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Circulating miRNA 20a, miRNA 140-5p, and VEGF as Predictive Biomarkers of Metastasis in Liquid Biopsy of Breast Cancer Patients

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ABSTRACT

MicroRNAs (miRNAs) are critical modulators in breast carcinogenesis. Metastasis remains the underlying cause of breast cancer-related mortality. We sought to elucidate the predictive potential of circulating miR-20a and miR-140-5p as non-invasive liquid biopsy biomarkers. This study enrolled 50 breast cancer patients (25 primary non-metastatic and 25 metastatic patients), and 15 control subjects. The expression of miR-20a and miR-140-5p was measured using qRT-PCR. Serum level of vascular endothelial growth factor (VEGF) was determined by ELISA. The predictive value of the studied markers was examined by Receiver-operating characteristics (ROC) curve analysis. MiR-20a was significantly upregulated, miR-140-5p downregulated, together with elevated serum VEGF levels in all breast cancer patients in comparison to controls and the metastatic compared to non-metastatic group ($p<0.001$ for each). MiR-20a, miR-140-5p, and VEGF exhibited significant predictive value for metastasis (AUC of 1 in all), with high specificity and sensitivity. MiR-20a and miR-140-5p expression were associated with positive lymph node metastasis ($p<0.05$) and correlated with VEGF levels ($p<0.0001$). In conclusion, **o**ur findings suggest that circulating miR-20a and miR-140-5p are promising non-invasive predictive biomarkers to discriminate between metastatic and locally-confined breast cancer. They may also hold a promise as targets for miRNA-based treatments.

Keywords: *miR-20a; miR-140-5p; VEGF, breast cancer; liquid biopsy.*

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1. INTRODUCTION

Female breast cancer is the leading cause of cancer incidence worldwide with approximately 2.3 million new cases **[1]**. According to GLOBOCAN 2020, breast cancer ranks first among women for incidence and mortality in the vast majority of countries **[1]**. Despite the recent advances in the management of breast cancer, metastatic disease represents the central clinical challenge and the underlying cause of breast cancer-related deaths **[2]**. Nevertheless, 5–10% of cases are metastatic at diagnosis and around 30% of women presented with the early-stage disease will ultimately progress later and develop metastatic lesions **[3]**. Accordingly, ongoing research is currently focusing on unveiling the mechanisms underlying breast cancer metastasis to identify biomarkers that could eventually predict metastasis at an early stage.

The era of precision medicine entails the development of novel approaches as an urgent need for screening, early detection, tailoring treatment choice, and monitoring response to therapy. Liquid biopsies, such as blood samples,

have attracted considerable attention in oncology as a minimally invasive and easily accessible tool that can identify the disease at an early stage and monitor disease progression **[4]**. The study of circulating cell-free microRNAs (miRNAs) in cancer patients' serum has emerged as promising novel non-invasive biomarkers for early detection of cancers and predicting their molecular characteristics **[5]**. MiRNAs are short, non-coding RNA sequences (19–23 nucleotides) that play a critical role in the regulation of gene expression, post-transcriptionally, in many biological systems via targeting messenger RNA (mRNA), resulting in either translational repression or RNA degradation **[6]**. More than 60% of human protein-coding genes are predicted to have binding sites for miRNAs in their 3′-untranslated region (3′-UTR) **[6]**. MiRNAs are critical modulators in cellular pathways and hence, dysregulation of miRNAs is intricately linked to various diseases including cancer **[5]**.

Compelling evidence has revealed the pivotal role of miRNAs in the regulation of hallmarks of cancer including proliferation, angiogenesis, apoptosis, invasion, metastasis, and tumor stemness **[7]**. Altered miRNA expression can occur in cancer as a result of various mechanisms such as transcriptional deregulation, epigenetic alterations, or inhibition of specific miRNA processing **[8]**. MiRNAs can play different roles in oncogenesis by functioning as oncogenes or as tumor suppressors; as they are upregulated or downregulated in many malignancies **[9]**. Moreover, miRNAs contribute to the maintenance of tumor-initiating properties **[10]** and can also act as modulators in the pathways of the metastatic cascade, including angiogenesis, sustained proliferation, and epithelial-mesenchymal transition (EMT) **[11]**. It was previously shown that mammary epithelial cells from malignant tumors can release miRNAs into peripheral circulation so that their molecular profiling has the potential to predict different breast cancer features including metastasis **[10]**. Owing to their structural stability, ease of isolation, and detection, miRNAs expression in peripheral blood has been considered as promising biomarkers for breast cancer **[12]**.

Angiogenesis is one of the hallmarks of cancer serving a key role in tumor growth and metastasis **[13, 14]**. It is a complex mechanism that allows tumor development at the early stages of carcinogenesis through the formation of new blood vessels either from the existing vasculature or from endothelial progenitors derived from bone marrow **[15]**. Notably, miRNAs can post-transcriptionally regulate the genes responsible for the angiogenic switch **[14]**, including miR-20a **[13]** and miR-140-5P **[16]**.

