Candidate circulating microRNAs as potential diagnostic and predictive biomarkers for the monitoring of locally advanced breast cancer patients

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Abstract
This study aimed at investigating the expression of candidate microRNAs (miRs), at initial diagnosis, during neoadjuvant chemotherapy, and after the tumor resection in locally advanced breast cancer patients. Plasma samples were collected from locally advanced breast cancer patients (n = 30) and healthy subjects (n = 20) for the detection of candidate miRs' expression using the real-time quantitative polymerase chain reaction. At initial locally advanced breast cancer diagnosis, the expression of miR-21, miR-181a, and miR-10b was significantly increased, whereas that of miR-145 and let-7a was significantly decreased, compared to the healthy individuals. The diagnostic accuracy of miR-21 was superior to both carcinoembryonic antigen and carcinoma antigen 15-3 as diagnostic biomarkers for locally advanced breast cancer. By the end of the treatment, the expression of altered miRs rebound to control values. The expression levels of candidate plasma miRs are useful diagnostic biomarkers, as well as monitoring a proper response for locally advanced breast cancer patients to the treatment. Furthermore, miR-10b and miR-21 can be considered as predictive biomarkers for progression-free survival.

Keywords
MicroRNAs, locally advanced breast cancer, neoadjuvant chemotherapy, tumor resection, real-time polymerase chain reaction

Introduction
Breast cancer is the most commonly diagnosed cancer in women worldwide, and the principal cause of cancer-related mortality in over 100 countries.\textsuperscript{1,2} In Egypt, the incidence of breast cancer ranks second after liver cancer, with 23,081 newly diagnosed cases in 2018, representing 35.1\% of all female cancers (23,081/65,693), and 9,254 deaths that represent 23.8\% of all female cancer deaths (9,254/38,814).\textsuperscript{3} Locally advanced breast cancer (LABC) is classified as stage III breast cancer, according to the guidelines from the US National Comprehensive Cancer Network, without distant metastasis.\textsuperscript{4}

MicroRNAs (miRs) are endogenous short non-coding transcripts (~23 nucleotides RNAs) that play important gene-regulatory roles in animals and plants by pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression.\textsuperscript{5} MiRs act as potential oncogenes (oncomiRs) or tumor suppressors...
and their altered expression in biological fluids was linked to the existence of many cancers, including breast cancer.6

MiR-10b, which is encoded by a highly conserved genomic region located near the homeobox D (HOXD) cluster on chromosome 2, is linked to a range of functions, including regulation of angiogenesis and promotion of cell invasion.7 It induces its tumor-promoting properties primarily through direct interaction with downstream targets, including homeobox D10 (HOXD10), neurofibromatosis type 1 (NF1), Krüppel-like factor 4 (KLF4), and phosphatase and tensin homolog (PTEN). Inhibiting miR-10b expression in breast cancer models can effectively suppress cancer proliferation, migration, and invasion.8–11 MiR-21 is located on chromosome 17 (17q23.1) in the 11th intron of the transmembrane protein 49 (TMEM49) gene. MiR-21 is an oncomiR, which promotes the development of tumors through inhibiting tumor suppressor genes. It targets many different gene transcripts, such as programmed cell death 4 (PDCD4), PTEN, hypoxia-inducible factor (HIF1α), tissue inhibitor of metalloproteinases-3 (TIMP3), or tropomyosin-1 (TM1). Also, the expression of Bcl2 (an anti-apoptotic protein) can be induced by miR-21 binding to its 3′ UTR, decreasing, therefore, apoptosis and increasing proliferation by dysregulating the Bax/Bcl2 ratio. Furthermore, existing evidence demonstrates that transforming growth factor-beta (TGF-β) stimulation increases the miR-21 expression in cancer cells, which in turn up-regulates the process of epithelial-mesenchymal transition (EMT). This is associated with the induction of breast cancer stem cell (BCSC)-like phenotype and increase of HIF1α levels.12–18 MiR-155 is a multifunctional miR that is encoded by chromosome 21. It is an important oncogenic miR in human cancers, including breast cancer. Its up-regulation promotes proliferation and metastasis through target genes, such as RAS homolog family member A (RHOA), C-X-C chemokine receptor type 4 (CXCR4), SRY-box transcription factor 1 (SOX1), tumor protein (p53), and forkhead box O3 (FOXO3).19–25 The up-regulation of the miR-181a gene, which is located on chromosome 1, promotes metastasis and invasion of human cancers, as well as inhibiting apoptosis of breast cancer cells through target genes, such as ataxia telangiectasia mutated (ATM).26,27 MiR-145 is located on chromosome 5 (Sq32-33) and inhibits proliferation, angiogenesis, and metastasis of human breast cancer through multiple target genes, including epidermal growth factor receptor (EGFR), c-myc, sex-determining region Y-box2 (SOX2), vascular endothelial growth factor (VEGF), neural cadherin (NCAD), HIF2α, transmembrane glycoprotein Mucin1 (MUCIN 1), human epidermal growth factor receptor (HER3), and rho-associated coiled-coil kinase (ROCK1). Down-regulation of miR-145 induces metastasis in human breast cancer.28–32 Let-7a, a member of the let-7 family, is regulated by p53 and is, therefore, a tumor suppressor miR. Its up-regulation inhibits proliferation, angiogenesis, and metastasis in human breast cancer through target genes, including high mobility group AT-hook 2 (HMGA2), h-ras, k-ras, c-myc, cyclin D2, and PBX homeobox 3 (PBX3), whereas its down-regulation induces breast cancer proliferation, angiogenesis, and metastasis. Also, its loss or down-regulation is associated with cancer aggressiveness.33–41

