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ORIGINAL ARTICLE

Aberrant Expression of Circulating MicroRNA-373 and MicroRNA-660 as Biomarkers for Diagnosis of Breast Cancer in Egyptian Females

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ABSTRACT

Background: *Breast cancer (BC) is a leading cause of cancer-related mortality in women worldwide. Early detection significantly improves outcomes, yet current diagnostic methods, such as mammography and biopsy, have limitations, particularly in detecting early-stage disease. Circulating microRNAs (miRNAs) have emerged as promising non-invasive biomarkers for cancer detection.* ***Objective:*** *This study aims to evaluate the expression of serum miR-373 and miR-660-5p in breast cancer patients and their association with clinicopathological parameters.* ***Methodology:*** *This is a case-control study was conducted on 25 breast cancer patients and 25 age-matched healthy controls. Serum miRNA-373 and miRNA-660-5p levels were measured using quantitative real-time polymerase chain reaction (qPCR). Clinical and pathological data, including tumor stage, histological type, and molecular markers, were collected. Statistical analysis was performed to assess the differences in miRNA expression and their correlations with clinicopathological parameters.* ***Results:*** *Breast cancer patients exhibited significantly higher serum levels of miR-373 and miR-660-5p compared to controls ($p < 0.001$ and $p = 0.030$, respectively). MiR-373 expression was significantly associated with advanced tumor stages and nodal involvement ($p = 0.008$). However, miR-660-5p showed no significant correlation with clinicopathological features. The combined use of miR-373 and miR-660-5p improved diagnostic accuracy, with an area under the curve (AUC) of 0.859.* ***Conclusion:*** *Serum miR-373 and miR-660-5p are potential non-invasive biomarkers for breast cancer detection. MiR-373, in particular, shows promise in identifying advanced disease stages. Combining these miRNAs enhances diagnostic accuracy, offering a potential tool for early breast cancer diagnosis.*

INTRODUCTION

Breast cancer remains one of the leading causes of cancer-related mortality among women worldwide, originating primarily in the breast's lobules or ducts. This complex and heterogeneous disease is driven by a combination of genetic, hormonal, and environmental factors, contributing to its various subtypes, which respond differently to available treatments[1]. Several tumor markers have been suggested for the evaluation and management of breast cancer including estrogen and progesterone receptors (ER/PR)[2]. Early detection is crucial for improving patient outcomes, as it significantly enhances the chances of successful intervention and survival. However, current diagnostic tools, including mammography, ultrasound, and biopsy, while effective, have limitations in terms of accessibility, cost, invasiveness, and detection accuracy, especially in early-stage cancers and among younger women with dense breast tissue[3]. Consequently, there is a growing need for novel, non-invasive diagnostic methods that can offer high sensitivity and specificity for early breast cancer detection [4-5].

The investigation of circulating microRNAs (miRNAs) as potential cancer biomarkers is one field with considerable potential. miRNAs are small, typically 22 nucleotides in length, non protein coding RNAs which post transcriptionally regulate gene expression by specifically binding to the 3' UTR of the target messenger RNAs (mRNAs) and causing either their transcriptional repression or degradation[6]. The process of creation of miRNAs is rather intricate and their over or under expression can disrupt normal cellular economy leading to events such as tumor formation and its stages. With regard to breast miRNAs, each can act as an oncogene or a tumor suppressor that regulate critical processes involved in proliferation,

apoptosis, metastasis and hormone receptor signaling[7].

Serum circulating miRNAs, found in the blood and other body fluids like saliva and in urine are easy to detect and highly stable which makes most of them a great biomarker for non-invasive diagnosis[3]. This stability comes from encapsulation of miRNAs in exosomes and aren't inflammatory cells or their complexing with the protein Argonaute2 which protects them from being degraded by RNAse[8]. This in turn means that circulating miRNAs have the potential for use in the diagnosis of breast cancer and monitoring of the progression of the disease.