MiR-20a, located at 13q31.3, belongs to the miR-17 family of the oncogenic miR-17-92 cluster. It serves a key role in various functions related to carcinogenesis including EMT, migration, invasion, and senescence in colorectal cancer **[17]**, as well as regulation of glioma cell proliferation and invasion **[18]**. In addition, up-regulation of miR-20a can promote the growth of cervical carcinoma cells **[19]**, non-small cell lung carcinoma **[20]** and promotes prostate cancer invasion and migration **[21]**. Although dysregulation of miR-20a expression was observed in multiple cancers, yet its efficacy as a prognostic circulating biomarker in breast cancer has not been extensively addressed.

MiR-140, located on chromosome 16q22.1, produces two different single-stranded molecules, 140-3p and 140-5p, which have multiple targets that regulate cell cycle transition, cell proliferation, and apoptosis **[22]**. MiR-140-5p was shown to be downregulated in many malignancies, suggesting its function as a tumor suppressor gene. The molecular role of miR-140-5p in the initiation and progression of the diversity of tumors have been demonstrated including gastric cancer **[23]**, non-small cell lung cancer **[24]**, cervical cancer **[25]**, and ovarian cancer **[26**]; yet the role of circulating miR-140-5p needs to be elucidated.

It is noteworthy that the vascular endothelial growth factor (VEGF) is a major contributor to cell proliferation and migration and is one of the most potent angiogenic factors. High expression of VEGF has been observed in body fluids in a variety of cancers **[27]**. Besides, VEGF plays a key role in the progression of breast cancer and is associated with worse survival **[22]**. VEGF is

also a crucial target protein of miRNA regulation in angiogenesis **[28]**.

The emerging molecular knowledge on the important role of miRNAs in breast carcinogenesis warrants to be translated into advancement in the clinical management of breast cancer patients and as potential targets for the development of novel anticancer drugs. Microarray expression profiling comparing the miRNA expression in normal tissues, benign and malignant tumors revealed that several miRNAs are aberrantly expressed in both benign and malignant breast tissues in comparison to normal tissues, including miR-20a and miR-140; indicating that they may have a role in proliferation and early stages carcinogenesis **[29]**. However, research studies of the circulating miRNAs associated with breast cancer have been limited so far. Accordingly, the rationale for our study was to elucidate the prognostic and predictive potential of circulating miRNAs 20-a and 140-5p as minimally-invasive biomarkers to discriminate metastatic from locally confined breast cancer. Moreover, the correlations between these angiogenesis-related miRNAs with VEGF protein levels were studied to establish the possibility of using them as surrogate biomarkers to stratify the patients according to their angiogenic profile.

2. SUBJECTS and METHODS

2.1. Subjects

A series of 50 female patients diagnosed with invasive breast cancer were recruited from Clinical Oncology Department, Ain Shams University Hospitals, Cairo, Egypt. They were divided into two groups: the first group included 25 primary non-metastatic treatment naïve patients not receiving any preoperative radiotherapy or chemotherapy, and the second group comprised 25 breast cancer patients suffering from metastasis. Patients suffering from liver or kidney diseases, diabetes mellitus, cardiovascular disorders, or any inflammatory diseases were excluded from the study. Demographic data including age at diagnosis and family history of cancer; and clinicopathological characteristics including tumor size, nodal involvement, metastasis, histological type, ER, PR and HER-2/*neu* status, and Ki-67 proliferative index were retrieved from hospital medical records. The American Joint Committee on Cancer (AJCC) Tumor, Node, Metastasis (TNM) classification system was used for staging breast cancers.

All participants signed a written informed consent which informed them that their blood samples will be utilized for medical research and briefly described the aim of the study. The study was approved by the Ethical Committee of Research, Faculty of Pharmacy, Ain Shams University (32/2021), and was carried out in agreement with the recommendations and regulations of the Helsinki Declaration.

2.2. Methods

2.2.1. Blood sampling

Peripheral blood samples were collected from the study population on plain vacutainer tubes for serum preparation. The serum was then divided into 2 aliquots, 1 aliquot for miRNA extraction and the other for VEGF assay, and stored at –80°C until analysis.

2.2.2. miRNA extraction and quantification

Total RNA was extracted from 200 µl of serum using miRNeasy mini kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. The quality and quantity of the RNA extract were performed with Beckman dual spectrophotometer (USA). Complementary DNA (cDNA) was synthesized by the use of the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California), followed by storage at -20 °C.

