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ORIGINAL RESEARCH ARTICLE

Pilot Study of Serum MicroRNA-21 as a Diagnostic and Prognostic Biomarker in Egyptian Breast Cancer Patients

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Abstract

Background MicroRNAs are small RNA molecules that bind to complementary sequences of target messenger RNAs and down-regulate their translation to protein or degrade them. MicroRNAs play critical roles in many different cellular processes. Hence, aberrant microRNA expression is common in a variety of disorders, including cancer.

Patients and Methods In this work, we quantified serum microRNA-21 (miR-21) expression levels in 30 breast cancer patients, 30 cancer-free individuals with risk factors for developing breast cancer, and another 30 controls without risk factors, in order to test the role of miR-21 as a possible diagnostic and prognostic biomarker in breast cancer.

Results Our results indicated that miR-21 expression was elevated in asymptomatic high-risk individuals (2.98-fold) compared with healthy non-risk controls ($p < 0.001$), and was increased in almost all sera of cancer patients (12.72-fold) compared with healthy controls ($p < 0.001$). Higher levels of serum miR-21 were also correlated with tumors of higher grades, more nodal involvement, distal metastasis and advanced clinical stages ($p < 0.01$). Furthermore, over-expression levels declined towards normal after surgical tumor resection ($p < 0.001$).

Conclusion In conclusion, our findings demonstrate that serum miR-21 expression profile may serve as a potential non-invasive diagnostic and prognostic biomarker for breast cancer.

Key Points

Serum microRNA-21 (miR-21) was remarkably elevated in asymptomatic high-risk individuals compared with healthy non-risk controls.

Increased circulating miR-21 was detected in breast cancer patients, in particular those with more advanced disease. Over-expression levels declined towards normal after surgical tumor resection.

Serum miR-21 expression profile could be used for breast cancer screening, diagnosis, and prognosis; in particular, it may have value in early detection of breast cancer.

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1 Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths in women worldwide, accounting for 23 % of all new cancer cases and 14 % of total cancer deaths [1]. In Egypt, it is the most common cancer among women, accounting for approximately 37.6 % of cancers; however, it is often diagnosed at advanced stages of the disease [2]. Therefore, identification of non-invasive biomarkers is important in achieving early BC diagnosis and improving patient outcomes [3].

MicroRNAs (miRNAs) are a class of very short ribonucleic acid (RNA) molecules which control gene expression by either promoting translational repression or degradation of messenger RNAs (mRNAs) [4, 5]. MiRNAs have been implicated in multiple cellular processes including differentiation, proliferation, migration, and apoptosis [6, 7]. Altered expression of miRNAs has been found in every malignancy examined and appears to play an important role in tumorigenesis [8]. Detection of extracellular miRNAs in blood and other body fluids and the feasibility of their quantification provide evidence that these tiny molecules may serve as promising molecular biomarkers for cancer [9–11].

MicroRNA-21 (miR-21) is considered an oncogenic miRNA 'oncomir' since it promotes tumor growth, invasion, angiogenesis, and metastasis by targeting and suppressing several apoptotic and tumor suppressor genes, including the programmed cell death 4 (*PDCD4*), tropomyosin 1 (*TPM1*), phosphatase and tensin homolog (*PTEN*) tumor suppressor, cell division cycle 25 homolog A (Cdc25a), reversion-inducing cysteine-rich protein with kazal motifs (RECK), mammary serine protease inhibitor (*MASPIN*) genes, and tissue inhibitor of metalloproteinase 3 (*TIMP3*) [6, 8, 12, 13]. Thus, it is one of the most prominent miRNAs implicated in the genesis and progression of human cancer [14]. Therefore, the aim of this study was to test the potential value of serum miR-21 as a diagnostic and prognostic biomarker for BC.

