Circulating MicroRNA-21 As A Promising Marker for Early Detection of Breast Cancer and Disease Progression in Egyptian Females

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Abstract

Background: The traditional imaging techniques and the currently accepted markers such as serum CA15.3 have limited specificity and sensitivity to identify early stages breast cancer patients. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level via mRNA degradation and/or translational repression. They can modulate up to 60% of protein-coding genes in the human genome, one of which is miRNA-21. MiRNA-21 and its alterations play critical roles in breast cancer.

Aim of Study: The study aimed at assessing the diagnostic and prognostic role of miRNA-21 in breast cancer females.

Subjects and Methods: This case-control study was conducted on (150) breast cancer female patients and their age matched (50) healthy controls. All subjects recruited in the present study were subjected to measurement of serum microRNA-21 expression using quantitative real time PCR (qRT-PCR) in comparison to serum CA15.3.

Results: Serum miRNA-21 expression is upregulated in breast cancer patients compared to controls ($Z=4.4, p<0.01$), with disease advanced TNMstages ($H=24, p<0.001$) and negative estrogen receptor status ($Z=2.2, p<0.05$). At cut off level $1.07 (2^{\Delta\Delta Ct})$, miRNA-21 had 100% sensitivity and 90% specificity in discriminating patients with breast cancer from healthy controls. Meanwhile, CA15.3 had 70% sensitivity and 60% specificity in distinguishing between the two groups. At cut off level $3.33 (2^{\Delta\Delta T})$ miRNA-21 expression had 86.7% sensitivity and 100% specificity in discriminating patients with early breast cancer from patients with advanced disease. While serum CA15.3 had 80% sensitivity and 86.7% specificity in discriminating between the same patients groups.

Conclusion: Serum miRNA-21 levels are significantly higher in breast cancer patients compared to healthy subjects. Increased miRNA-21 expression levels correlated with disease stages, estrogen receptor presence reflecting prognosis and influencing constructing treatment modalities.

Key Words: Breast cancer – miRNA-21 – CA15.3 – qRT-PCR.

Introduction

BREAST cancer is the most common cancer in women and accounts for 29% of all cancers diagnosed every year worldwide. It is the second leading cause of cancer death in women, exceeded only by lung cancer, but it is the first in women under the age of 55 [1]. Therefore, the early diagnosis of breast cancer plays a critical role in its prognosis. Although mammograms are currently the best test for breast cancer screening, yet they have high false positive rates [2]. Hindered by their low sensitivity, the known serum markers such as CA 15.3 and BR27.29 are not used for screening of breast cancer [3]. This implies further investigations using expensive breast imaging and invasive biopsy. Therefore, the development of a more sensitive approach for early breast cancer diagnosis, particularly from benign lesions, is needed to supplement and/or complement existing detection methods [4].

The fundamental proposed regulatory role of miRNAs in a variety of biological processes such as cell proliferation, differentiation, and apoptosis suggests that differential expression of these transcripts may be exploited as a novel source of circulating molecular biomarkers [5]. The levels of circulating miRNAs packaged in exosomes differ between cancer patients and healthy donors and thus measuring circulating miRNAs levels may be useful in detection of early cancer and significantly contribute to treatment success [6]. More importantly, change in contents and amounts of secreted miRNAs are associated with disease stage and
regulation of the malignant phenotype in many cancers including colorectal cancer [7], lung cancer [8] and breast cancer [9,10].

MiRNA-21 is one of the oncogenic up-regulated miRNAs and its gene is located on chromosome 17q23.1 [11]. Several studies reported its significant up-regulation in breast cancer tissues up to 10-13 folds compared to the normal adjacent tissues and that higher level of exosomal miRNA-21 is significantly associated with the presence of circulating tumor cells [12,13].

In this context, the present study aimed at assessing the possible role of miRNA-21 as a marker for breast cancer and its association with disease progression in Egyptian females.