To improve the treatment quality and the survivability rate of breast cancer patients, high accuracy in the early detection is crucial.42 Even though mammography and core needle biopsy are the most reliable detection methods, they are not sensitive or comfortable enough for women to select as routine examinations for breast cancer. Also, several limitations still exist for the tumor markers: carcinoembryonic antigen (CEA) or carcinoma antigen 15-3 (CA 15-3) because of their low sensitivity in early detection.43 Accordingly, this study aimed at analyzing the expression of a panel of some circulating oncomiRs and tumor suppressor miRs, including miR-10b, miR-21, miR-145, miR-155, miR-181a, and let-7a, as non-invasive molecular biomarkers for the initial detection of LABC, treatment response, and predicting relapse or metastasis.

Subjects and methods

Subjects

The study comprised 30 newly diagnosed female patients (aged from 30 to 61 years) with LABC selected from the Outpatient Clinics at the National Cancer Institute (Cairo University, Cairo, Egypt) before receiving neoadjuvant chemotherapy. Diagnosis of all patients was based on standard clinical criteria, including echo-Doppler study, bilateral digital mammography, complementary ultrasound, histological study, digitalized X-ray examination of the chest, bone scan, and immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor 2 (HER2), as well as the serum tumor markers CEA and CA 15-3. The characteristics of patients at baseline are depicted in Table 1. In addition, 20 clinically healthy volunteer female subjects (visitors to the Outpatient Clinics at the Egyptian National Cancer Institute) were selected to include an age distribution that was comparable to the patient group. None of the healthy controls had been previously diagnosed with any malignancies. Furthermore, they had no family history of breast cancer.

Ethics approval and consent to participate

Ethical approval was granted by the Institutional Review Board of the Egyptian National Cancer
The study was conducted following the Helsinki Declaration, and informed written consents were obtained from all participants.

**Study design**

In this study, the first blood sample (pre-treatment) was collected from breast cancer patients at baseline before the beginning of the neoadjuvant chemotherapy. The second blood sample (inter-treatment) was collected from the patients after they received four cycles of adriamycin/cyclophosphamide (intravenous infusion of 60/600 mg/m², one cycle/3 weeks). One week later, after collecting the second blood sample, the patients received weekly Taxol® (Paclitaxel) (intravenous infusion of 80 mg/m²) for 12 consecutive weeks (patients with +ve HER2/neu expression received intravenous infusions of trastuzumab (6 mg/kg) once every 3 weeks along with Taxol®). One week later, after the last Taxol® dose, all patients underwent tumor resection and the third blood sample was collected 1 week later (post-treatment). The change in the level of candidate miRs in the plasma was evaluated.

**RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction analysis of selected miRs**

Total RNA, including miRs, was isolated from plasma using the miRNeasy Mini kit (Qiagen, Hilden, Germany). MiRs were converted to cDNA by the miScript II real-time (RT) kit (Qiagen) using the Biometra Thermal Cycler (Analytik Jena, Upland, CA, USA). The relative expression of the candidate miRs (miR-10b, miR-21, miR-155, miR-181a, miR-145, and let-7a) was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using the miScript SYBR Green PCR kit (Qiagen) and the miScript Primers assays (Qiagen) in the ViiA 7 RT PCR System (Applied BioSystems, Life Technologies, Carlsbad, CA, USA). The primer sequences were as follows: miR-10b (hsa-miR-10b-3p; ACAGAUCGAUUCUAAGGGGAU), miR-21

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**Table 1. Clinicopathological data of LABC patients at baseline (pre-treatment) included in the study.**

| Clinical features | n = 30 | % |
|-------------------|--------|---|
| Age of patients (years) |        |   |
| <40               | 9      | 30|
| >40               | 21     | 70|
| Menopause         |        |   |
| Pre-menopause     | 11     | 36.7|
| Post-menopause    | 19     | 63.3|
| Body mass index   |        |   |
| <30               | 12     | 40 |
| >30               | 18     | 60 |
| Stage             |        |   |
| III               | 30     | 100|
| Lymph nodes       |        |   |
| Positive          | 19     | 63.3|
| Negative          | 11     | 36.7|
| Family history    |        |   |
| Positive          | 5      | 16.7|
| Negative          | 25     | 83.3|
| Estrogen receptor (ER) |    |   |
| Positive          | 21     | 70 |
| Negative          | 9      | 30 |
| Progesterone receptor (PR) |   |   |
| Positive          | 21     | 70 |
| Negative          | 9      | 30 |
| HER2/neu          |        |   |
| Positive          | 11     | 36.7|
| Negative          | 19     | 63.3|
| Subtypes          |        |   |
| Luminal A (LA) (+ve ER; +ve PR; -ve HER2) | 15 | 50 |
| Luminal B (LB) (+ve ER; +ve PR; +ve HER2) | 6 | 20 |
| HER2/neu (+ve ER; -ve PR; +ve HER2) | 5 | 16.7 |
| Triple negative   |        |   |
| (-ve ER; -ve PR; -ve HER2) | 4 | 13.3 |

LABC: locally advanced breast cancer; HER2: human epidermal growth factor receptor 2.
(hsa-miR-21-3p; CAACACCAG UCGAUGGGCUGU), miR-155 (hsa-miR-155-3p; CUCUACAUAAUAGCA UAAACA), miR-181a (hsa-miR-181a-3p; ACCAUCG ACCGUUGAUUGUACC), miR-145 (hsa-miR-145-3p; GGAUUCCUGGAAAUACUGUUC), let-7a (hsa-let-7a-3p; CUAUACAUCUACUGUCUUC), and the housekeeping miR-16 (hsa-miR-16-3p; CCAGUAAU ACUGUGCUGCUGA). The amplification protocol consisted of an initial denaturation step at 95°C for 15 min, followed by 40 cycles (94°C for 15 s, 55°C for 30 s, and 70°C for 30 s). The relative expression of selected miRs was normalized to an internal control (miR-16) and relative to a calibrator (respective miRs from normal plasma sample) and was calculated using the comparative threshold cycle method of Schmittgen and Livak.44

Serum CEA and CA 15-3 analyses
CEA and CA 15-3 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits provided by Cell Biolabs, Inc. (San Diego, CA, USA).

Bioinformatics
Selected miRs related to breast cancer were retrieved from previously published studies deposited in the National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus (GEO) through the GEO2R application (https://www.ncbi.nlm.nih.gov/geo/geo2r/) with the following accession numbers: GSE70754, GSE41526, and GSE22981.

Statistical analysis
The Shapiro–Wilk test for normality (p > 0.05) showed that all data were non-parametric. The quantitative results were expressed as median, 25th, and 75th percentile (quartiles) values. The non-parametric Mann–Whitney U test was used for comparing results between two independent groups. Wilcoxon test for multiple comparisons of non-parametric data was applied, followed by Friedman’s test to compare repeated measurements. Spearman’s correlation was used to measure statistical dependence between two variables. Receiver operating characteristic (ROC) curves were computed; the area under the curve (AUC) and cut-off values were calculated to assess the diagnostic accuracy of the markers. The cross-tabulation analysis was carried out, and the significance (χ²) and likelihood ratio (LR) were calculated from the chosen cut-off values. All statistics were analyzed using SPSS Statistical Software version 20.0 (SPSS Inc., Chicago, IL, USA).

