Clinical Utility of Circulating MicroRNA-21 in Breast Cancer
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ABSTRACT
Background: Breast cancer remains the most frequent type of cancer in females worldwide, with nearly 1.7 million new cases diagnosed in 2012. In Egypt, breast cancer is the most common malignancy among females accounting for 18.3% of total cancer cases in Egypt. Unfortunately, the traditional imaging techniques as well as the currently accepted markers such as serum CEA and CA 15.3 cannot adequately identify early stage patients. MiRNAs are a class of endogenous non-coding RNAs ~22 nucleotides in length, which regulate gene expression primarily at the post-transcriptional level and thus are involved in many cellular processes, such as cell proliferation, differentiation, and apoptosis. One of which is miRNA-21 and its alterations have been shown to play critical roles in breast cancer.

Materials and Methods: This is a pilot retrospective case-control study. We quantified serum microRNA-21 expression levels using qRT-PCR in 30 breast cancer patients and another 10 controls in comparison to CA 15-3 as a conventional marker of breast cancer used in clinical practice.

Results: MiRNA-21 expression levels in early diagnosis of breast cancer patients with a superior both sensitivity and specificity of 100% specially in comparison with CA 15.3 that displayed 70% sensitivity and 60% specificity.

Conclusion: MiRNA-21 levels are significantly higher in breast cancer patients compared to healthy subjects. Furthermore, we found that increased miRNA-21 expression levels correlated with progression of breast cancer stages.

Keywords: Breast cancer, miRNA-21, CA15.3

INTRODUCTION
Breast cancer remains the most frequent type of cancer in females worldwide, with nearly 1.7 million new cases diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women (1). In Egypt, breast cancer is the most common malignancy among females accounting for 18.3% of total cancer cases in Egypt (2).

Unfortunately, the traditional imaging techniques such as mammography and ultrasonography, as well as, the currently accepted markers such as serum CEA and CA 15.3 cannot adequately identify early stage patients. Therefore, there is an urgent need to search for better markers with higher sensitivity to vastly improve breast cancer diagnosis, staging and treatment (3). MiRNAs are a class of endogenous non-coding RNAs ~22 nucleotides in length, which regulate gene expression primarily at the post-transcriptional level and thus are involved in many cellular processes, such as cell proliferation, differentiation, and apoptosis. Deregulated expression of specific miRNAs that function as tumor suppressors or oncogenes is associated with the pathogenesis of cancers (4). MiRNA-21 is one of the oncogenic up-regulated miRNAs and its gene is located on chromosome 17q23.1. It has been found differentially expressed in breast cancer and normal tissues. It is significantly up-regulated in breast cancer tissues up to 10-13folds compared to the normal adjacent tissues, thus serving as a potential marker of breast cancer. Assessment of miRNA-21 in serum sample has the advantages of simple collection, less invasiveness, and easy monitoring (5).

MATERIALS AND METHODS
Study Participants
This study was conducted on 30 female patients attending the Surgery Department and Out-patient Clinics at El Demerdash Hospitals, Ain Shams University during the period from August 2014 till December 2015. Patients were classified into two groups. Group I (cancer patients) which was further classified into two subgroups, subgroup (Ia) early stages breast cancer (stage I and II) and subgroup (Ib) advanced stages breast cancer (stage III and IV), according to TNM stage classification. While group II included 10 apparently healthy female subjects served as a healthy control group. All studied individuals were subjected to full history, 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.

Sample Collection and Processing
Serum samples were collected from all participants for the assay of CA15.3. Plasma samples were collected from all participants for the assay of...
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circulating miRNA-21.

**Serum CA15.3 Assay:**
The analysis of serum CA15.3 was done on ARCHITECTi2000SR auto analyzer (Abbott Laboratories Diagnostics).

**MiRNA-21 Assay:**

**RNA Extraction**
The total RNA, including small RNA, was isolated from serum using the QiagenmiRNeasy Serum/Plasma Kit following the protocol supplied by the manufacturer.

**Reverse Transcription**
The TaqManTM MicroRNA Reverse Transcription kit (Applied Biosystems) and miRNA-21 specific stem–loop primers (Applied Biosystem, assay) were used for miRNA reverse transcription (RT) reaction. RT primer for small nuclear miRNA 16 (Applied Biosystems) was used as an endogenous control.

**qRT-PCR Analysis**

Real-time polymerase chain reaction (qRT-PCR) technology was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Mature miR-21 was quantified using the TaqMan_ MicroRNA assay (Applied Biosystems, assay), TaqMan_ endogenous control assay for miRNA-16(Applied Biosystems, assay), and Taqman_ Universal PCR master mix II, No UNG (Applied Biosystems).

**Data Analysis**
The fold change of miRNA expression in each patient sample or asymptomatic at-risk individual relative to the average expression in healthy controls were calculated based on the threshold cycle (CT) value using the equation of relative quantity = 2-DDCT method, where DDCT = (CT miRNA-21-CT miRNA-16)BC or HC.