**Subjects and Methods**

**A- Subjects:**

This study was conducted on one hundred and fifty (150) female patients attending the Out-Patient Clinics and Surgery Department at El-Demerdash Hospitals, Ain Shams University in the period from March 2016 till July 2017. Subjects were divided into two groups; Group I (cancer patients) who were further subdivided into two subgroups, Subgroup (IA) early stages breast cancer (stage I and II) including (75) patients and Subgroup (IB) advanced stages breast cancer (stage III and IV) including (75) patients, according to TNM stage classification [14]. While Group II included fifty (50) apparently healthy female subjects serving as a healthy control group.

**Inclusion criteria:**

Confirmed diagnosis of breast cancer by biopsy, histopathological assessment and TNM classification.

**Exclusion criteria:**

- Patients with previous breast cancer treatment who had received chemotherapy, radiotherapy, or operation were excluded from the study groups.
- Patients with benign breast diseases as fibroadenoma, ductal epithelial hyperplasia and fibrocystic disease of the breast.

All studied individuals were subjected to full history taking, clinical examination with special emphasis on breast examination and mammogram. For patients only, radiological investigations as bone scan, CT scan and/or MRI as well as breast biopsy were done for histopathological examination and steroid receptors study.

An informed consent was taken from all subjects included in the study before history taking, physical examination and blood sample withdrawal. The procedures applied in this study were approved by The Ethical Committee of Human Experimentation of Ain Shams University, and are in accordance with the Helsinki Declaration of 1975.

**B- Sampling:**

Under complete aseptic conditions, five milliliters of venous blood were collected by venipuncture from each subject and were divided into; three milliliters evacuated in sterile EDTA-treated vacutainers then immediately transferred to the laboratory and centrifuged at (1900xg for 10 minutes). Plasma was separated into new aliquote tubes and stored at –80°C for the subsequent assay of circulating miRNA-21. Two milliliters were withdrawn in a plain tube vacutainer and left to clot for 15 to 20 minutes, then serum was separated by centrifugation (1500xg for 10 minutes), aliquoted and stored at –20°C for the subsequent assay of CA15.3.

**C- Methods:**

1- **Analytical methods:**

The assay of serum CA 15.3 was done on ARCHITECTi2000SR autoanalyzer (Abbott Laboratories Diagnostics, Division Abbott Park, IL 60064 USA) using reagents supplied by the manufacturer based on immunochemiluminescent assay. Assay of miRNA-21 was done by quantitative real time polymerase chain reaction (qRT-PCR).

**Assay of serum CA15.3:**

ARCHITECT CA 15-3 assay is a chemiluminescent microparticle immunoassay for the quantitative determination of DF3 antigen in human serum and plasma on the ARCHITECTi2000SR System using 115D8 and DF3 monoclonal antibodies (mAbs). It is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex. In the first step, sample, wash buffer and mAb (115D8) coated paramagnetic microparticles are combined. DF3 present in the sample binds to the 115D8 coated microparticles. After washing, mAb DF3 acridinium-labeled conjugate is added in the second step. Pre-Trigger and Trigger solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amounts of DF3 defined antigen in the sample and the RLUs detected by the ARCHITECTi2000SR optical system [15].
**Assay of serum MicroRNA-21:**

**MiRNA-21 RNA extraction:**

The total RNA, including small RNA, was isolated from plasma using the miRNeasy Mini Kit provided by Qiagen (Qiagen incorporation, 28159 Avenue, Stanford Valencia, CA 91355, USA) following manufacturer protocol.

**Measurement of RNA purity and concentration:**

3 μL of RNA solution were added to 72 μL of diethylpyrocarbonate (DEPC) water (dilution 1: 25) followed by vortex. Sample was read at 260 nm and 280nm using the Nanodrop spectrophotometer supplied by (Thermo Fisher Scientific, 3411 Silverside Road Wilmington, USA). Protein was detected at 230nm. Samples were considered with good RNA purity if A260/A280 ratio is 1.8-2.

**Reverse transcription:** Following manufacturer protocol, Reverse Transcription (RT) was performed on the extracted RNA prepared in the previous step using TaqMan MicroRNA Reverse Transcription kit. MiRNA-21 specific stem-loop primers and small nuclear miRNA- 16 were used as an endogenous control, all provided by Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Dr Foster City, California, 94404, USA).