However, among them, miR-373 and miR-660-5p stand out and have the potential to be used as diagnosis biomarkers in breast cancer[9-11]. The abnormal level of these miRNAs in the blood has been associated with breast cancer, hence their importance in this type of non-pervasive examination. Such measurement and analysis of these miRNAs may assist in understanding the molecular pathways of breast carcinoma and may improve the tumor's early diagnostic accuracy.

The current objective of this study is to determine the levels of miR-373 and miR-660-5p in the serum of female patients with breast cancer and to assess any possible relationships with clinicopathological parameters. This study includes examination of the circulatory miRNA for establishing the diagnostic aspects of such type of cancer thereby improving detection and management of the ailment.

METHODOLOGY

Ethical Approval:

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP). Ethical approval was obtained from the local ethics committee in the Faculty of Medicine, Assiut University, Assiut, Egypt (Approval NO. 17101468). Informed consent was obtained from all participants, detailing their rights, study purpose, procedures, risks, and benefits.

Study Population:

This is a case-control study was conducted with 50 females recruited between November 2022 and December 2023 and divided into two groups; **Group A (Cases Group)**: comprised 25 Female patients with breast cancer admitted to the Department of Medical Oncology, South Egypt Cancer Institute. Inclusion criteria for the case group included a documented diagnosis of breast cancer based on physical examination, imaging, and histopathology, no prior history of exposure to chemotherapy, radiotherapy, hormonal therapy, other malignancies, or chronic inflammatory diseases such as chronic hepatitis, rheumatoid arthritis (RA), or systemic lupus erythematosus (SLE), age of 20 years old or more and ability to provide blood samples. **Group B (Control Group)**: comprised 25 healthy Females with age matched to case group. Inclusion criteria for the control group were negative breast cancer diagnosis in the routine national breast cancer screening program and no history of other malignancies or chronic inflammatory diseases. Participants who did not meet the inclusion criteria were excluded from the study.

Data Collection:

The clinical data included breast cancer staging based on The American Joint Committee on Cancer (AJCC) TNM staging system (8th edition). Stages were categorized by Tumor size (T), lymph Node involvement (N), and Metastasis (M), as outlined by Zhu and Doğan[12]. Histopathological grading was performed using the Nottingham grading system, which assesses tumors based on tubule formation, nuclear pleomorphism, and mitotic count. The tumor types analyzed were ductal carcinoma (the most common type) and lobular carcinoma[13]. Molecular classification was based on immunohistochemistry, which identified four primary types: Luminal A, Luminal B, HER2-enriched, and Triple-negative[14]. The Ki-67 marker, a proliferation indicator, was measured. A level of ≥30% was considered indicative of high cellular proliferation[15]. In addition to age, breast cancer history in first-degree relatives and the menopausal status was documented, as it influences hormonal exposure and breast cancer risk[16].

Serum Preparation and RNA Extraction

Serum Preparation:

Venous blood (5 ml) was collected from each participant in serum separator tubes (SST) and left to clot at room temperature for 30 minutes. The samples were centrifuged at 4000 rpm for 10 minutes, and the serum was carefully transferred to storage tubes and stored at -80°C until RNA extraction.

RNA Extraction:

MiRNA was extracted using the MiRNeasy Mini Kit (QIAGEN, Catalog Number 217004, Germany). QIAzol Lysis Reagent was added to the serum sample, followed by chloroform addition and centrifugation to separate the phases. The aqueous phase was then mixed with ethanol and passed through RNeasy Mini spin column. Several wash steps using Buffer RWT and RPE were performed to purify the RNA. The final RNA was eluted with RNase-free water and stored at -80°C. RNA concentrations were determined using a nanodrop spectrophotometer.

Reverse Transcription and cDNA Preparation

Reverse transcription of miRNA was performed using the miRCURY LNA Reverse Transcription Kit (QIAGEN, Catalog Number 339340, Germany). A reaction mixture of 5x miRCURY RT Buffer, 10x RT Enzyme Mix, RNase free water, and RNA was prepared and incubated at 42°C for 1 hour. The inactivation was done at 95°C for 5 minutes, and the cDNA was stored at -20°C.