**Statistical Analysis**
IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2015) was used for data analysis. As the data for miRNA-21 relative expression levels and CA 15.3 did not fit a Gaussian distribution, miRNA-21 and CA 15.3 levels were characterized by their median and range from the 25th to the 75th percentile, rather than their mean and coefficient of variation. The expression of miRNA-21 and CA 15.3 levels were calculated for different groups using the Mann–Whitney U (MW) and the Kruskall-Wallis (KW) tests. Spearman’s rank order correlation analysis was performed. P values were considered statistically significant below 0.05. The receiver operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was calculated to assess the best sensitivity and specificity for prediction of the optimum cutoff values of case-control status for serum miR-21 expression level and CA 15.3 level.

The study was approved by the Ethics Board of Ain Shams University.

**RESULTS**

Serum levels of CA 15.3 and miRNA-21 expression levels among studied group I and II: It revealed a highly statistically significant difference between the two groups as regard CA 15.3 and miRNA-21 (Z= 2.3 and 4.4, respectively with p < 0.01) using Wilcoxon’s Rank Sum test for non-parametric data as shown in table (1) and figure (1).

A statistically significant difference 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 shown in table (2) and figure (2).

Statistical comparison between CA15.3 and miRNA-21 in patients with different stages of breast cancer, subgroup Ia (stages I and II) and subgroup Ib (stages III and IV) as compared to each other using Wilcoxon’s Rank Sum test for non-parametric data: Table (3) recorded a statistically significant difference in CA 15.3 levels when each two groups were compared versus each other (stage I versus II, Z= 3.2, p <0.01), (stage II versus III, Z= 2.8, p <0.01), (stage III versus IV, Z= 3.2, p <0.01), (stage II versus IV, Z= 3.5, p <0.01), (stage I versus III, Z= 3.0, p <0.05) and (stage I versus IV, Z= 3.1, p <0.01). However, regarding miRNA-21 expression levels, it recorded a statistically significant difference on comparing stage III versus stage IV breast cancer (Z= 2.6, p <0.01), stage II versus stage IV breast cancer (Z= 3.5, p <0.01) and stage I versus stage IV breast cancer (Z= 2.6, p <0.01). However, no statistically significant difference was recorded regarding miRNA-21 expression levels on comparing other stages versus each other.

Correlation study between CA15.3 levels and miRNA-21 expression levels in breast cancer patients (group I) 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= 0.56, p <0.001) (Figure3). A statistically significant difference between miRNA-21 expression levels in breast cancer patients with positive ER compared to breast cancer patients with negative ER was found, (Z= 2.2, p < 0.05).
Receiver operating characteristic (ROC) curve analysis was applied to assess the diagnostic performance of CA15.3 and miRNA-21 in discriminating patients with breast cancer (group I) from healthy controls group (group II). At the best chosen cut-off level of 12 U/mL for CA 15.3, the diagnostic sensitivity, specificity, PPV, NPV and total efficacy were 70%, 60%, 84%, 40% and 67.5%, respectively with AUC 0.734. At the best chosen cut-off level of 1.09 (2-ΔΔCT) for miRNA-21 expression levels, the marker displayed a 100% diagnostic sensitivity, specificity, PPV, NPV and total efficacy with AUC 1.000 figure (4) and table (4).

**Table (1):** Descriptive and Comparative Statistics of the Serum levels of CA 15.3 and miRNA-21 Expression Levels in Group I and Group II Using Wilcoxon's Rank Sum Test for Non-Parametric Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (n=30) M(Q1-Q3)</th>
<th>Group II (n=10) M (Q1-Q3)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-15.3 (U/mL)</td>
<td>19.7 (9.8-71.3)</td>
<td>11.0 (7.4-16.3)</td>
<td>2.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>miRNA-21 (2-ΔΔCt )</td>
<td>3.1 (2.2-5.2)</td>
<td>1.02 (0.93-1.06)</td>
<td>4.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

![Figure (1): Median values of CA 15.3 (U/mL) and miRNA-21 (2-ΔΔCt) in breast cancer patients versus healthy control.](image)

**Table (2):** Statistical Comparison of CA15.3 and miRNA-21 Expression Levels among the Subgroups of Breast Cancer Patients Using Kruskall-Wallis Test for Non-Parametric Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subgroup Ia</th>
<th>Subgroup Ib</th>
<th>H</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage I</td>
<td>Stage II</td>
<td>Stage III</td>
<td>Stage IV</td>
</tr>
<tr>
<td></td>
<td>(n=6) M (Q1-Q3)</td>
<td>(n=9) M (Q1-Q3)</td>
<td>(n=7) M (Q1-Q3)</td>
<td>(n=8) M (Q1-Q3)</td>
</tr>
<tr>
<td>CA15.3(U/mL)</td>
<td>16.5 (11.9-31.0)</td>
<td>10.0 (7.5-17.1)</td>
<td>21.0 (8.8-50.0)</td>
<td>218.0 (53.3-556.5)</td>
</tr>
<tr>
<td>miRNA-21 (2-ΔΔct )</td>
<td>1.6 (1.2-1.9)</td>
<td>2.6 (2.3-3.0)</td>
<td>3.5 (3.2-4.8)</td>
<td>7.4 (5.9-8.7)</td>
</tr>
</tbody>
</table>

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Fig. (2): Median values of CA 15.3 (U/mL) and miRNA-21\(^{(2^{-\Delta\Delta C_t}})}\) in patients with different stages of breast cancer.