2 Materials and Methods

2.1 Study Participants

The study included 90 women: 30 pre-operative BC patients, 30 healthy individuals with associated risk factors for developing breast cancer (RC), and another 30 healthy controls without risk factors (HC). Cancer-free individuals were matched with the cancer patients group with respect to age, gender, timing of sampling, and duration of specimen storage. All participants had no history of pregnancy or lactation within the last year, heart disease, renal problems, diabetes mellitus, or any concurrent inflammatory condition. They were evaluated for the presence of at least one of the following risk factors predisposing to BC: early menarche <12 years, late menopause >53 years, nullipara, late first full-term pregnancy >30 years, no history of lactation, presence of atypical hyperplasia on breast biopsy, or use of hormonal contraceptives or hormonal replacement therapy. In addition, a BC risk assessment tool version 6 according to Cuzick and Tyrer, and National Cancer Institute models were used to quantitatively estimate the risk of all participants to develop BC [15]. Patients were recruited from the General Surgery Clinic, Suez Canal University Hospital, Ismailia, Egypt, during the period between January 2012 and August 2013. Patients had histologically confirmed primary BC and no history of receiving chemotherapy or radiotherapy. The Medical Research Ethics Committee of the Faculty of Medicine, Suez Canal University approved the study and all patients provided written informed consent.

2.2 Histopathological Assessment

Breast tumor specimens were evaluated by a pathologist to determine tumor type, size, pathological grade (according to the Elston and Ellis modification of the Scarff-Bloom-Richardson classification) [16], and lymph node involvement. The clinical stage at the time of surgery was classified according to the American Joint Committee on Cancer (AJCC) tumor–node–metastasis (TNM) classification system [17]. Molecular intrinsic subtype of tumor was assessed based on the receptor status, for the presence or absence of estrogen receptor (ER) and progesterone receptor (PR), and the expression of human epidermal growth factor receptor 2 (HER2) protein [18]. The Nottingham Prognostic Index (NPI) [19] and Immunohistochemical Prognostic Index (IHPI) [20] were used for prognostic assessment. In addition, the predicted risk of recurrence for each patient was calculated according to the European Society of Medical Oncology (ESMO) clinical recommendations for follow-up of primary BC [21].

2.3 Sample Collection and Processing

Fasting serum samples were collected from all participants. Paired pre-operative and post-operative (6 h and 2 weeks) serum samples were obtained from BC patients to compare serum miR-21 levels before and after surgical tumor resection. Samples were collected in Vacutainer Serum Separator Tubes II (Becton Dickinson, Plymouth, UK), divided into aliquots, and stored at −80 °C. Samples were allowed to undergo one freeze–thaw cycle only.

2.4 RNA Extraction

The total RNA, including small RNA, was isolated from serum using the Qiagen miRNeasy Serum/Plasma Kit (Qiagen, Catalog no. 217184) following the protocol supplied by the manufacturer. RNA concentration was determined using the Nano Drop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA). The wavelength-dependent extinction coefficient '33' was taken to represent the micro-component of all RNA in the solution [22].

2.5 Reverse Transcription

The TaqMan™ MicroRNA Reverse Transcription kit (Applied Biosystems, P/N 4366596, Egypt) and miRNA-21 specific stem–loop primers (Applied Biosystem, assay ID 000397, Egypt) were used for miRNA reverse transcription (RT) reaction. RT primer for small nuclear RNA U6 (RNU6B) (Applied Biosystems, assay ID 001093, Egypt) was used as an endogenous control. For each serum sample, 10 ng of total RNA was used in a 15-μL reaction mixture containing 5 μL of RNA extract, 0.15 μL of 100 mM of each deoxynucleotide triphosphate, 1 μL of MultiScribe® reverse transcriptase (50 U/μL), 1.5 μL of 10× RT buffer, 0.19 μL of RNase inhibitor (20 U/mL), 3 μL of gene-specific TaqMan® primer and 4.16 μL of nuclease-free water. RT was carried out in a Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min, then held at 4 °C. A negative control was included in each experiment, using all the reagents except reverse transcriptase, to ensure that polymerase chain reaction (PCR) products were not due to contamination by genomic DNA [23].