**PCR amplification:** Amplification was performed on RT-PCR system using the TaqMan MicroRNA Assays kits provided by Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Dr Foster City, California, 94404, USA) in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines Figs. (1,2) [16].

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![Figure 1](miRNA_16.png)
**Fig. (1): Amplification Plot for miRNA-16.**

![Figure 2](miRNA_21.png)
**Fig. (2): Amplification Plot for miRNA-21.**
Detection and calculation of results: Results were reported in relative quantification. The relative expression level (fold change) for miRNA-21 in each sample was calculated by the comparative cycle threshold \( 2^{-\Delta \Delta Ct} \) method using miRNA-16 as an endogenous reference control. The cycle threshold \( (C_t) \) was determined for (miRNA-21 and miRNA-16) in each experimental sample using Step One PlusTM Software v2.1. The \( \Delta C_t \) in each experimental sample was calculated as \( \Delta C_t = (C_t_{\text{miRNA-21}} - C_t_{\text{miRNA-16}}) \). The average (mean) \( C_t \) was calculated for samples of the control samples group. Then \( \Delta \Delta C_t \) was calculated for every sample (malignant and control). Finally, the relative expression level (fold change) for miRNA-21 in each sample was calculated using \( (2^{-\Delta \Delta Ct}) \) formula \( \Delta \Delta C_t = \Delta C_t \) of every sample-average \( \Delta C_t \) of control samples [17].

2- Statistical methods: All statistical analyses were done using software version IBM SPSS (Statistical Package for the Social Sciences) statistics (Version 25.0, IBM Corp., USA, 2017-2018). Descriptive statistics of various studied parameters were expressed as percentage for qualitative data, median (M) and Inter-Quartile Range (IQR) which extends between the 25th and 75th percentiles (Q1 and Q3, respectively) for quantitative non-parametric data. Comparative statistics were done using the Wilcoxon’s rank sum test for non-parametric data. Correlation analysis was performed using Spearman’s rank correlation coefficient \( (r_s) \) for non-parametric data. Receiver Operator Characteristic (ROC) curve was constructed and optimal cut-off values for plasma miRNA-21 and serum CA15.3 were established and the Area Under the Curve (AUC) was calculated by the best sensitivity and specificity where the right angle at the upper left corner is the best diagnostic threshold (cut-off) of the parameter being varied. In all statistical analyses, \( p \)-value >0.05: Non significant; \( p \)-value <0.05: Significant; \( p \)-value <0.01 or 0.001: Highly significant.

Results

Descriptive statistics of the demographic and clinical parameters in Group I (breast cancer patients) and Group II (healthy controls) are shown in Tables (1,2).

Table (2) and Fig. (3) show the comparative statistics of the serum levels of CA 15.3 and miRNA-21 expression levels between the studied Groups I & II. CA 15.3 and miRNA-21 showed highly significant statistical increase in Group I compared to Group II (Z=2.3 and 4.4, respectively with \( p<0.01 \)).
As seen in Tables (3,4), a highly significant statistical increase was recorded among patients with different stages of breast cancer regarding CA 15.3 (H=15.7, \( p < 0.001 \)) and miRNA-21 (H=24, \( p < 0.001 \)) using Kruskall-Wallis test for non-parametric data.

As demonstrated in Table (5), a statistically significant increase in miRNA-21 expression levels was found in breast cancer patients with negative ER compared to breast cancer patients with positive ER, \( (Z=2.2, p < 0.05) \). On the other hand, no statistically significant difference was found in the expression levels of miRNA-21 between breast cancer patients as regards PR status \( (Z=1.8, p > 0.05) \). As regards CA 15.3 serum levels, no statistically significant difference was recorded between breast cancer patients as regards ER and PR status \( (Z=0.93 \text{ and } 0.4, \text{ respectively with } p > 0.05) \).

Our correlation study between CA 15.3 levels and miRNA-21 expression levels in breast cancer patients (Group I) is shown in Table (6) using Spearman’s rank correlation coefficient test. It revealed a highly statistically significant positive correlation between CA 15.3 levels and miRNA-21 expression levels \( (r_s=0.56, p < 0.001) \).