Quantitative Real-Time PCR (qPCR)

To measure the expression levels of the miRNA, a quantitative PCR assay was performed using the miRCURY LNA SYBR Green Kit (QIAGEN, Catalog Number 339345, Germany), and primer assay kit (QIAGEN, Catalog Number 339306, Germany) were used for targeting miRNA-373-5p, miR-660-5p, and the housekeeping miRNA-16-5p. With the SYBR Green Master Mix, newly suspended primers, cDNA, RNase-free water and ROX dye a reaction mix was assembled according to manufacturer instructions into a PCR plate. The Applied Biosystems 7500 Fast RT-PCR system was set for an initial heat activation at 95°C for 2 mins, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 56°C for 60 seconds.

Data Analysis:

Sample Size Calculation

Sample size calculation was done based on previous study of Hosseini Mojahed[17], by using G Power 3.1.9.2 program. Main outcome variable was Difference in miRNA expression levels between patients and controls. Statistical t-test was used, and power was 0.80.

Data Normalization:

miRNA-16 served as the endogenous control, and the ΔCt method was used to calculate the relative expression of miRNA-373-5p and miR-660-5p. Fold changes were calculated using the 2^-ΔΔCt method[18].

Statistical Analysis

Data analysis was performed using SPSS version 27. Categorical data were presented as frequencies and percentages, while numerical data were evaluated using the Shapiro-Wilk test. Depending on distribution, data were presented as means with standard deviations or medians with ranges. Statistical tests included the independent sample t-test, Mann-Whitney U test, Kruskal-Wallis test, chi-square test. ROC curve analysis was performed to assess the discriminative ability of miRNA-373 and miRNA-660, and statistical significance was set at P value < 0.05.

RESULTS

Table 1 shows that there was no statistically significant difference between cases and controls in terms of age, menopausal status, or family history of breast cancer ($p > 0.05$).

Table 1: Demographic Data of Breast Cancer Patients and Controls

Variables	Cases (n=25)	Controls (n=25)	P-Value
Age in years	52.20±13.13 (25-78)	47.96±9.56 (30-66)	0.198*
Menopausal Status			0.999**
Premenopausal	14 (56.0%)	14 (56.0%)	
Postmenopausal	11 (44.0%)	11 (44.0%)	
Family History			0.999**
Positive	3 (12.0%)	2 (8.0%)	
Negative	22 (88.0%)	23 (92.0%)	

Data expressed as Mean ± SD or frequency (%).
*Independent Sample T test; **Chi-square test.

Table 2 provides a summary of the histopathological type and grade, TNM staging, and molecular markers of breast cancer patients, indicating that the majority of patients were in Stage II (36%) or Stage III (40%), and most had invasive ductal carcinoma (92%).

Table 2: Histopathological Type and Grade, TNM Staging, and Molecular Markers of Breast Cancer Patients

Variables	Total (n=25)	%
Histopathological Type		
Invasive Ductal Carcinoma	23	92.0%
Invasive Lobular Carcinoma	2	8.0%
Histopathological Grade		
Grade II	19	76.0%
Grade III	6	24.0%
T Staging		
T1	6	24.0%
T2	14	56.0%
T3	5	20.0%
N Staging		
N0	7	28.0%
N1	5	20.0%
N2	11	44.0%
N3	2	8.0%
M Staging		
M0	22	88.0%
M1	3	12.0%
Final TNM Staging		
Stage I	3	12.0%
Stage II	9	36.0%
Stage III	10	40.0%
Stage IV	3	12.0%
Early Stages (I, II)	12	48.0%
Advanced Stages (III, IV)	13	52.0%
Estrogen Receptors		
Positive	17	68.0%
Negative	8	32.0%
Progesterone Receptors		
Positive	15	60.0%
Negative	10	40.0%
HER2 Status		
Positive	4	16.0%
Negative	21	84.0%
Ki-67 Index		
<30% (Low to Intermediate)	8	32.0%
≥30% (High Proliferative)	17	68.0%
Molecular Subtypes		
Luminal A	14	56.0%
Luminal B	3	12.0%
HER2+ or Enriched	1	4.0%
Triple Negative	7	28.0%

Figure 1 illustrates that the median expression of miRNA-373 was significantly higher in breast cancer patients compared to controls (11.41 (0.57-506.12) vs. 1.01 (0.57-1.95), $p < 0.001$), and the median expression of miRNA-660 was also significantly elevated in patients compared to controls (2.61 (0.14-289.70) vs. 1.29 (0.15-2.71), $p = 0.030$).