Results
Data represented in Figures 1 and 2 show a significant up-regulation in the relative expression of plasma miR-10b, miR-21, and miR-181a levels (p < 0.01, p = 0.001, and p < 0.05, respectively), along with a significant increase in serum CA 15-3 and CEA levels (p < 0.05 and p < 0.01, respectively) in LABC patients at initial diagnosis (pre-treatment), whereas a significant down-regulation was demonstrated in the expression of miR-145 and let-7a (p < 0.01), compared to healthy controls. However, a gradual significant decrease was recorded in the plasma miR-10b and miR-21 levels, as well as the breast tumor size, whereas a gradual significant increase in the plasma let-7a level was demonstrated among the different treatment stages of LABC patients. By contrast, a non-significant change was recorded in miR-145 and miR-181a, as well as serum CA 15-3 and CEA levels of LABC patients after receiving four cycles of adriamycin/cyclophosphamide (inter-treatment), compared to their levels at initial diagnosis (pre-treatment), whereas a significant increase was recorded in miR-145 level, while miR-181a, CA 15-3 and CEA levels were significantly decreased following Taxol® treatment and tumor resection (post-treatment), compared to their respective pre- and inter-treatment levels. Most interestingly, a rebound to the control level in the expression of candidate miRs and tumor markers of LABC patients was observed at the end of the treatment.

ROC curve analysis revealed significant AUC values for candidate plasma miRs, including miR-10b, miR-21, miR-181a, miR-145, and let-7a, as well as serum CA 15-3 and CEA (0.73, 0.78, 0.70, 0.72, 0.71, and 0.75, respectively). At the optimal cut-off values for each marker, the sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy percent were calculated and presented in Table 2. Absolute specificity (100%) values were recorded for both miR-10b and miR-21, whereas the highest diagnostic accuracy was recorded for miR-21.

Table 3 illustrates significant correlations between different plasma miR profiles (miR-10b, miR-21, miR-145, and let-7a), serum tumor marker (CA 15-3), and tumor size in LABC patients at initial diagnosis (pretreatment). MiR-10b had a significant positive correlation with plasma miR-21, serum CA 15-3, and tumor size (r = 0.62, 0.54, and 0.4, respectively), and a significant negative correlation with miR-145 and let-7a (r = 0.57 and 0.54, respectively). However, plasma miR-21 had a positive correlation with serum CA 15-3 and tumor size (r = 0.73 and 0.74, respectively), and a negative correlation with plasma miR-145 and let-7a (r = 0.88 and 0.58, respectively). Plasma miR-145
Figure 1. Expression of plasma miRs was determined by qRT-PCR in serially collected blood samples of locally advanced breast cancer (LABC) patients at three time intervals (pre-treatment at baseline; inter-treatment after receiving four cycles of adriamycin/cyclophosphamide; post-treatment after tumor resection). Mann–Whitney U test for non-parametric data was applied to compare pre- and post-treatment patients versus control subjects (significant at *p < 0.05, **p < 0.01, and ***p < 0.001). Wilcoxon test for multiple comparisons was applied followed by Friedman's test to compare LABC patients at the three different treatment stages (different letters denote significance between stages).
Figure 2. Statistical significance of serum tumor markers (CEA and CA 15-3) and tumor size in locally advanced breast cancer (LABC) patients at three time intervals (pre-treatment at baseline; inter-treatment after receiving four cycles of adriamycin/cyclophosphamide; post-treatment after tumor resection). Mann–Whitney U test for non-parametric data was applied to compare pre- and post-treatment patients versus control subjects (significant at *p < 0.05 and **p < 0.01). Wilcoxon test for multiple comparisons was applied followed by Friedman’s test to compare LABC patients at the three different treatment stages (different letters denote significance between stages).

Table 2. Diagnostic values (sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy %) of significant biomarkers to distinguish LABC patients at baseline (pre-treatment) from control subjects.

| Parameter   | AUC  | p  | Cut-off | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Diagnostic accuracy (%) |
|-------------|------|----|---------|-----------------|-----------------|--------|--------|------------------------|
| miR-10b     | 0.73 | 0.01 | 2.52    | 53.3            | 100             | 100    | 58.8   | 72                     |
| miR-21      | 0.78 | 0.01 | 4.94    | 63.3            | 100             | 100    | 64.5   | 78                     |
| miR-181a    | 0.70 | 0.05 | 1.51    | 50              | 80              | 78.9   | 51.6   | 62                     |
| miR-145     | 0.70 | 0.05 | 0.78    | 45              | 83.3            | 69.4   | 64.3   | 68                     |
| let-7a      | 0.72 | 0.01 | 0.52    | 50              | 93.3            | 73.7   | 83.3   | 76                     |
| CA 15-3 (U/mL) | 0.71  | 0.05 | 22.5    | 60              | 75              | 78.3   | 55.6   | 66                     |
| CEA (ng/mL) | 0.75 | 0.01 | 2.05    | 56.7            | 75              | 77.3   | 53.6   | 64                     |