The expression levels of miR-20a, miR-140-5p, were measured using specific TaqMan™ MicroRNA Assays Kits (miR-20a assay ID 000580, catalog number 4427975; miR-140-5p assay ID 00187, catalog number 4427975; Applied Biosystems, Foster City, California) utilizing the Rotor-Gene Q real-time polymerase chain reaction (RT-PCR) cycler with specific TaqMan™ Universal Master MiX II. The expression level of the studied miRNAs was normalized using reference miR-16 (TaqMan™

assay catalog number 00039, Applied Biosystems, Foster City, California) as the internal control **[30]** and analyzed by the $2^{-\Delta\Delta Ct}$ method **[31]**.

2.2.3. VEGF assay by ELISA technique

Serum VEGF and CA 15-3 levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits: Human Vascular Endothelial Growth Factor ELISA Kit (Catalog number E0080Hu, Bioassay technology laboratory, England/China) and Cancer Antigen 15-3 ELISA Kit (Catalog number EL1-1279; Monocent, USA). ELISA procedures were conducted using Hyprep automated ELISA system (Hyperion Inc, Miami, FL) following instructions of the manufacturer.

2.2.4. Statistical analysis

Data were analyzed with IBM© SPSS© Statistics version 26 (IBM© Corp., Armonk, NY). Numerical data are presented as mean and standard deviation (SD) and between-group differences are compared using the independent-samples t-test. Categorical variables are presented as numbers and percentages, while the Pearson chi-squared test or Fisher's exact test were used to comparing differences between groups. Correlations were examined using Pearson's correlation analysis. Receiver-operating characteristics (ROC) curve analysis was employed to test the predictive value of different biomarkers.

3. RESULTS

The clinical characteristics of breast cancer patients are shown in **Table 1**. The mean age at diagnosis was 54.16±5.3 years for the primary non-metastatic treatment naïve patients, 53.72±10.3 years for the metastatic patients, and 53.2±3.1 years for the control group. The majority of patients showed ER-positive (88%), PR positive (90%), and Her2/*neu* negative (90%) tumors. Positive lymph node involvement was observed in 72% of patients. Regarding tumor size, T2 was observed in 48% and T3 in 24% of the patients. For metastatic breast cancer patients, the most common sites of metastasis were bone (n= 13), contralateral breast (n= 8) and lungs (n= 4).

3.1. Expression levels of miR-20a, miR-140-5p, and VEGF levels in serum of breast cancer patients and controls

As shown in **Table 2**, serum expression levels of miR-20a were significantly elevated in breast cancer patients (8.661±4.854) compared to the control group (1.049±0.086) at $p< 0.0001$. Serum expression levels of miR-140-5p, show significant downregulation in breast cancer patients (0.512±0.265) in comparison to the control group (1.048±0.079) at $p< 0.0001$. Serum levels of VEGF were significantly higher in breast cancer patients (1012.0±364.6) compared to the control group (257.3±114.2) at $p< 0.0001$.

3.2. Expression levels of miR-20a, miR-140-5p, and VEGF levels in serum of metastatic and non-metastatic breast cancer patients

MiR-20a showed significantly higher up-regulation in the metastatic breast cancer group (13.160±1.960) than the non-metastatic group (4.162±1.445) at $p< 0.0001$ as shown in **Fig. 1A**. Regarding expression levels of miR-140-5p in both groups, it showed significantly lower down-regulation in the metastatic breast cancer group (mean 0.265±0.054) than the non-metastatic group (0.759±0.113) at $p< 0.0001$ as shown in **Fig. 1B**. Moreover, serum VEGF was significantly higher in metastatic breast cancer patients (1292.0±318.7) compared to the non-metastatic group (732.0±80.2) at $p< 0.0001$ as shown in **Fig. 1C**. As depicted in **Table 3**, no association was observed between metastatic and non-metastatic groups regarding hormone receptor status. CA 15-3 levels and Ki-67 were significantly elevated in metastatic breast cancer patients (39.9 ± 26.3 and 15.9 ± 8, respectively) compared to non-metastatic patients (27.3±11.6 and 23.7±7.1, at $p< 0.05$, $p< 0.0$, respectively).

Table 1. Clinical characteristics of breast cancer patients

Clinicopathological parameters	Mean ± SD/ Count (%)
Age (years)	53.9 ± 10.3
Family history of breast cancer	7 (14.0%)
Involved side	
Right	25 (50.0%)
Left	23 (46.0%)
Bilateral	2 (4.0%)
Stage	
Stage II	15 (30%)
Stage III	10 (20%)
Stage IV	25 (50%)
ER	
Negative	6 (12.0%)
Positive	44 (88.0%)
PR	
Negative	5 (10.0%)
Positive	45 (90.0%)
HER2/neu	
Negative	46 (92.0%)
Positive	4 (8.0%)
Tumor grade	
T1	7 (14.0%)
T2	24 (48.0%)
T3	12 (24.0%)
T4	7 (14.0%)
Lymph Node State	
N0	14 (28.0%)
N1	25 (50.0%)
N2	11 (22.0%)