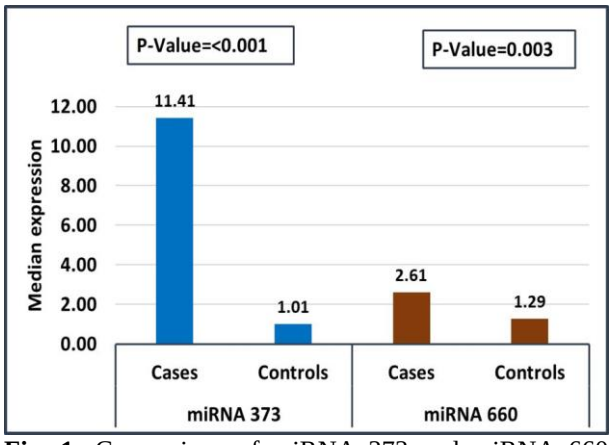

Fig. 1: Comparison of miRNA 373 and miRNA 660 expression between patients with breast cancer and controls

Table 3 shows no significant associations between the expression of miRNA-373 and miRNA-660 with menopausal status, family history, histopathological type, or grade. However the miRNA-373 expression was significantly higher with increasing staging of lymph node ($p = 0.008$) and advanced stages disease ($p = 0.001$), while miRNA-660 did not show a significant association with TNM staging or molecular markers. Also, Figure 2 shows statistically significant higher expression of miRNA 373 in breast cancer patients with lymph node involvement (N positive) in comparison to those without lymph node involvement (N negative) ($p = 0.009$).

Table 3: Association between Demographic Data, Histopathological Type and Grade, TNM Staging, Molecular Markers, and Expression of miRNA-373 and miRNA-660 in Breast Cancer Patients

Variables	miRNA-373 (Median, range)	P-Value	miRNA-660 (Median, range)	P-Value
Menopausal Status		0.934		0.547
Premenopausal	10.78 (0.57-253.06)		2.82 (0.15-57.21)	
Postmenopausal	11.57 (0.57-506.12)		1.40 (0.14-289.70)	
Family History		0.180		0.277
Positive	19.07 (19.07-73.68)		5.09 (2.61-37.05)	
Negative	9.68 (0.57-506.12)		1.53 (0.14-289.70)	
Histopathological Type		0.581		0.548
Invasive Ductal Carcinoma	11.41 (0.57-506.12)		1.50 (0.14-289.70)	
Invasive Lobular Carcinoma	9.83 (0.59-19.07)		8.81 (2.61-15.01)	
Histopathological Grade		0.899		0.203
Grade II	11.41 (0.57-506.12)		1.57 (0.14-289.70)	
Grade III	10.86 (1.50-19.07)		13.39 (0.91-37.05)	
T Staging		0.211		0.544
T1	7.54 (0.57-19.07)		1.95 (0.38-4.46)	
T2	10.31 (0.57-253.06)		3.29 (0.14-289.70)	
T3	53.94 (0.59-506.12)		5.34 (0.14-36.46)	
N Staging		**0.008**		0.560
N0	1.50 (0.57-11.57)		1.30 (0.15-15.01)	
N1	4.93 (0.57-19.07)		2.61 (0.38-57.21)	
N2	32.52 (1.54-506.12)		1.57 (0.14-289.70)	
N3	36.50 (19.07-53.94)		20.90 (5.34-36.46)	
M Staging		0.315		0.242
M0	10.78 (0.57-506.12)		2.09 (0.14-289.70)	
M1	19.07 (5.59-253.06)		16.31 (1.50-36.46)	
Final TNM Staging		**0.001**		0.335
Early Stages (Stage I, II)	2.21 (0.57-19.07)		1.95 (0.15-57.21)	
Advanced Stages (Stage III, IV)	32.52 (01.54-506.12)		5.09 (0.14-289.70)	
Estrogen Receptors		0.838		0.641
Positive	11.57 (0.57-253.06)		3.02 (0.14-37.05)	
Negative	10.78 (0.59-506.12)		1.24 (0.14-289.70)	
Progesterone Receptors		0.890		0.868
Positive	11.57 (0.57-253.06)		3.02 (0.14-37.05)	
Negative	10.78 (0.59-506.12)		1.43 (0.14-289.70)	
HER2 Status		0.711		0.120
Positive	10.39 (0.60-19.07)		1.08 (0.38-2.61)	
Negative	11.41 (0.57-506.12)		4.46 (0.14-289.70)	
Ki-67 Index		0.062		0.683
<30% (Low to Intermediate)	1.52 (0.57-53.94)		7.90 (0.14-57.21)	
≥30% (High Proliferative)	15.49 (0.57-506.12)		1.57 (0.14-289.70)	
Molecular Subtypes of Breast Cancer		0.976		0.432
Luminal A (HR+/HER2-)	12.33 (0.57-253.06)		4.77 (0.14-37.05)	
Luminal B (HR+/HER2+)	11.57 (0.60-19.07)		1.29 (0.38-2.61)	
HER2+ or Enriched (HR-/HER2+)	9.21		0.88	
Triple Negative (HR-/HER2-)	11.41 (0.59-506.12)		1.57 (0.14-289.70)	