LABC: locally advanced breast cancer; AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value; CA: carcinoma antigen; CEA: carcinoembryonic antigen.
showed a significant positive correlation with plasma let-7a (r = 0.69), and a significant negative correlation with serum CA 15-3 and tumor size (r = 0.70 and 0.60, respectively). Plasma let-7a demonstrated a significant negative correlation with serum CA 15-3 and tumor size (r = 0.43 and 0.42, respectively). Finally, serum CA 15-3 had a significant positive correlation with tumor size (r = 0.48).

Using the cut-off value of 4.94 for plasma miR-21, it was possible to significantly sort out 60%, 33.3%, 0%, and 0% of LABC patients with luminal A (LA), luminal B (LB), HER2, and triple-negative (TN) subtypes, respectively. Using the cut-off value of a positive/negative family history, it was possible to significantly sort out 0%, 0%, 40%, and 75% of LABC patients with LA, LB, HER2, and TN subtypes, respectively, having a positive family history, out of 100%, 100%, 60%, and 25% of LABC patients with LA, LB, HER2, and TN subtypes, respectively, having a negative family history (Table 4).

Using the cut-off values of 2.52, 4.94, and 22.5 for miR-10b, miR-21, and CA 15-3, respectively, it was possible to significantly sort out 81.8%, 72.7%, and 72.7%, respectively, of LABC patients without lymph nodes involvement with lower cut-off values, out of 73.7%, 84.2%, and 78.9%, respectively, of LABC patients with positive lymph nodes having higher cut-off values (Table 5). Also, using the cut-off values of some combined markers, such as CA 15-3+miR-10b, CA 15-3+miR-21, and CA 15-3+miR-181a, we found that 75%, 75%, and 65% of healthy controls had both markers below their cut-off values, respectively, whereas 66.7%, 70%, and 76.7% of LABC patients had one or both markers above their specified cut-off values, respectively. In addition, the cut-off values of CEA + miR-10b, CEA + miR-21, and CEA + miR-181a showed that 75%, 75%, and 60% of healthy controls had both markers below their cut-off levels, respectively, whereas 73.3%, 76.7%, and 76.7% of LABC patients had one or both markers above their cut-off levels, respectively (Table 5). Also, using the cut-off values of some combined markers, such as CA 15-3+miR-10b, CA 15-3+miR-21, and CA 15-3+miR-181a, we found that 75%, 75%, and 65% of healthy controls had both markers below their cut-off values, respectively, whereas 66.7%, 70%, and 76.7% of LABC patients had one or both markers above their specified cut-off values, respectively.

### Table 3. Significant correlations between different parameters in LABC patients at baseline (pre-treatment).

| Parameter | Correlated parameters | Correlation coefficient (r) | p-value |
|-----------|-----------------------|-----------------------------|---------|
| miR-10b   | miR-21                | 0.62                        | 0.001   |
|           | miR-145               | -0.57                       | 0.01    |
|           | let-7a                | -0.54                       | 0.01    |
|           | CA 15-3               | 0.54                        | 0.01    |
|           | Tumor size            | 0.4                         | 0.05    |
| miR-21    | miR-145               | -0.88                       | 0.001   |
|           | let-7a                | -0.58                       | 0.001   |
|           | CA 15-3               | 0.73                        | 0.001   |
|           | Tumor size            | 0.74                        | 0.001   |
| miR-145   | let-7a                | 0.69                        | 0.001   |
|           | CA 15-3               | -0.7                        | 0.001   |
|           | Tumor size            | -0.6                        | 0.001   |
| let-7a    | CA 15-3               | -0.43                       | 0.05    |
|           | Tumor size            | -0.42                       | 0.05    |
| CA 15-3   | Tumor size            | 0.48                        | 0.01    |

LABC: locally advanced breast cancer; CA: carcinoma antigen. Spearman correlation for non-parametric data was applied.