ER: Estrogen receptor; PR: Progesterone receptor; HER2/neu, human epidermal growth factor receptor; T; tumor size; N, nodal involvement

Table 2. Comparison of cases of breast cancer and controls

Variable	Breast Cancer (n=50) Mean	SD	Control (n=15) Mean	SD	P-value†
Age (years)	53.9	10.3	53.2	3.1	0.787
miRNA 20a relative expression	8.661	4.854	1.049	0.086	<0.0001
miRNA 140-5p relative expression	0.512	0.265	1.048	0.079	<0.0001
VEGF (pg/ml)	1012.0	364.6	257.3	114.2	<0.0001

SD = standard deviation, † Independent-samples t-test.

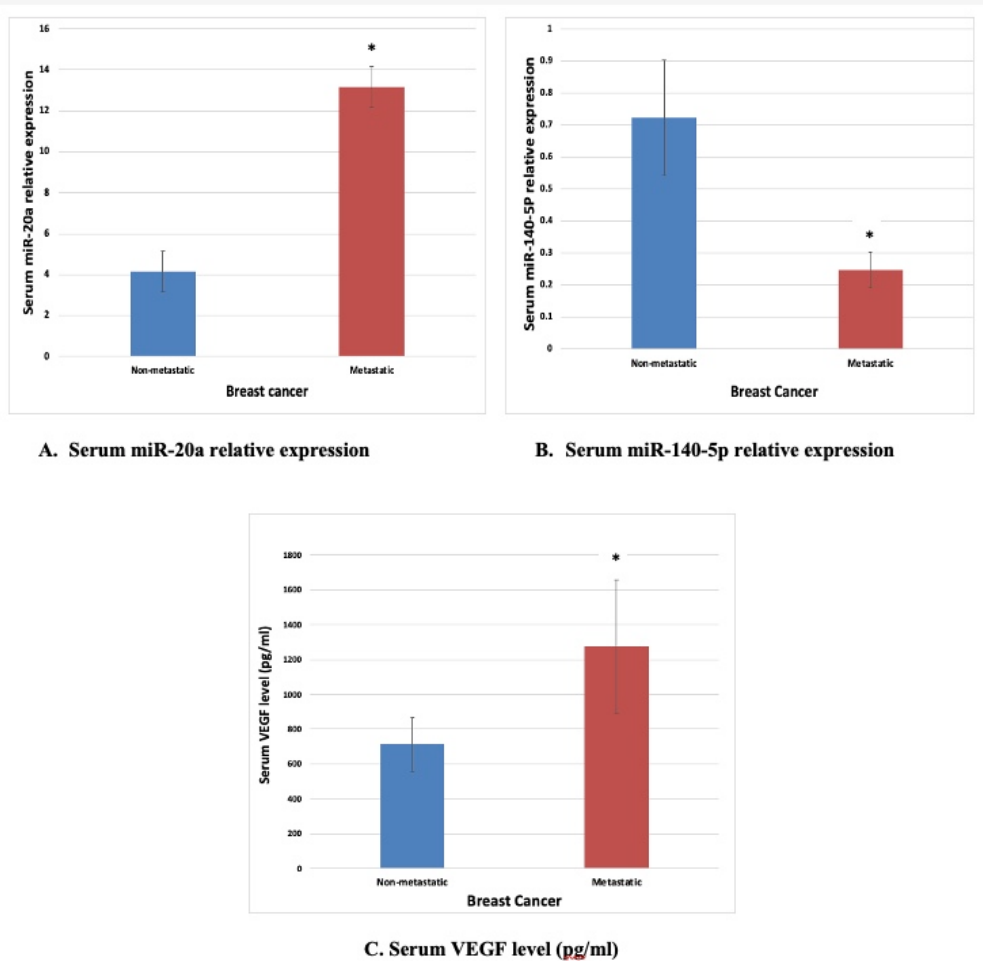

Fig. 1. Serum levels of the studied biomarkers in patients with metastatic or non-metastatic breast cancer: A. Serum miRNA 20a relative expression, B. Serum miRNA 140-5p relative expression and C. Serum VEGF level (pg/ml). Data is presented as mean ± SD. * Statistically significant at p<0.001.