Mann-Whitney U Test used to compare median between two groups.
Kruskal-Wallis test used to compare median between more than two groups.

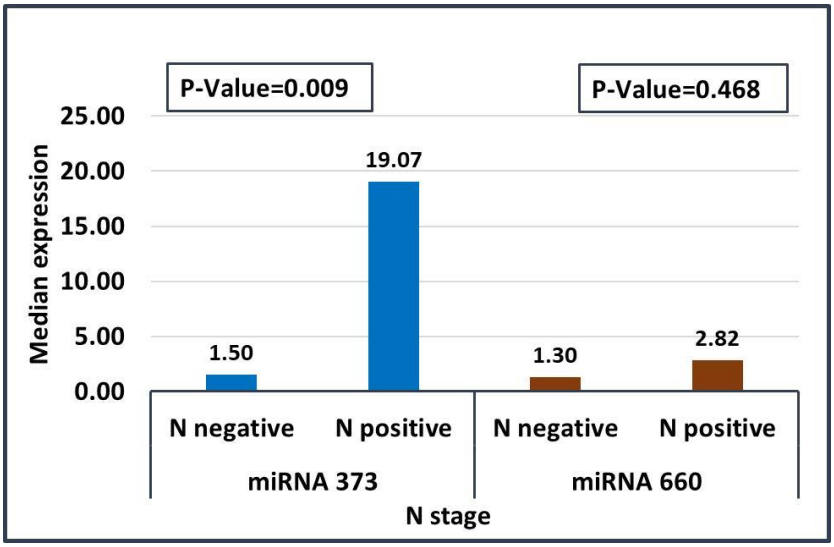

Fig. 2: N positive and N negative stage and expression of miRNA 373 and miRNA 660

Table 4 and Figure 3 demonstrate that the combined use of miRNA-373 and miRNA-660 achieved an accuracy of 88.0%, a sensitivity of 84.0%, and a specificity of 92.0% in predicting breast cancer, surpassing the diagnostic accuracy of either marker alone.