### Table 4. Cross-tabulation showing the ability of plasma miR-21 and family history to sort out tumor subtypes of LABC at baseline (pre-treatment).

| Marker        | Cut-off | LA (N = 15) | LB (N = 6) | HER2 (N = 5) | TN (N = 4) | χ²     | LR     |
|---------------|---------|-------------|------------|--------------|------------|--------|--------|
| miR-21        | <4.94   | N 9         | 2          | 0            | 0          | 0.05   | 11.60  |
|               | ≥ 4.94  | N 6         | 4          | 5            | 4          |        |        |
| Family history| Positive| N 0         | 0          | 2            | 3          | 0.01   | 15.8   |
|               | Negative| N 15        | 6          | 3            | 1          |        |        |
specified cut-off values, respectively. By contrast, the cut-off values of miR-145 + let-7a revealed that all LABC patients (100%) had one or both markers below their cut-off values, whereas 35% of healthy controls had both markers above their cut-off values. However, the cut-off values of miR-10b + miR-21 + miR-181a showed that 80% of healthy controls had all markers below their cut-off levels, whereas 76.7% of LABC patients had at least one marker above its cut-off value. The highest diagnostic accuracy for all combinations (78%) was reported for miR-181a with either miR-10b or miR-21, or all of the three combinations (Table 6).

A 5-year disease-free survival (DFS) follow-up for LABC patients starting after tumor resection (0 weeks) was performed. A DFS of 83.3% was obtained, where 25 out of 30 patients were free from cancer relapse or metastasis, whereas five out of 30 patients (16.7%) suffered from breast tumor relapse and metastases to the lungs, liver, or bones (Figure 3). Using the cut-off values of 2.52 and 4.94 for miR-10b and miR-21, respectively, it was possible to significantly sort out 92% (miR-10b level lower than the cut-off value of 2.52) and 92.3% (miR-21 level lower than the cut-off value of 4.94) of breast cancer patients who were free from cancer relapse or metastasis, out of 60% (miR-10b level above the cut-off value of 2.52) and 75% (miR-21 level above the cut-off value of 4.94) of breast cancer patients who suffered from breast tumor relapse and metastases (Table 7).

**Discussion**

This study aims at evaluating the diagnostic and predictive role of the expression of some candidate miRs, including miR-10b, miR-21, miR-155, miR-181a, miR-145, and let-7a, in LABC. In agreement with previous findings, we demonstrated that the expression of the oncomiR-10b, oncomiR-21, and oncomiR-181a was significantly increased in LABC patients, whereas tumor suppressor miR-145 and let-7a expressions were significantly down-regulated, compared to healthy subjects. Similarly, the dysregulation of serum miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-191, and miR-382 in breast cancer patients has been reported by Mar-Aguilar et al., who concluded that these seven miRs can be used as a biomarker in breast cancer. Also, both miR-10b and miR-21 were reported to induce the invasion and proliferation of MCF-7 breast cancer cells by suppressing tumor suppressor genes. Recently, Zhang et al. demonstrated that the overexpression of actin fiber–associated protein 1-antisense RNA1 (AFAP1-AS1), a newly discovered lncRNA, could promote triple-negative breast cancer (TNBC) cell proliferation and invasion through competitive binding to miR-145 that in turn up-regulates MutT homolog-1 (MTH1) expression and TNBC cell proliferation.

The routinely used markers for the diagnosis of breast cancer are CEA, CA 15-3, and CA 27-29. However, they are not accurate biomarkers, as they have limited sensitivity and specificity. Furthermore, they are rarely elevated before gross disease and are not seen in many patients with metastases. ROC curve analysis revealed that miR-21 has a higher sensitivity than that for the routinely used tumor markers: CEA and CA 15-3 for the diagnosis of LABC. Furthermore, all of the miRs, including miR-10b, miR-21, miR-181a, miR-145, and let-7a, showed better specificity value than that for CEA and CA 15-3. However, better sensitivity and specificity were reported when combining different miRs (Table 7), demonstrating the distinctive expression patterns of candidate miRs in this study, and suggesting their values as non-invasive diagnostic molecular biomarkers for breast cancer.

