been reviewed by Nathan and Schmid (111), and the US Food and Drug Administration has approved the use of atezolizumab (Tecentriq, Genentech/Roche) plus the chemotherapy nab-paclitaxel (Abraxane, Celgene) as first line treatment for unresectable, metastatic, PD-L1-positive TNBC (112).

Increased immune activation through interferon type I (IFN-I) signaling has been shown to increase the efficacy of

Table V. Circulating microRNAs in TNBC.

First author, year	MicroRNA expression level		Clinical associations	Biological functions	(Refs.)
	High hsa-miR-	Low hsa-miR-			
Heneghan <i>et al</i> , 2010	Let-7				(79)
Zeng <i>et al</i> , 2013		30a	Greater sensitivity and specificity for diagnosis vs. CEA and CA15-3		(80)
^a Eichelser <i>et al</i> , 2013	34a, 93, 373				(81)
Eichelser <i>et al</i> , 2014	373exosomal		Aggressiveness	Downregulation of ER, apoptosis inhibition	(82)
Sahlberg <i>et al</i> , 2015	18b, 103, 107, 652		Poor OS and RFS, probability of tumor relapse	Target ER, EMT, DNA repair, metastasis	(75)
Shin <i>et al</i> , 2015		199a-5p	Tumor stage	EMT. Targets GRP78	(83)
^a Mishra <i>et al</i> , 2015	106a-5p, 454-3p	195-5p, 495	Diagnosis in PBMCs	Targets HB-EGF, EREG	(84)
^a Huo <i>et al</i> , 2016	21-5p, 194-5p, 205-5p, 375,	376c-3p, 382-5p, 411-5p	Recurrence		(85)
Madhavan <i>et al</i> , 2016	144, 200a, 200b, 200c 210,		Metastasis and OS predictors		(78)
Chen <i>et al</i> , 2016	215, 486-5p	199a-5p		Proliferation inhibition, apoptosis, EMT. Targets PIK3CD	(86)
Gao <i>et al</i> , 2017 ^b	155		Early stage	Targets RAD51	(87)
Li <i>et al</i> , 2017	93-3p, 105		Biomarker for TNBC, poor survival	Targets SFRP1, activates WNT	(36)
Zeng <i>et al</i> , 2017		34a, 34c	Poor OS, distant metastasis	Targets GIT1, FRA-1	(88)
Qattan <i>et al</i> , 2017	195		Early diagnosis	Targets GLUT3	(89)
Swellam <i>et al</i> , 2019		335	Poor DFS		(90)
Kahraman <i>et al</i> , 2018	34a-5p, 34b-5p	664b-3p	After NCT		(76)
Niedzwiecki <i>et al</i> , 2018		200c	Metastasis predictor		(91)
Braicu <i>et al</i> , 2018 ^a	200b	29c	Brain metastasis, poor survival	EMT regulation, metastasis	(77)
Bai <i>et al</i> , 2018	20a-5p		Promotes the migration and invasion of TNBC cells <i>in vitro</i>	Targets RUNX3 ^{low}	(92)

^aNot exclusive for TNBC; also applicable to other breast cancer subtypes. ^bHormonal receptor negative. The terms high and low refer to genes that are upregulated or downregulated, respectively, as compared with the control group in each study. CEA, tumor marker carcinoembryonic antigen; CA15-3, tumor marker cancer antigen 15-3; ER, estrogen receptor; EMT, epithelial-mesenchymal transition; OS, overall survival; RFS, relapse-free survival; DFS, disease-free survival; PBMCs, peripheral blood mononucleated cells; NCT, neoadjuvant chemotherapy; TNBC, triple-negative breast cancer.

anti-PD-1 immunotherapy (113). Thus, the stratification of patients with the IM subtype and/or a high rate of TILs may help to identify patients for whom checkpoint inhibitors would be more effective.

Based on the success of immunotherapy in melanoma and lung cancer, the association between miRNAs and the immune response in BC is worthy of consideration, particularly with regard to the use of checkpoint inhibitors (114).

PD-1 and circulating miRNAs in TNBC. PD-1 is a cell surface protein coded for by the *PDCD1* gene; it belongs to the immunoglobulin superfamily and the CD28 family of proteins. It is expressed in antigen-specific effector T cells and memory T cells. When it binds to its ligands (PD-L1 or PD-L2), it suppresses T-cell proliferation, the production of cytokines such as IFN- γ and cytotoxic activity, thus resulting in immunosuppression and immunotolerance. The level of PD-1 expression in T cells is regulated by the inflammatory response and infection. PD-L1 is expressed in a variety of cell types and tissues such as solid or hematologic neoplasms; when PD-L1 is expressed in cancer cells it suppresses immunosurveillance. Consequently, various drugs have been developed to counter these effects (115).

The molecular mechanisms that direct the regulation of PD-1 expression, including *cis*-DNA regulatory elements, transcription factors, epigenetic regulation and changes in DNA methylation patterns and histone modifications, have been described (116); nevertheless, how miRNA-mediated regulation occurs remains largely unknown. The miRDB database (<http://mirdb.org>) predicts 61 miRNAs that could potentially regulate *PDCD1*. Based on the miRNAs predicted to bind to the *PDCD1* gene in two databases (miRDB and TargetScan version 7.2; <http://www.targetscan.org>; accessed June 25, 2019), we found that the circulating miRNA hsa-miR-195-5p, previously detected in TNBC, was a likely candidate as a regulator of *PDCD1* expression (target score 76, context score percentile 86).