As shown in Table (7), serum CA 15.3 at the best chosen cut-off level of 12U/mL had a 70% diagnostic sensitivity, 60% specificity, 84% PPV, 40% NPV and 67.5% total efficacy. At the best chosen cut-off level of 1.07 \( (2^{-\Delta \Delta C_t}) \) for miRNA-21 expression levels, the marker displayed a 100% sensitivity, 90% specificity, 96.8% PPV, 100% NPV and 97.5% efficacy.
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Table (7): Diagnostic performance of CA 15.3 and miRNA-21 in discriminating Group I from Group II.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 15.3 (U/mL)</td>
<td>12</td>
<td>70</td>
<td>60</td>
<td>84</td>
<td>40</td>
<td>67.5</td>
</tr>
<tr>
<td>miRNA-21 (2ΔΔCt)</td>
<td>1.07</td>
<td>100</td>
<td>90</td>
<td>96.8</td>
<td>100</td>
<td>97.5</td>
</tr>
</tbody>
</table>

Table (8): Diagnostic performance of CA 15.3 and miRNA-21 in discriminating Group I from Group II.

<table>
<thead>
<tr>
<th>Parameter Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive Predictive Value (%)</th>
<th>Negative Predictive Value (%)</th>
<th>Diagnostic Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 15.3 (U/mL)</td>
<td>20.3</td>
<td>80</td>
<td>86.7</td>
<td>85.7</td>
<td>81.3</td>
</tr>
<tr>
<td>miRNA-21 (2ΔΔCt)</td>
<td>3.33</td>
<td>86.7</td>
<td>100</td>
<td>100</td>
<td>88.2</td>
</tr>
<tr>
<td>CA 15.3 (U/mL)</td>
<td>8/3.33</td>
<td>93.3</td>
<td>100</td>
<td>100</td>
<td>93.8</td>
</tr>
<tr>
<td>miRNA-21 (2ΔΔCt)</td>
<td>2ΔΔCt</td>
<td>93.8</td>
<td>100</td>
<td>93.8</td>
<td>96.7</td>
</tr>
</tbody>
</table>

Table (8): Diagnostic performance of CA 15.3 and miRNA-21 in discriminating between patients with early breast cancer (Subgroup IA) and patients with advanced breast cancer (Subgroup IB).

Discussion

Clinical trials using circulating miRNAs as cancer biomarkers are being carried out in the United States and other countries [18]. In recent years, with the advent of gene expression profiling technologies, an increasing number of studies have revealed the genetic association between miRNAs and different cancers such as breast cancer [9,10]. Even though there were several reports using circulating miRNA markers for breast cancer detection, they were quite inconsistent [19-24]. In view of the previous observations, our study aimed at assessing the clinical utility of circulating miRNA-21 in breast cancer patients and correlating its level with the routinely used marker CA15.3.

Results of the present study revealed significantly increased levels of serum CA15.3 in breast cancer patients compared to healthy controls. This goes in agreement with Zhang et al., [25], Shao et al., [26] and Fejzić et al., [27], who proved that serum levels of CA 15.3 increased significantly in patients with breast cancer more than healthy controls. They added that, CA 15.3 is expressed on the luminal surface of the normal glandular breast secretory epithelium and its expression and secretion are increased with malignant cell transformation.

Our study also revealed that serum levels of miRNA-21 expression were significantly higher in breast cancer patients than in healthy controls. These results were in agreement with the findings of a study by Gao et al., [12] on 89 breast cancer female patients who had a histologically confirmed diagnosis as compared to 55 healthy controls. The latter research group concluded that miRNA-21 is a useful diagnostic serum marker in breast cancer. Moreover, Zhang et al., [28] measured serum expression levels of miRNA-21 in 106 breast cancer participants whose diagnosis was confirmed by histopathology and they had not received chemotherapy, radiotherapy, or operation. Their results confirmed a statistically significant increase in patients compared to healthy participants. They concluded that elevated serum expression levels of miRNA-21 have great clinical value as a biomarker in breast cancer. Moreover, Si et al., [29] found that the level of miRNA-21 was significantly higher not only in serum samples but also in tissue samples of breast cancer patients compared to their healthy controls.