Table (3): Statistical Comparison between CA15.3 and miRNA-21 in Patients with Different Stages of Breast Cancer, Subgroup Ia (stages I and II) and Subgroup Ib (stages III and IV) as Compared to each Other Using Wilcoxon's Rank Sum Test for Non-Parametric Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CA 15.3 (U/mL)</th>
<th>miRNA-21 ((2^{-\Delta\Delta C_t}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z</td>
<td>P</td>
</tr>
<tr>
<td>Stage I/Stage II</td>
<td>3.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage II/Stage III</td>
<td>2.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage III/Stage IV</td>
<td>3.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage II/Stage IV</td>
<td>3.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage I/Stage III</td>
<td>3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Stage I/Stage IV</td>
<td>3.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Fig. (3): Statistical Correlation Study between the Various Studied Parameters in Group I.
Table (4): Diagnostic Performance of CA 15.3 and miRNA-21 in Group I versus Group II

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cutoff</th>
<th>Diagnostic Sensitivity (%)</th>
<th>Diagnostic Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Diagnostic Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA15.3(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 (2^\Delta\Delta CT)</td>
<td>1.09</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**DISCUSSION**

This study was conducted to evaluate the clinical utility of the circulating miRNA-21 in patients with breast cancer in comparison to CA 15-3 as a conventional marker of breast cancer. CA 15-3 was measured by immunoochemiluminescence technique and MiRNA-21 was quantified using an RT-PCR technique (5). MiRNA-21, used as an endogenous control, was consistently expressed in all study groups and was not influenced by BC status, and this was in accordance with multiple studies which used MiRNA-16 as a normalizer in cancer (5,6).

Our study revealed that serum levels of miRNA-21 expression was found to be over-expressed breast cancer patients than in healthy controls meanwhile, CA 15.3 lacks this criterion. These results were in agreement with the findings of a study by Gao et al., who reported an increase in miRNA-21 in BC patients compared with HC group while CA 15.3 was elevated in patients with metastasis (5). Similar over-expression levels were reported in the circulation of BC patients whose diagnosis was confirmed by histopathology, patients had not received chemotherapy, radiotherapy, or operation (6). Level of miRNA-21 was significantly higher not only in serum samples but also in tissue samples of breast cancer compared to their healthy controls (7). This aberrant over-expression of miR-21 in cancer could be caused by genomic alterations involving the miR-21 gene, modulation of miRNA gene expression through epigenetic mechanisms, or defects in miRNA biogenesis (8).

Our study also showed that miRNA-21 expression levels can successfully differentiate between early stages (I and II) and late stages of breast cancer (IIIandIV). Similarly, when Toraih et al., and Han et al., they 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 (8,9). These results were consistent with the oncogenic role of miRNA-21 as it is one of the most important miRNAs associated with cell migration and the invasiveness of breast cancer cells (9).

Another important finding in the current study was the significant high miRNA-21 expression levels in negative ER receptors breast cancer patients, which represents a valuable prognostic and predictive importance in management protocols of these patients. This agrees with previous research works done by Lee et al., and Wang et al., who revealed ER negativity.
expression was significantly associated with high miRNA-21 expression (10,11). 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 (11).

The ROC curve analysis, in this study, revealed that serum miRNA-21 expression could differentiate BC patients from cancer-free individuals with 100% specificity and sensitivity and could even distinguish patients with TNM stage III and IV from patients with earlier stages of BC, with 100% specificity and 86.7% sensitivity. This was consistent with multiple studies which reported a high diagnostic value of serum miR-21 for BC (6,8,9).

The utility of miRNA profiles as potential diagnostic marker for BC has been gaining interest (9). Our study had some limitations, including small sample size and a limited ability to generalize our results since all our patients were Egyptian females. Despite these limitations, our study provided initial data about the up-regulation of serum miRNA-21 in BC patients and suggested the diagnostic value of serum miR-21 in BC patients with metastasis. Clearly, our results should be further validated by a prospective study in a multicenter clinical trial.

CONCLUSION
MiRNA-21 levels are significantly higher in breast cancer patients compared to healthy subjects. Furthermore, we found that increased miRNA-21 expression levels correlated with progression of breast cancer stages.

Our findings indicate that serum miRNA-21 may serve as a novel potential diagnostic and prognostic marker for recurrence and survival of breast cancer patients before resection.

REFERENCES