Table 4: Diagnostic Accuracy of miRNA-373 and miRNA-660 in Predicting Breast Cancer

Validity Measures	miRNA-373	miRNA-660	Combined (miRNA-373 + miRNA-660)
AUC (95% CI)	0.800 (0.663-0.900)	0.678 (0.531-0.803)	0.859 (0.732-0.941)
Cut-off	>1.46	>2.71	>1.46 + >2.71
Accuracy (%)	86.0%	70.0%	88.0%
Sensitivity (%)	80.0%	48.0%	84.0%
Specificity (%)	92.0%	92.0%	92.0%
Positive Predictive Value (%)	90.9%	85.7%	91.3%
Negative Predictive Value (%)	82.1%	63.9%	85.2%
P-Value	**<0.001**	**0.026**	**<0.001**

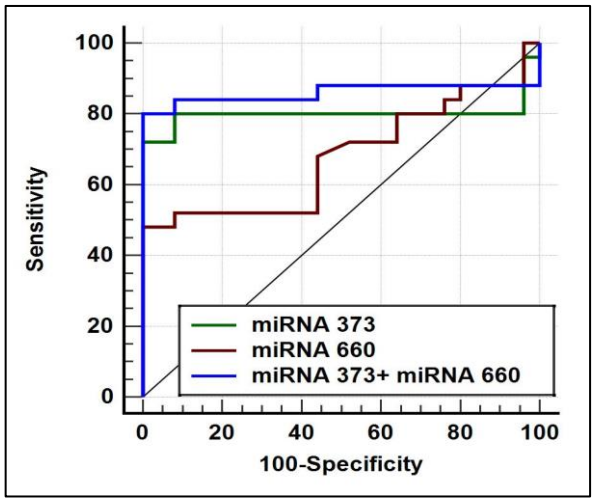

Fig. 3: ROC Curve for Diagnostic Ability of miRNA-373 and miRNA-660 in Predicting Breast Cancer

DISCUSSION

The findings of this study significantly reveal that miRNAs, particularly miR-373 and miR-660, hold promise as non-invasive biomarkers for breast cancer diagnosis and prognosis. This investigation aligns with a substantial number of studies that explore miRNA dysregulation in cancer, demonstrating that miR-373 and miR-660 are not only upregulated in breast cancer patients but also associated with disease progression and metastasis[19-21].

The role of miR-373 in the pathology of breast cancer is more intricate; miR-373 act as both oncogene and tumor suppressor. In our investigation, miR-373 level was significantly upregulated in breast cancer patients as compare to control, thus making it an attractive target for development of a diagnostic tool (P < 0.001). This is in agreement with Chen and colleagues'[19] report that a 4.38-fold increase in miR-373

level occurs in breast cancer patients who have lymph node metastasis and with results obtained by Eichelser et al.,[20] who found miR-373 to be elevated in triple negative breast cancer (TNBC). As well as, Wang et al.,[22] reported that miR-373 encourages epithelial-mesenchymal transition (EMT) and metastasis while Fan et al.,[23] and Cheng et al.,[5]discovered that miR-372, member of miR-520/373 family, induces cellular proliferation and progression of cancer. Additionally, Bakr et al.,[10] also reported that tissues express higher level of miR-373 in patients with larger tumor size and positive lymph nodes and hence it is likely to have prognostic as well as diagnostic value.

Nonetheless, certain studies show findings that contradict some other studies. For instance, Swellam et al.,[24] reported that suggesting that miR-373 alone might not be as robust but could be valuable as part of a panel. Similarly, Qu et al.,[25] and Keklikoglou et al.,[26] have provided evidence indicating the potential tumor-suppressor activity of miR-373 in corresponding situations. Wei et al.,[27] reported diverse functions of miR-373 in cancer. Understanding miR-373 biology, its double face in cancer, can promote cancer through miR-373-TXNIP-HIF1alpha-TWIST axis[19] and suppress by microRNA-373 targeting oncogenes[25-26] highlights the complications in the functioning of miRNA as an oncogenic microRNA does in breast cancer. The function of miR-373 as an oncogene or tumor suppressor may depend on the cellular context and genetic background [28].

Our results confirm that the expression of miRNA-373 does not correlate with menopausal status, family history, histopathological type or grade of disease stage in breast cancer patients .In agreement with Müller et al.,[29] found no correlation between miRNA-373 levels and any other clinical parameters in HER2 positive breast cancer. As well as, Bakr et al.,[10] where hormonal status seemed not to have any significant effect on the levels of miRNA-373, although the size of the tumor and lymph node involvement were factors that were positively correlated with levels of miRNA-373. Despite a result towards elevated expression levels of miRNA-373 in patients with a family history of breast cancer (P = 0.180) this finding did not reach statistical significance, but it was in support of Liu et al.,[30] where they reported the association between miRNA-373 and breast cancer genetic predisposition.