The underlying mechanism of connection between miRNA-21 and breast cancer is the location of miRNA-21 gene on chromosome 17q23.2. This region is frequently amplified in breast tumors and
that genetic amplification in tumor tissues is correlated with high expression of miRNA-21 [30]. Another possible cause of miRNA-21 up-regulation is due to the presence of a CpG island region, 2 kb upstream the mature miRNA-21 sequence that could be hypomethylated in breast cancer causing up-regulation of its expression [31].

The first study that examined the circulating miRNA-21 expression in an Egyptian population was done by Toraih et al., [32] followed by other studies including Motawi et al., [33] who reported increased expression of serum levels of miRNA-21 in 30 patients with different stages of breast cancer compared to the levels found in healthy control women. The study referred their results to the oncogenicity of miRNA-21 which promoted tumor growth, invasion, angiogenesis, and metastasis by targeting and suppressing several apoptotic and tumor suppressor genes, including PDCD4, TPM1, PTEN and MASPIN.

Our current study showed a highly statistically significant increase in CA15.3 levels among the four different stages of breast cancer in patients group compared to healthy participants. This was similar to results of Nisman et al., [34] and Shao et al., [26], who confirmed the significant association of CA15.3 with TNM stages of breast cancer. Higher serum levels of CA15.3 were more often detected in patients with advanced stage as the elevated levels are related to the tumor burden.

As for the miRNA-21 expression levels, our study marked the highly statistically significant rise in its expression among different TNM stages of the disease. Moreover, miRNA-21 expression levels can successfully differentiate between early stages (I & II) and late stages of breast cancer (III & IV). These results were supported by Toraih et al., [32] and Han et al., [35], who suggested that miRNA-21 is a sensitive and specific non invasive biomarker of breast cancer as well as an indicator for the invasiveness and reported that the serum level of miRNA-21 was significantly high in breast cancer at all TNM stages with significant rise with progression of patients stage. At the same time, Rodríguez-Martínez et al., [13] stated that during neoadjuvant treatment, exosomal miRNA-21 expression levels directly correlated with tumor size and higher levels of exosomal miRNA-21 significantly associated with the presence of circulating tumor cells and thus liquid biopsies based on exosomal miRNA-21 can be a complementary clinical tool for improving breast cancer diagnosis and prognosis.

Another important finding in the current study was the significant high miRNA-21 expression levels in negative ER breast cancer patients, which represents a valuable prognostic and predictive importance in management protocols of these patients. This agrees with a previous research work done by Lee et al., [36] and Wang et al., [37], who reported that ER negativity expression was significantly associated with high miRNA-21 expression. However, in our study, no statistically significant difference was detected between patients with positive and negative PR in the expression of miRNA-21. Similar results were reported by Si et al., [29] and Wang et al., [37], who found no association between high miRNA-21 expression levels and PR status. The high expression of miRNA-21 in tumor stroma is associated with a much poorer clinical outcome in ER and/or PR negative patients. This is attributed to miRNA-21 targeting PTEN in Triple-Negative Breast Cancer (TNBC) tissue which leads to down-regulation of the tumor suppressor gene PTEN. These findings suggested that anti-miRNA-21-based therapies could be a valuable treatment in breast cancer especially in TNBC cases [38].

Few studies examined the relation between CA15.3 and ER/PR status in breast cancer patients to evaluate the prognostic value of CA 15.3 and its correlation with the molecular subtypes of breast cancer [26]. In our study, no statistically significant change was detected in serum levels of CA 15.3 with the ER and PR status. Our results are almost similar to the results shown by Rasmy et al., [39], who measured CA 15.3 serum levels in 280 cancer breast females with 233 ER positive and 196 PR positive patients. They found only a borderline significant correlation between CA 15.3 levels and ER expression. In addition, there was no significant correlation between CA 15.3 levels and PR expression.