Table 3. Comparison of hormone status in patients with metastatic or non-metastatic breast cancer

Variable		Non-Metastatic Breast Cancer (n=25)	Metastatic Breast Cancer (n=25)	P-value†	Odds ratio (OR)	95% CI for OR	P-value for OR‡
Estrogen receptor (ER)	*Negative*	4 (16.0%)	2 (8.0%)	0.497	2.19	0.36 to 13.22	0.393
	Positive	21 (84.0%)	23 (92.0%)				
Progesterone receptor (PR)	*Negative*	3 (12.0%)	2 (8.0%)	0.682	1.57	0.24 to 10.30	0.639
	Positive	22 (88.0%)	23 (92.0%)				
HER2/neu gene	*Negative*	25 (100.0%)	21 (84.0%)	0.059	10.67	0.54 to 209.66	0.119
	Positive	0 (0.0%)	4 (16.0%)				

Data are number (%).
OR = odds ratio, 95% CI = 95% confidence interval.
†. Fisher's exac t test.
‡. Z-test.

3.4. Predictive value of miR-20a, miR-140-5p, and VEGF

The ROC curve analysis indicated the significant predictive value for miR-20a, miR-140-5p, and VEGF in the prediction of metastasis over the well-established tumor markers CA 15-3 and Ki-67 with 100% specificity as shown in **Fig. 2A, 2B, 2C, 2D, and 2E**, respectively, and in **Table 4**. For CA 15-3, the AUC was 0.618 (95% CI: 0.469 to 0.751) with cut off value >39 and 44 % sensitivity while for Ki-67, the AUC was 0.773 (95% CI: 0.632 to 0.879) with cut off value ≤20 and 92% sensitivity. On the other hand, for miR-20a, the AUC was 1.000 (95% CI: 0.929 to 1.000) with cut off value >6.694 and 100% sensitivity while for miR-140-5p, the AUC was 1.00 (95% CI: 0.929 to 1.000) with cut off value ≤0.371 and 100% sensitivity. VEGF showed AUC 1.00 (95% CI: 0.929 to 1.000) with cut off value >800 and 100% sensitivity.

3.5. Association between biomarkers and lymph node involvement and tumor size

MiR-20a was significantly upregulated in N1/N2 patients (n= 36) (9.59±4.72) than N0 patients (n= 14) (6.28±4.51) at $p < 0.05$ while miR-140-5p was significantly down-regulated in N1 patients (n=36) (0.47±0.27) than N1/N2 patients (n= 14) (0.63±0.22) at $p< 0.05$ as shown in **Fig. 3**. However, CA 15-3, Ki-67, VEGF showed no significant association with lymph node involvement in the studied groups. Also, there was no relation between miR-20a relative expression, miR-140-5p relative expression, CA 15-3, Ki-67, or VEGF levels, and tumor size or hormone receptor status.

3.6. Correlations between various biomarkers in patients with breast cancer

Correlation analysis of the studied biomarkers is given in **Table 5**. MiR-20a relative expression was significantly positively correlated with Ki-67 levels ($r= 0.432$, $p<0.01$). Pearson's correlation also showed that miR-140-5p relative expression is negatively correlated with Ki-67 levels ($r= -0.388$, $p<0.01$) and strongly negatively correlated with miR-20a relative expression ($r= -0.873$, $p<0.0001$). Finally, VEGF was positivelyy correlated with Ki-67 levels ($r= 0.357$, $p<0.05$) and miR-140-5p relative expression ($r= -0.698$ $p<0.0001$), while it showed significant positive correlation with miR-20a relative expression ($r = 0.687$, $p<0.0001$)