2.6 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 (see Supplementary Table S1 in the electronic supplementary material [ESM]). Mature miR-21 was quantified using the TaqMan® MicroRNA assay (Applied Biosystems, assay ID 000397, catalogue no 4427975, Egypt), TaqMan® endogenous control assay for RNU6B (Applied Biosystems, assay ID 001093, catalogue no 4427975, Egypt), and Taqman® Universal PCR master mix II, No UNG (2×) (Applied Biosystems, P/N 4440043, Egypt). The PCR reactions were carried out in final volumes of 20 μL, including 1.33 μL RT product, 2× TaqMan® Universal PCR Master Mix, 1 μL TaqMan® small RNA assay. All reactions were run in duplicate and included a no-template control (with water instead of cDNA), and a no-reverse transcriptase control. The PCR was performed on an AB 7500HT instrument with SDS Software version 2.1.1 (Applied Biosystems, Egypt) as follows: 95 °C for 10 min followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.7 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 (C_T) value using the equation of relative quantity $= 2^{-\Delta\Delta CT}$method, where $\Delta\Delta C_T = (C_T$ miR-21$-C_T$ RNU6B$)_{BC \text{ or } RC} - (C_T$ miR-21$-C_T$ RNU6B$)_{mean\ HC}$ [24].

2.8 Statistical Analysis

Data was managed using the *Statistical Package for the Social Sciences (SPSS) for windows* software (version 20.0). As the data for miR-21 relative expression levels did not fit a Gaussian distribution, miR-21 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 miR-21 was calculated for different groups using the Mann–Whitney *U* (MW) and the Kruskall-Wallis (KW) tests [25]. 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 [26].

3 Results

3.1 Characteristics of the Study Groups

There was no significant difference between BC and RC groups regarding the number or types of risk factors for developing BC ($p > 0.05$) (see Supplementary Table S2 in the ESM). However, on quantitative risk assessment using a Tyrer-Cuzick model which predicts chances of developing BC over a given time span, BC patients had higher predicted lifetime and 10-year risk of developing BC than the RC group ($p < 0.05$) (see Supplementary Table S3 in the ESM). Clinicopathological features of BC patients are shown in Table 1. According to the NPI and IHPI scoring systems, 60 and 40 % of patients had poor prognosis, respectively. Regarding the estimated ESMO recurrence risk, two thirds of the BC patients had high recurrence

Table 1 Clinicopathological features of breast cancer patients ($N = 30$)

Variable	n (%)[a]
Age at diagnosis	
Mean age in years	42.9 ± 14.5
<35 years	8 (26.7)
≥35 years	22 (73.3)
Tumor side	
Right	18 (60)
Left	12 (40)
Location	
Unilateral	24 (80)
Multicentric	6 (20)
Histological type	
Ductal	20 (66.7)
Lobular	6 (20.0)
Others	4 (13.3)
Pathological grade[b]	
Grade 1	0 (0.0)
Grade 2	23 (76.7)
Grade 3	7 (23.3)
Lymphovascular invasion	16 (53.4)
Skin invasion	5 (16.7)
Nipple invasion	0 (0.0)
Tumor size	
Mean (SD) in mm	5.6 ± 1.6
T1	0 (0.0)
T2	16 (53.3)
T3	6 (20)
T4	8 (26.7)
Lymph node status	
N0	5 (16.7)
N1	10 (33.3)
N2	15 (50)
Metastasis	
M0	16 (53.3)
M1	14 (46.7)
Site of metastasis	
Bone	6 (42.9)
Visceral	8 (57.1)
Clinical stage[c]	
Stage II	8 (26.7)
Stage III	8 (26.7)
Stage IV	14 (46.6)
Estrogen receptors	
Negative	12 (40)
Positive	18 (60)
Progesterone receptor	
Negative	12 (40)
Positive	18 (60)
$HER2^+$ status	
Negative	24 (80)
Positive	6 (20)
Molecular class	
Luminal A	14 (46.7)
Luminal B	4 (13.3)
$HER2^+$	2 (6.7)
Basal-like	10 (33.3)

T tumor size, *N* lymph node status, *M* metastasis, *HER2*$^+$ human epidermal growth factor receptor 2, *Luminal A* ER$^+$, PR$^+$, HER2$^-$, *Luminal B* (triple positive), ER$^+$, PR$^+$, HER2$^+$, *HER2*$^+$ *subset* ER$^-$, PR$^-$, HER2$^+$, *Basal-like* (triple negative), ER$^-$, PR$^-$, HER2$^-$, *ER* estrogen receptor *PR* progesterone receptor

[a] Values are shown as mean ± SD or as number (percentage)

[b] Pathological grading, according to Elston and Ellis modification of Scarff–Bloom–Richardson classification

[c] Clinical staging was classified according to the American Joint Committee on Cancer (AJCC) tumor-lymph node-metastasis (TNM) staging system

risk >50 % and one third had intermediate recurrence risk between 10–50 %.