A positive correlation was established between oncomiRs (miR-10b and miR-21) and the tumor biology.
Table 6. Cross-tabulation showing the reliability of significant combined biomarkers to distinguish LABC patients at baseline (pre-treatment) from healthy subjects.

| Combined markers                  | Cut-off                  | Control | Pre-treatment | $\chi^2$ | LR   | PPV (%) | NPV (%) | Diagnostic accuracy (%) |
|-----------------------------------|--------------------------|---------|---------------|----------|------|--------|---------|-------------------------|
| CA 15-3 + miR-10b                 | Both low                 | N 15   | 10            | 0.01     | 8.63 | 80     | 60      | 70                      |
|                                   |                          | % 75   |               |          |      |        |         |                         |
|                                   | One or both high         | N 5    | 20            |          |      |        |         |                         |
|                                   |                          | % 25   |               |          |      |        |         |                         |
| CA 15-3 + miR-21                  | Both low                 | N 15   | 9             | 0.01     | 10.1 | 80.8   | 62.5    | 72                      |
|                                   |                          | % 75   |               |          |      |        |         |                         |
|                                   | One or both high         | N 5    | 21            |          |      |        |         |                         |
|                                   |                          | % 25   |               |          |      |        |         |                         |
| CA 15-3 + miR-181a                | Both low                 | N 13   | 7             | 0.01     | 8.8  | 76.7   | 65      | 72                      |
|                                   |                          | % 65   |               |          |      |        |         |                         |
|                                   | One or both high         | N 7    | 23            |          |      |        |         |                         |
|                                   |                          | % 35   |               |          |      |        |         |                         |
| CEA + miR-10b                     | Both low                 | N 15   | 8             | 0.01     | 11.7 | 81.5   | 65.2    | 74                      |
|                                   |                          | % 75   |               |          |      |        |         |                         |
|                                   | One or both high         | N 5    | 22            |          |      |        |         |                         |
|                                   |                          | % 25   |               |          |      |        |         |                         |
| CEA + miR-21                      | Both low                 | N 15   | 7             | 0.001    | 13.5 | 82.1   | 68.2    | 76                      |
|                                   |                          | % 75   |               |          |      |        |         |                         |
|                                   | One or both high         | N 5    | 23            |          |      |        |         |                         |
|                                   |                          | % 25   |               |          |      |        |         |                         |
| CEA + miR-181a                    | Both low                 | N 12   | 7             | 0.01     | 6.9  | 74.2   | 63.2    | 70                      |
|                                   |                          | % 60   |               |          |      |        |         |                         |
|                                   | One or both high         | N 8    | 23            |          |      |        |         |                         |
|                                   |                          | % 40   |               |          |      |        |         |                         |
| CA 15-3 + CEA + miR-10b           | All low                  | N 12   | 6             | 0.01     | 8.4  | 75     | 66.7    | 72                      |
|                                   |                          | % 60   |               |          |      |        |         |                         |
|                                   | At least one high        | N 8    | 20            |          |      |        |         |                         |
|                                   |                          | % 40   |               |          |      |        |         |                         |
| CA 15-3 + CEA + miR-21            | All low                  | N 12   | 6             | 0.01     | 8.4  | 75     | 66.7    | 72                      |
|                                   |                          | % 60   |               |          |      |        |         |                         |
|                                   | At least one high        | N 8    | 20            |          |      |        |         |                         |
|                                   |                          | % 40   |               |          |      |        |         |                         |
| CA 15-3 + CEA + miR-181a          | All low                  | N 10   | 4             | 0.01     | 8.01 | 72.2   | 71.4    | 72                      |
|                                   |                          | % 50   |               |          |      |        |         |                         |
|                                   | At least one high        | N 10   | 26            |          |      |        |         |                         |
|                                   |                          | % 50   |               |          |      |        |         |                         |
| miR-10b + miR-21                  | Both low                 | N 20   | 11            | 0.001    | 26.97| 100    | 64.5    | 78                      |
|                                   |                          | % 100  |               |          |      |        |         |                         |
|                                   | One or both high         | N 0    | 19            |          |      |        |         |                         |
|                                   |                          | % 0    |               |          |      |        |         |                         |
| miR-10b + miR-181a                | Both low                 | N 16   | 10            | 0.01     | 11.03| 83.3   | 61.5    | 72                      |
|                                   |                          | % 80   |               |          |      |        |         |                         |
|                                   | One or both high         | N 4    | 20            |          |      |        |         |                         |
|                                   |                          | % 20   |               |          |      |        |         |                         |
| miR-21 + miR-181a                 | Both low                 | N 16   | 7             | 0.001    | 16.38| 85.2   | 69.6    | 78                      |
|                                   |                          | % 80   |               |          |      |        |         |                         |
|                                   | One or both high         | N 4    | 23            |          |      |        |         |                         |
|                                   |                          | % 20   |               |          |      |        |         |                         |
| miR-145 + let-7a                  | Both high                | N 7    | 0             | 0.001    | 14.59| 69.8   | 100     | 74                      |
|                                   |                          | % 35   |               |          |      |        |         |                         |
|                                   | One or both low          | N 13   | 30            |          |      |        |         |                         |
|                                   |                          | % 65   |               |          |      |        |         |                         |
| miR-10b + miR-21 + miR-181a       | All low                  | N 16   | 7             | 0.001    | 16.38| 85.2   | 69.6    | 78                      |
|                                   |                          | % 80   |               |          |      |        |         |                         |
|                                   | At least one high        | N 4    | 23            |          |      |        |         |                         |
|                                   |                          | % 20   |               |          |      |        |         |                         |