The similarity of invasive ductal and lobular carcinomas (P = 0.581) is supported by the findings of Chen et al.,[19] where miRNA-373 was noted to be overexpressed in various histological types. Nonetheless, there was a notable approval of miRNA-373 concerning nodal involvement, P = 0.008, and advanced TNM stages, P = 0.001. Eichelser et al.,[20] also obtained similar results in breast cancer, especially in aggressive subtypes. The validated prognostic factor for breast cancer, expression of miRNA -373 was shown to differ significantly between early and Advanced stages of the disease as noted by Bakr et al.,[10]. Analysis of ER, PR, and HER2 also showed no significant association which is also consistent with earlier studies like Lowery et al.,[31].

In line with the results of our study, the authors noted a significant upregulation of miR-660 in the peripheral blood of breast cancer patients as compared to the healthy controls (P = 0.030), which is consistent with studies by Peng et al.,[21] and Nashtahosseini et al.,[32] that reported upregulation of miR-660-5p in breast cancer and its correlation with tumor aggression. A similar action as the one identified in the present study has been reported for miR-660 in breast cancer progression because of its targeting of critical oncogenic signaling networks, including the PI3K/AKT/mTOR pathway, which is a key pathway targeted by miR-660-5p to drive tumor formation as was noted by Peng et al.,[21]. Additionally, Villarreal-García et al.,[11] also showed that overexpression of miR-660-5p in breast cancer patients was associated with worse survival estimates that confirm its predictive power even more.

MiR-660 levels were found to be higher in breast cancer patients than in healthy ones; however, this expression did not correlate well with factors such as menopausal status, histopathological type or TNM staging and Ki-67 index, while Shen et al.,[33] also support our results, who found no significant association between breast cancer patients and the expression of miR-660-5p in relation to these markers. Those findings relate to the study carried out by Villarreal-García et al.,[22] where it was reported that miR-660-5p expression did not correlate significantly with these clinical parameters either. This may imply that miR-660 does not follow the path of other traditional breast cancer prognostic indicators such as hormonal status and proliferation indices. Even so, as was the case with Peng et al.,[21] miR-660 was shown to have a role in enhancing breast cancer through the PI3K/AKT/mTOR pathway, implying that in some instances, particularly advanced disease patients with limited treatment options, miR-660 could serve as a novel treatment target.

According to our results, miR-373 is a better breast cancer biomarker than miR-660. When combined, miR-373 and miR-660 improve diagnostic accuracy, suggesting a synergistic effect.

The miR-373 study confirms previous findings on its breast cancer relevance. Eichelser et al.,[20] also had reported significant diagnostic value with an AUC of 0.879 indicating the ability to discriminate normal subjects from patients with breast cancer. On the other hand, relatively, lower performance was noted for miR-660, which however, was able to retain a high level of specificity (92%), which is important in minimizing chances of false positivity. Nashtahosseini et al.,[32] reported MiR-660-5p on the other hand had moderate levels of sensitivity (79%) and specificity (61%) for the

reliable detection of breast cancer. Diagnostic accuracy assessment using the combination of MiR-373 and MiR-660 maximized the AUC to 0.859 (increased accuracy), which was further supported by the use of Mohamed et al.,[34], who found multi-miRNA panels to be beneficial in breast cancer detection improving sensitivity and specificity.

CONCLUSION

In conclusion, our study conclusively proves that miR-373 and miR-660 are promising candidates as diagnostic biomarkers among breast cancer patients with optimal performance was elicited by miR-373. This combination of these particular miRNAs will help in the early diagnosis and assessment of the risk of developing breast cancer, through a non invasive method that is an adjunct to conventional diagnosis. These findings need broader studies to either confirm or disband them, and investigation about the use of these miRNAs as a modality of preventing breast cancer should be undertaken.

Conflict of Interest: The authors declare that they have no competing interests.

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