3.2 Spectrophotometric Analysis for RNA Concentration

The amount of small RNA fraction in samples was directly correlated to the corresponding total RNA concentration ($r = 0.721$, $p < 0.001$, calculated by Pearson's correlation), and represented about 62.4 % of the total extracted RNA concentration.

3.3 Serum MicroRNA-21 Expression Profile in Cancer Patients

The endogenous control, RNU6B, was uniformly and stably expressed even in the presence of malignancy, with no significant difference between BC, RC, and HC groups ($p = 0.576$). In contrast, C_T values of miR-21 varied significantly among the studied groups; with the lowest C_T value, and hence the highest miRNA content, in tumor sera ($p < 0.001$) (see Supplementary Table S4 in the ESM). Serum miR-21 was significantly over-expressed in BC patients compared with both the asymptomatic RC individuals and healthy controls ($p < 0.001$) (Fig. 1). In BC patients, 90 % exhibited up-regulation of miR-21 with relative expression levels >1.0, and 80 % of samples displayed miR-21 over-expression (greater than 2-fold) compared with healthy controls. Interestingly, pre-

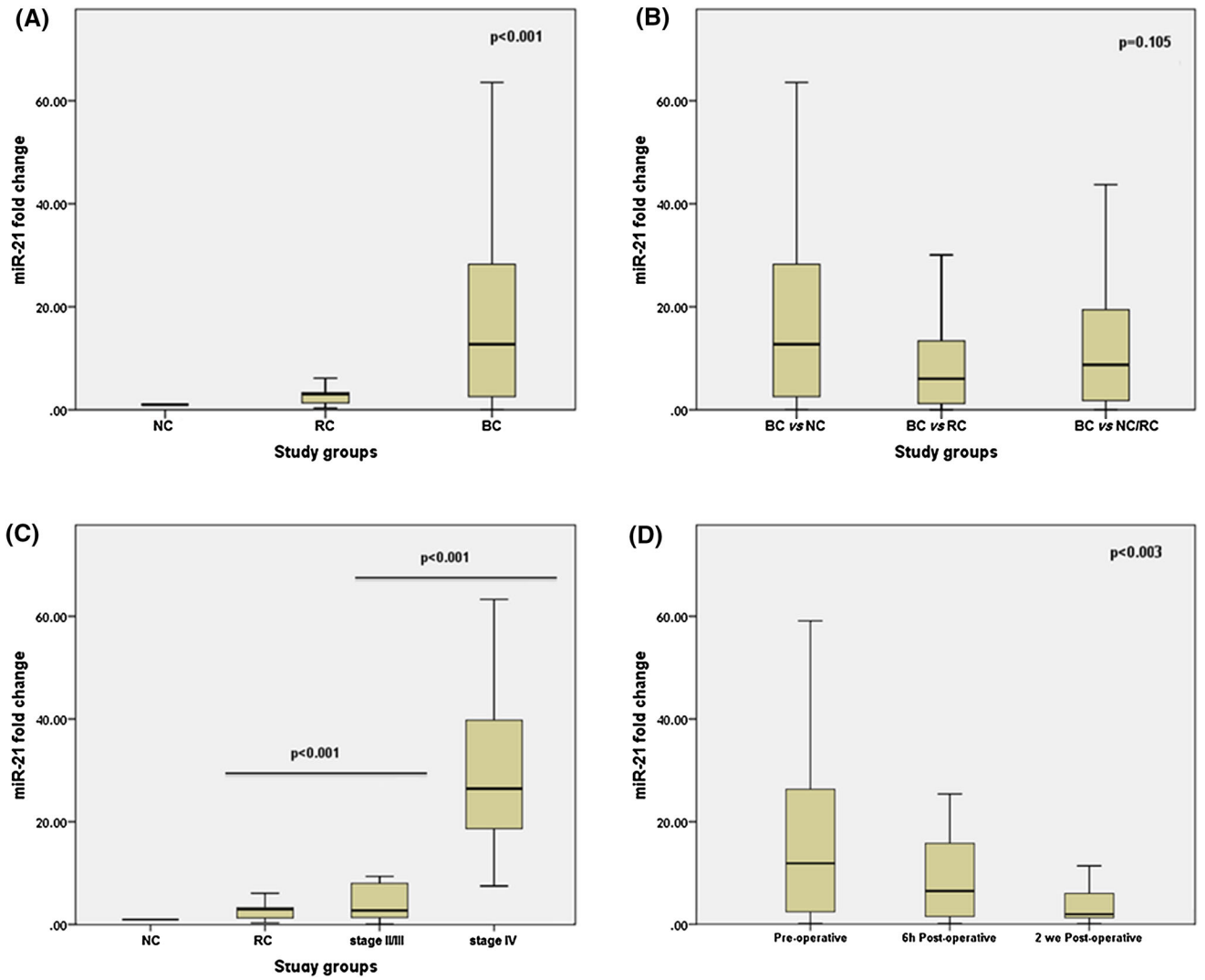

Fig. 1 Serum microRNA-21 expression profile in the study groups (30 breast cancer patients [*BC*], 30 high-risk controls [*RC*], and 30 healthy controls [*HC*]). Values are presented as medians. The *box* defines *upper* and *lower* quartiles (25 and 75 %, respectively) and the *error bars* indicate *upper* and *lower* adjacent limits. Fold-change for miRNA-21 was normalized to RNU6B and calculated using the delta-delta CT method [= 2 $(-\Delta\Delta C_T)$] compared with HC. Kruskal Wallis and Mann–Whitney tests were used. **a** The median miR-21 expression levels were significantly up-regulated 12.72-fold in BC and 2.98-fold in RC compared with HC. **b** Serum miR-21 was over-expressed in serum of BC compared with HC, RC, and all cancer-free individuals. **c** Expression levels were significantly higher in patients with metastasis (stage IV) compared with those with loco-regional disease (stage II/III). **d** Elevated pre-operative miR-21 levels significantly dropped by half 6 h after curative tumor resection, and declined more after 2 weeks

operative miR-21 over-expression levels in patients significantly dropped to half, 6 h after curative tumor resection, then declined more towards normal after 2 weeks ($p < 0.001$) (Fig. 1d). ROC curve analysis confirmed that serum miR-21 levels could discriminate between BC patients and cancer-free individuals with 86.7 % specificity and 66.7 % sensitivity, and could even distinguish patients with AJCC stage IV BC from patients with earlier stages with 87.5 % specificity and 92.9 % sensitivity (Fig. 2a, b).

3.4 Serum MicroRNA-21 Expression Profile in Asymptomatic High-Risk Individuals

The expression levels of serum miR-21 were remarkably up-regulated in asymptomatic high-risk individuals compared with healthy controls; median (1st–3rd quartiles) of 2.98 (1.33–3.33) ($p < 0.001$) (Fig. 1a). In the at-risk group, 19 out of 30 individuals exhibited elevated levels of miR-21 with relative expression levels >1.0, and 15 of the samples displayed miR-21 over-expression