LABC: locally advanced breast cancer; LR: likelihood ratio; PPV: positive predictive value; NPV: negative predictive value; CEA: carcinoembryonic antigen; CA: carcinoma antigen.
suppressor miRs (miR-145 and let-7a), whereas negative correlations were observed between the oncomiR-10b and oncomiR-21 and the tumor suppressors miR-145 and let-7a, pointing out to the important association of these biomarkers in breast cancer progression. In addition to family history, plasma miR-21 was able to sort out LABC tumor subtypes according to its expression. It has been previously reported that the expression of miR-10b, miR-21, miR-145, and let-7a was closely associated with the clinical and pathologic features of breast cancer, such as lymph node status, tumor size, and expression of sex hormone.\textsuperscript{49,50,52,57}

By the end of the treatment, the altered miRs rebound to control levels. Similarly, Khalighfard et al.\textsuperscript{52} demonstrated that the plasma miR-10b level is significantly decreased, whereas let-7a was significantly increased in breast cancer patients after mastectomy, chemotherapy, and radiotherapy, compared to their baseline level. Similar findings were also reported for miR-21, miR-145, and miR-181a.\textsuperscript{45,47,50,52,58–60} Also, Badr and colleagues\textsuperscript{61,62} found that breast cancer patients with decreased miR-21 expression following treatment had better clinical outcomes that can increase survival.

It has been reported that plasma miR-34a can predict chemotherapeutic resistance associated with higher expression levels in non-responsive locally advanced Egyptian breast cancer patients.\textsuperscript{63} Our study shows that LABC patients suffering from breast tumor relapse or metastases to the lungs, liver, or bones during a 5-year follow-up after tumor resection had miR-10b and miR-21 levels above their cut-off values. This finding indicates that monitoring miR-10b and miR-21 levels in patients at the end of treatment can predict breast cancer relapse, metastasis, or even recovery. This result is consistent with that of Liu et al.,\textsuperscript{64} who reported that miR-21 is a useful non-invasive biomarker to predict response to chemotherapy in HER2-positive breast cancer. Similarly, Wang et al.\textsuperscript{65} revealed that increased miR-10b expression might predict poor survival in patients with breast cancer.

In conclusion, our study verified three up-regulated oncomiRs (oncomiR-10b and oncomiR-21) and the tumor suppressors miR-145 and let-7a, pointing out to the important association of these biomarkers in breast cancer progression. In addition to family history, plasma miR-21 was able to sort out LABC tumor subtypes according to its expression. It has been previously reported that the expression of miR-10b, miR-21, miR-145, and let-7a was closely associated with the clinical and pathologic features of breast cancer, such as lymph node status, tumor size, and expression of sex hormone.\textsuperscript{49,50,52,57}

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In conclusion, our study verified three up-regulated oncomiRs (oncomiR-10b, oncomiR-21, and oncomiR-181a) and two down-regulated tumor suppressor miRs (miR-145 and let-7a) as potential candidate non-invasive diagnostic biomarkers of LABC, with better specificity (miR-21) and sensitivity (miR-10b, miR-21, miR-181a, miR-145, and let-7a) than the routinely used tumor markers: CEA and CA 15-3. Moreover, the mutual combination of miRs together, either alone or with CEA and CA 15-3, increases the accuracy of diagnosis. Besides, reducing oncomiRs level and increasing that of tumor suppressor miRs by the end of the treatment can be considered as a good tool to assess the proper response to the treatment. Also, our results revealed that the oncomiR-10b and oncomiR-21 may be considered promising predictive biomarkers for progression-free survival. However, large prospective clinical studies are warranted to confirm our preliminary results.
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