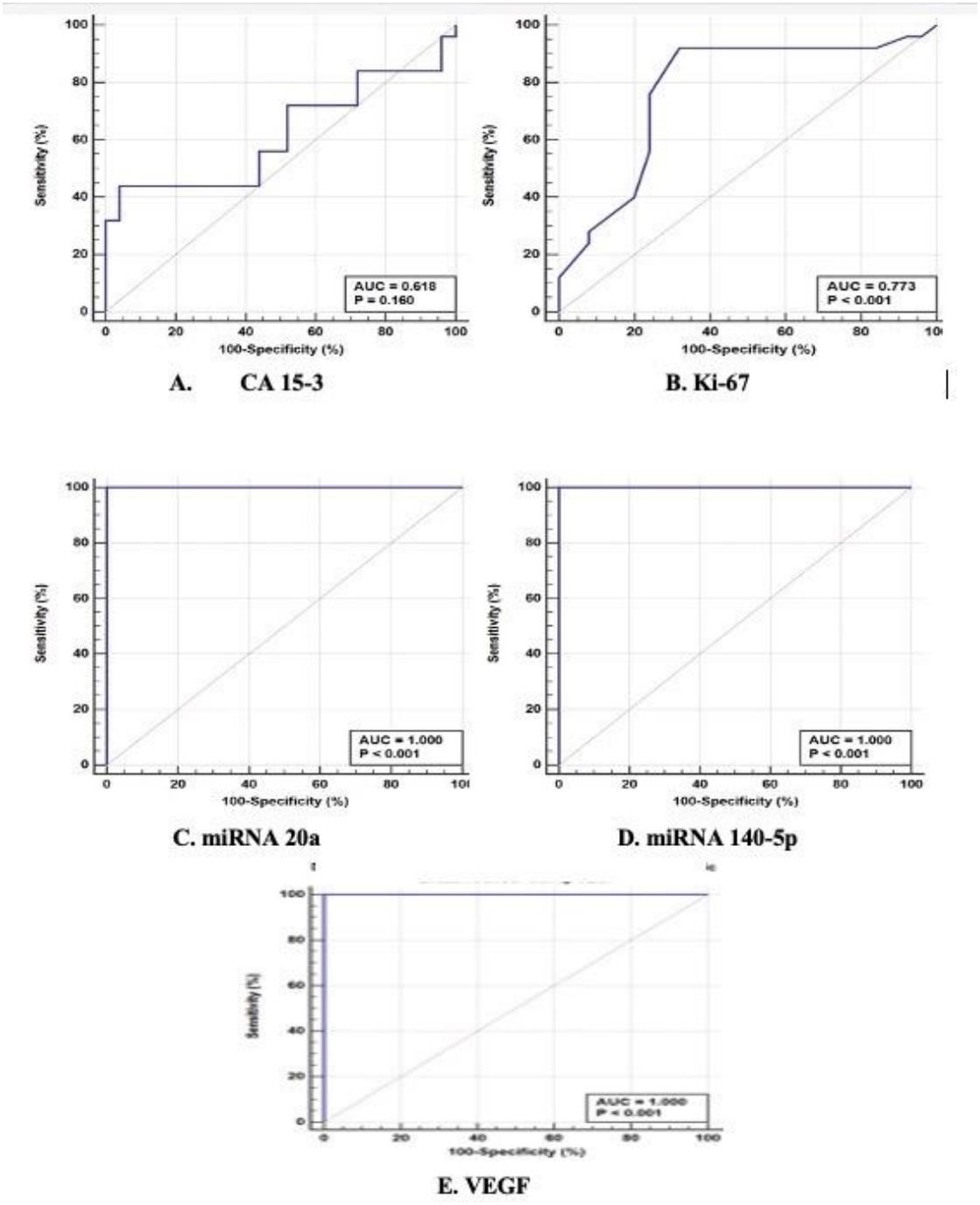

Fig. 2. Receiver-operating characteristic (ROC) curve for discrimination between metastatic and non-metastatic breast cancer using the studied biomarkers: A. CA 15-3 levels, B. Ki-67 levels, C. miRNA 20a, D. miRNA 140-5p and E. VEGF

Table 4. Receiver-operating characteristic (ROC) curve analysis for discrimination between metastatic and non-metastatic breast cancer

	Biomarker				
ROC metric	**CA 15-3**	**Ki-67**	**miRNA 20a**	**miRNA 140-5p**	**VEGF**
AUC	0.618	0.773	1.000	1.000	1.000
SE	0.0838	0.0717	0.000	0.000	0.000
95% CI	0.469 to 0.751	0.632 to 0.879	0.929 to 1.000	0.929 to 1.000	0.929 to 1.000
z statistic	1.404	3.805	-	-	-
P-value (AUC_0=0.5)	0.1603	0.0001	<0.0001	<0.0001	<0.0001
Youden index J	0.40	0.60	1.00	1.00	1.00
Associated criterion (Cut-off)	>39	≤20	>6.694	≤0.371	>800
Sensitivity (%)	44	92	100	100	100
95% CI	24.4 – 65.1	74.0 – 99.0	86.3 – 100.0	86.3 – 100.0	86.3 – 100.0
Specificity (%)	96	68	100	100	100
95% CI	79.6 – 99.9	46.5 – 85.1	86.3 – 100.0	86.3 – 100.0	86.3 – 100.0

ROC = receiver-operating characteristic curve, AUC = area under the ROC curve, SE = standard error, 95% CI = 95% confidence interval, J-index = ([sensitivity + specificity] – 1).

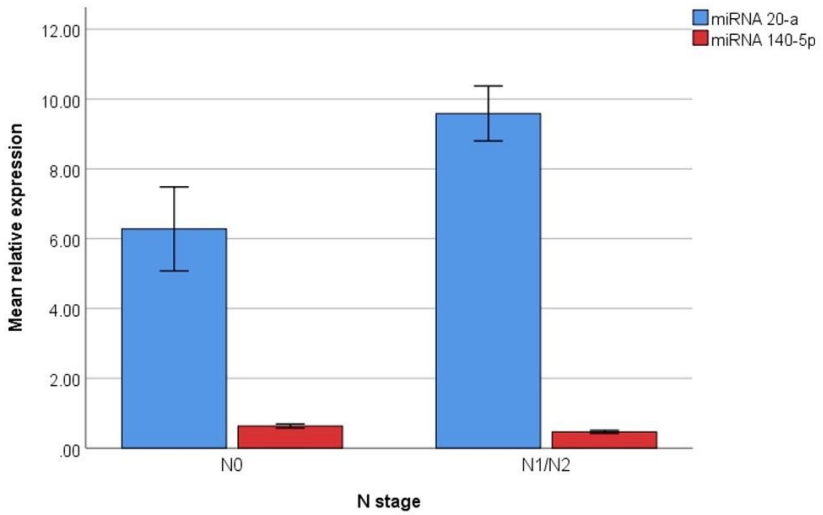

Fig. 3. Serum miRNA 20a and miRNA 140-5p relative expression in patients with or without LN involvement.