The downregulation of hsa-miR-195-5p has previously been described in the peripheral blood mononuclear cells of patients with TNBC; however, only a limited number of patients was included in this analysis (84); by contrast, Qattan *et al.* (89) observed the upregulation of hsa-miR-195 in patients with TNBC in a study with a larger number of samples. However, Yang *et al.* (38) found that the higher levels of hsa-miR-195 in BC were associated with improved prognosis. Nonetheless, PD-L1 has been identified as the target of hsa-miR-195.

PD-L1 and circulating miRNAs in TNBC. PD-L1 is a cell membrane protein that binds to PD-1 on effector T cells and transduces immunosuppressor signals. Expression of PD-L1 on the surface of tumor cells is critical for these cells to escape immunosuppression (117).

Based on the data in Table V and PL-D1 as the checkpoint gene for immune response, we performed an *in silico* analysis using DIANA-Tools (<http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software>) and miRTarBase v7.0 (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>). The following results were obtained: PD-L1 gene is regulated by several miRNAs and has 20 conserved target sites for hsa-miR-140-3p.2, hsa-miR-382-3p*, hsa-miR-320c,

hsa-miR-320d, hsa-miR-4429, hsa-miR-320b, hsa-miR-320a, hsa-miR-377-3p, hsa-miR-20b-5p, hsa-miR-519d-3p, hsa-miR-106b-5p, hsa-miR-93-5p*, hsa-miR-526b-3p, hsa-miR-17-5p, hsa-miR-106a-5p*, hsa-miR-20a-5p*, hsa-miR-142-5p, hsa-miR-5590-3p, hsa-miR-653-5p and hsa-miR-155-5p* (TargetScan version 7.2; http://www.targetscan.org/vert_72/; accessed on June 25, 2019), as well as poorly conserved target sites for >900 hsa-miRNAs.

A study examining the effect of PD-L1 expression in human dermic lymphatic endothelial cells (HDLECs) has been conducted, in which miRNA-mediated regulation of the PD-L1 gene was observed (118). In that study, the treatment of HDLECs with IFN- γ and TNF- α proinflammatory cytokines synergistically upregulated PD-L1, and altered the expression of several miRNAs that are able to reduce the expression of PD-L1. The miRNA found to be the most strongly upregulated was hsa-miR-155, which serves a key role in the immune system and cancer. Furthermore, the 3'-UTR of PD-L1 was demonstrated to have two functional binding sites for hsa-miR-155, and endogenous hsa-miR-155 was shown to control the kinetics and PD-L1 levels induced by treatment with IFN- γ and TNF- α . Similar findings in dermal fibroblasts confirm that a IFN- γ and TNF- α -induced hsa-miR-155/PD-L1 pathway is not exclusive to HDLECs. These results show that hsa-miR-155 contributes to the inflammation-induced control of PD-L1 expression in primary cells (118).

There are various studies that mention hsa-miR-155; a search of PubMed (accessed January 10, 2020) found 191 reports for 'miR-155 AND breast cancer'; 22 reports for 'miR-155 AND triple negative breast cancer'; and 3 reports for 'circulating miR-155 AND triple negative breast cancer'. Together, these studies demonstrate the well-acknowledged role of hsa-miR-155 in the early detection, tumor initiation, and cancer development and progression of BC (81,87,119).

CTLA4 and miRNAs in TNBC. CTLA4 is a transmembrane type 1 protein with 223 amino acids (120) coded for by the *CTLA4* gene. It belongs to the immunoglobulin receptor superfamily of proteins, which also includes CD28, ICOS, PD-1, BTLA and TIGIT (121). CTLA4, PD-1, BTLA and TIGIT are negative regulators that are crucial to T-cell mediated immunity (120,121).

In T cells, CTLA4 and CD28 bind to the same ligands (CD80 and CD86) on antigen-presenting cells, but have opposing effects; the interaction of CTLA4 with its ligands inhibits the T-cell response, whereas when CD28 binds to its ligands the T-cell response is activated. CTLA4 has greater affinity for CD80/CD86 than does CD28 (122).

The *CTLA4* gene is regulated by several miRNAs and has highly conserved target sites for hsa-miR-155-5p, hsa-miR-496.1 and hsa-miR-142-3p.2, in addition to >400 poorly conserved target sites (based on DIANA-miRPath and miRTarBase; accessed September 15, 2017). Of those highly conserved miRNAs, hsa-miR-155 is known to be a circulating hsa-miRNA in TNBC (87).

5. Conclusions

Circulating miRNAs are molecules with significant diagnostic, prognostic and even predictive potential as biomarkers

for cancer, due to the relative ease and non-invasive means by which they can be obtained. Nevertheless, it is necessary to standardize pre-analytical and analytical details to obtain results that have greater reproducibility, specificity and sensitivity, and to improve patient selection. hsa-miR-195 has been detected in the circulation and interacts with immune checkpoint molecules, as shown by the predictive analysis that identified PDCD1 as a potential target, and its regulation of PD-L1 has been demonstrated. Similarly, hsa-miR-155 also interacts with checkpoint genes involved in the immune response and has been reported to be a circulating miRNA during the early stages of TNBC. Analyzing the expression of these circulating miRNAs in greater detail is thus suggested, as well as their association with clinical responses in patients with TNBC treated with immunotherapy, to assess their potential role as predictive non-invasive biomarkers. Further studies of miRNA-mRNA-protein interactions and the signaling and/or metabolic pathways in which these miRNAs participate are also required.

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Authors' contributions

PPS and HAVS wrote, edited and critically revised the manuscript. MERT was responsible for manuscript design, obtaining financial support, as well as critical revision, editing and writing of the manuscript. All authors read and approved the final manuscript. They also agree to be accountable for all aspects of the work while ensuring that questions related to the accuracy or integrity of any of its parts are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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