On the other hand, Atoum et al., [40] reported that ER positive status (39 cases) was strongly correlated with elevated CA15.3 level among the studied 72 female patients (39/72 ER+/PR+). However, Shao et al., [26] reported that the elevation of CA15.3 levels was significantly greater in patients with ER negative when measured in 432 patients with histopathological molecular subtypes (luminal A, luminal B, Her-2 positive and TNBC). This discrepancy in results can be attributed to the difference in the sample size enrolled in the other study or the histopathological type of the disease.

Our study also revealed that miRNA-21 expression levels are highly significantly positively
correlated with CA 15.3 levels which enforce its possible future role in diagnosis and follow-up of breast cancer patients. This agreed with the results of Han et al., [38], who found positive correlation between the two markers and proves our suggestion for the possible role of miRNA-21 in the disease.

The diagnostic performance of CA 15.3 was studied by ROC curve analysis, where at cut-off level of 12U/mL, CA 15.3 is able to differentiate between breast cancer cases and the control group with 70% sensitivity, 60% specificity, 40% NPV, 84% PPV and 67.5% efficacy. These results were comparable to those of Thriveni et al., [41], who stated that at a cut-off 35U/mL, a rather lower diagnostic sensitivity 51.1% but a higher specificity of 100%. In other studies done by Toraih et al., [32] and Han et al., [38], miRNA-21 in breast cancer patients revealed lower diagnostic sensitivity (66.7%) and specificity (86.7% and 88.8%), respectively for the detection of breast cancer patients from healthy controls.

At our best chosen cut off 1.07 (2^–ΔΔCt), miRNA-21 proved an outstanding diagnostic performance for early detection of breast cancer patients with 100% sensitivity, 90% specificity, 96.8% PPV, 100% NPV and 97.5% efficacy providing a new tool for the early effective management of the disease. Zhang et al., [28] observed a diagnostic performance of 77.4% sensitivity, 67.9% specificity at cut off 0.453 (2^ΔΔCt), ROC curve analysis was constructed to differentiate between patients with early breast cancer and patients with advanced breast cancer. CA 15.3 at cut-off level 20.3U/mL was able to differentiate between patients with early and advanced breast cancer with 80% sensitivity, 86.7% specificity, 85.7% PPV, 81.3% NPV and 83.3% efficacy. At different cut off 25U/mL chosen by Incoronato et al., [42] in their study, the diagnostic sensitivity was 75% and the specificity was 76% which proved low sensitivity of CA 15.3 for early detection of breast cancer.

This difference in diagnostic performance may be explained by the difference in sample size of the study, distribution of study population among different TNM stages of the disease and histopathological subtypes of the disease.

MiRNA-21 expression levels showed better performance in discriminating between patients with earlier stages (TNM stage I and II) of breast cancer and patients with advanced breast cancer (TNM stage III and IV) with 86.7% sensitivity, 100% specificity, 100% PPV, 88.2% NPV and 93.3% efficacy at cut off 3.33 (2^ΔΔCt). This agreed with Toraih et al., [32], who proved that miRNA-21 distinguishes patients with advanced breast cancer from patients with earlier stages with 87.5% sensitivity and 92.9% specificity.

In our current study on applying multi-ROC curve analysis for assessment of the diagnostic performance of combination of CA 15.3 at a cut-off level 8.0U/mL and miRNA-21 at a cut-off level of 3.33 (2^–ΔΔCt) for discriminating between patients with early breast cancer (TNM stage I and II) and patients with advanced breast cancer (TNM stage III and IV), it revealed a sensitivity of 93.3%, specificity 100%, PPV 100%, NPV 93.8% and efficacy 96.7%. Therefore, the combination showed better diagnostic sensitivity and efficacy than each marker alone.

MiRNA-21 expression levels are significantly higher in breast cancer patients compared to healthy subjects. This finding supports the introduction of miRNA-21 in the panel for screening and early detection of breast cancer. Furthermore, our study proved that increased miRNA-21 expression levels correlated with progression of breast cancer stages. Together with its increased expression levels with negative ER status, this enforces its possible role as a prognostic marker in breast cancer follow-up and disease management protocols.

Our study provided the initial data for Egyptian population which might be further validated on a larger scale by a prospective study in a multicenter clinical trial.

Declaration:
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