Table 5. Correlations between various biomarkers in patients with breast cancer

Variable		CA 15-3	Ki-67	miRNA 20a relative expression	miRNA 140-5p relative expression
Ki-67	*Pearson r*	-0.191			
	P-value	0.185			
miRNA 20a relative expression	*Pearson r*	0.193	0.432**		
	P-value	0.179	0.002		
miRNA 140-5p relative expression	*Pearson r*	-0.279	-0.388**	-0.873**	
	P-value	0.050	0.005	<0.0001	
VEGF	*Pearson r*	0.123	0.357*	0.687**	-0.698**
	P-value	0.3964	0.011	<0.0001	<0.0001

*. Statistically significant at the P < 0.05 level.
**. Statistically significant at the P < 0.01 level.

4. DISCUSSION

MiRNAs have emerged as important regulators of tumor progression and metastasis in breast cancer through mediating angiogenesis, EMT, the Warburg effect, and the tumor microenvironment **[32]**. However, their effective clinical role as diagnostic, prognostic, predictive, and therapeutic guidance tools still require further analysis. Given the high incidence of breast cancer in women, with the development of distant metastases accounting for 90% of breast cancer deaths **[3]**, it is imperative to understand the underlying mechanisms of its metastatic progression and identify biomarkers to improve the outcome of these patients.

Angiogenesis is a pivotal process for breast cancer progression and metastases **[13, 14]**. Oncogenic miRNAs have been found to stimulate the angiogenic process by regulating the expression of different proteins involved in angiogenesis, thereby promoting breast cancer metastasis **[13]**. In this study, the expression of miR-20a was significantly up-regulated in breast cancer samples in comparison to healthy subjects. This finding is in agreement with a systematic review and metanalysis showing upregulated levels of circulating miR-20a in patients with glioblastoma, non-small-cell lung cancer, gastric cancer, prostate cancer, and lymphoma compared with normal healthy control **[33]**. In addition, we observed higher levels in patients with metastatic breast cancer in comparison to non-metastatic patients. It was previously shown that miR-20a induces angiogenic effects in breast cancer cell lines and contributes to the growth of an abnormal vascular mesh **[13]**. It was shown that miR-20a targets RUNX3 in triple-negative breast cancer cells, promoting their growth **[34]**. Another study showed that miR-20a-5p/HMGA2

axis is involved in LncRNA HOTAIR mediated cell growth, invasion, apoptosis, and migration in breast cancer **[35]**. MiR-20a was also shown to be involved with MAPK1 and c-Myc to regulate breast carcinogenesis and resistance, in a regulatory feedback loop **[36]**.

Additionally, miR-20a may contribute to breast tumorigenesis by promoting genomic damage and instability through loss of autophagy **[37]**. Noteworthy, the expression of miR-20a in our study was found to be positively correlated to VEGF protein levels. This finding is in agreement with previous data showing that miR-20a expression is related to a high-risk angiogenic profile related to VEGF, as indicated by an increase in mean vessel size, high expression of VEGF, and the presence of microvascular proliferation **[13]**.

MiR140-5p is a tumor suppressor gene that has been reported to be down-regulated in diverse cancers including lung cancer, gastrointestinal cancers, and prostate cancer. In alignment, this study showed reduced expression of circulating miR-140-5p in breast cancer samples in comparison to the control group. Moreover, the metastatic group exhibited lower expression of miR-140-5p in comparison to the non-metastatic group. These findings are in agreement with Lu *et al.* study **[16]**, which showed lower expression of miR-140-5p in breast cancer tissue samples and metastatic breast cancer compared with the adjacent normal tissues and non-metastatic cancer, indicating that circulating levels of miR-140-5p can reflect its tissue expression.

It was previously shown that miR-140-5p expression is reduced in breast cancer stem cells (BCSCs), and its mimics could enhance the sensitivity of BCSCs to doxorubicin through targeting the Wnt1/ABCB1 pathway **[38]**. MiR-140-5p has several immune-related targets that regulate cell cycle transition and cell proliferation. It was reported to suppress the proliferation of esophageal cancer and regulate the cell invasion through controlling Slug expression **[16]**. MiR-140-5p acts also as a tumor suppressor in ovarian cancer **[26]** and inhibits cervical cancer cell phenotypes via downregulating FEN1 **[25]**. MiR-140 was also shown to antagonize the effect of Retinoblastoma depletion on the process of carcinogenesis **[39]**. Our study showed a significant negative correlation between miR-140-5p expression and VEGF protein levels. This can be explained by the finding of a previous study demonstrating that miR-140-5p inhibited breast cancer cells invasion and angiogenesis via targeting VEGF-A 3′-UTR **[16]**. Similarly, miR-140-5p was shown to inhibit angiogenesis of larynx carcinoma **[40]** and non-small cell lung cancer cells **[24]** through targeting VEGF-A.

Serum VEGF levels were significantly elevated in the enrolled breast cancer patients in comparison to healthy controls, which is in line with previously published studies **[22, 41]**. In addition to its role in tumor angiogenesis, VEGF exerts autocrine functions that promote migration and invasion of breast cancer **[42]**. Moreover, higher levels were observed in metastatic breast cancer patients in comparison to the non-metastatic group. VEGF signaling is involved in the survival and dissemination of breast cancer cells that may be independent of angiogenesis by enabling evasion of apoptosis and progression towards invasive and metastatic disease **[43]**.

In this study, miR-20a, miR-140-5p, and VEGF could significantly discriminate between metastatic and non-metastatic breast cancer patients with 100% specificity and sensitivity indicating their potential value in identifying breast cancer patients that are at higher risk for recurrence and development of distant metastasis.

As regards to the association between the studied biomarkers and clinicopathological characteristics, miR-20a expression was

significantly upregulated and miR-140-5p was downregulated in breast cancer patients with lymph node involvement; suggesting their association with tumor aggressiveness. Similarly, a previous study showed that the expression of miR-20a was significantly higher in tumor tissues with more extensive nodal involvement. Although higher expression of miR-20a was previously noted in ER-negative tumors with high grade **[13]**, however, no significant association was noted in this study between miR-20a, miR-140-5p, or VEGF and hormone receptor status, tumor size, or histological grade.

Increased Ki-67 expression is known to be associated with aggressive and highly proliferative disease **[44]**. This might explain the observed positive correlation of Ki-67 to miR-20a expression and its negative correlation to miR-140-5p expression. This finding is in line with Sakurai *et al* study that illustrated a panel of oncogenic miRNAs positively associated with high Ki-67 while tumor suppressor miRNAs were found to be inversely correlated to Ki-67 **[45]**. Ki-67 was also found to be positively correlated to VEGF levels. This may be explained by the fact that the tumor proliferative status can reflect the invasiveness and metastatic potential of a cancer cell **[46]**. A previous study also showed a significant association between Ki-67 and VEGF receptor expression in breast cancer, indicating that tumors with high cell proliferation also have increased angiogenesis **[47]**.

Taken together, our results contribute to the evolving understating of the involvement of molecular deregulation of miRNAs in breast carcinogenesis and provide candidate biomarkers that may contribute to the optimization of breast cancer management. Nonetheless, we are limited by the small sample size and thus further prospective studies on larger cohorts including patients with benign lesions are warranted to confirm the clinical validity of our findings. However, this study may represent a step to establish a clinically useful panel of circulating miRNAs that can be used in the clinical oncology practice as prognostic or predictive tools. The correlation between these miRNAs with VEGF protein levels raises the intriguing possibility of using them as biomarkers of high-risk angiogenic profiles.

Conclusions

Our results provide valuable insights and further advance the potential value of circulating miRNAs in liquid biopsy of breast cancer patients. We, herein, highlight the clinical utility of miR-20a and miR-140-5p as non-invasive prognostic and predictive tools to discriminate between metastatic and non-metastatic breast cancer. This will in turn aid in the personalized clinical management of breast cancer patients. These results might also be relevant for using miR-20a and miR-140-5p as targets for the development of miRNA-based antiangiogenic treatments for breast cancer.

List of Abbreviations

miRNA, MicroRNA; VEGF, Vascular endothelial growth factor; ROC, Receiver-operating characteristics; 3′-UTR, 3′-untranslated region; RT-qPCR, real-time quantitative polymerase chain reaction; SD, standard deviation; CI, confidence interval; LN, lymph node; ER: Estrogen receptor; EMT, epithelial-mesenchymal transition; PR: Progesterone receptor; HER2/neu, human epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; cDNA, Complementary DNA; AUC, the area under the curve.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethical Committee, Faculty of

Pharmacy, Ain Shams University, and conducted according to the regulations and recommendations of the Declaration of Helsinki. Written informed consents were signed and collected from all study participants.

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data analyzed during this study are all included in the main manuscript.

Competing interests

No competing interests were declared by the authors.

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