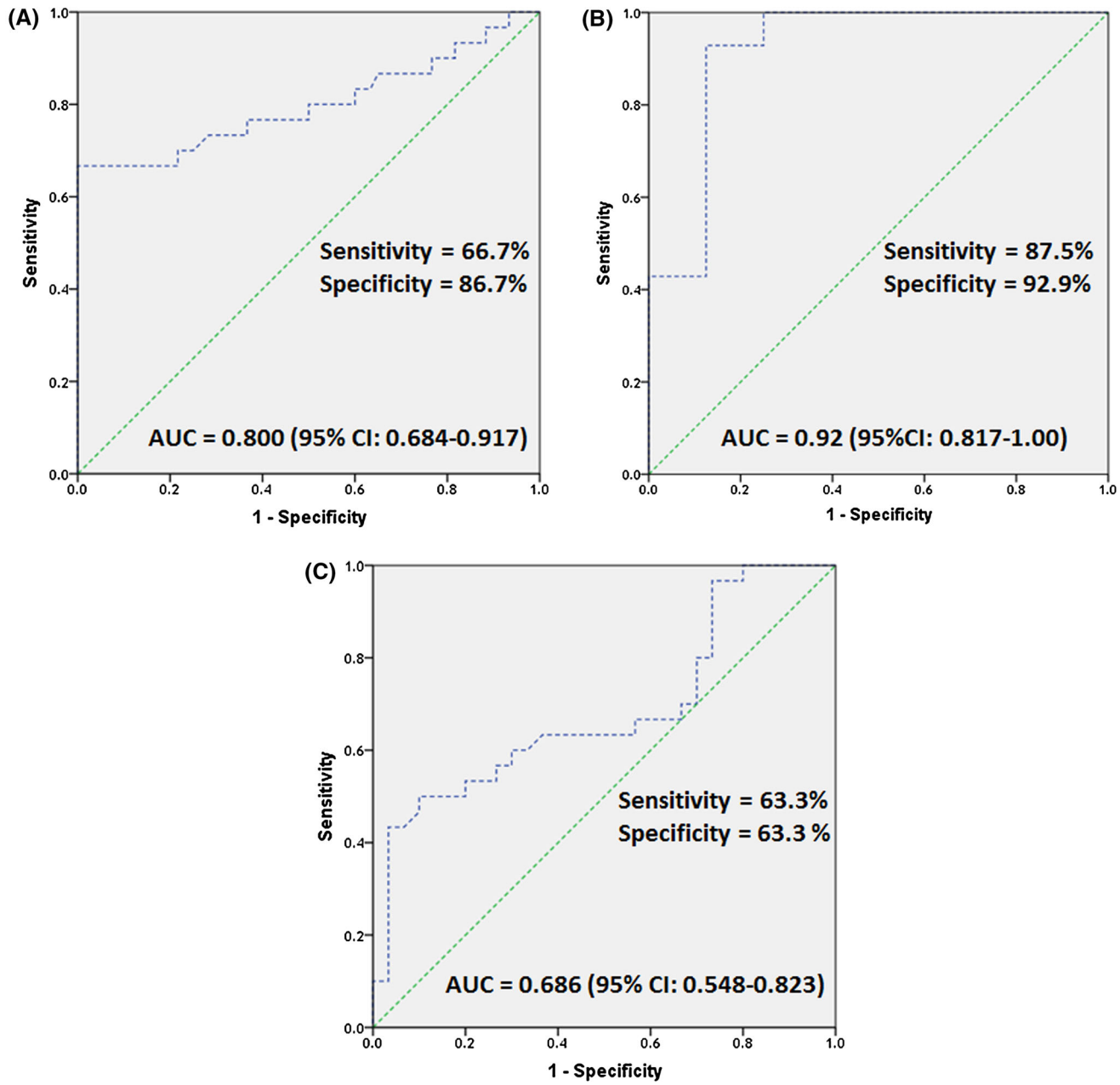

Fig. 2 Receiver operating characteristics (*ROC*) curve analysis for evaluating miR-21 diagnostic performance. **a** Breast cancer (*BC*) patients versus all cancer-free individuals: the AUC was 0.80 at the optimal cut-off value of $\Delta C_T = -7.02$ (3.48-fold change). **b** BC patients with stage metastasis versus patients with earlier stages of BC: AUC was 0.92 at $\Delta C_T = -8.2$ (7.89-fold change). **c** Asymptomatic high-risk individuals versus healthy controls: AUC was 0.686 at $\Delta C_T = -6.33$ (2.16-fold change). *AUC* area under the curve, *CI* confidence interval

of >2.0-fold compared with healthy controls (Table S4). In addition, the ROC curve for RC versus HC showed that serum miR-21 could predict asymptomatic high-risk individuals with 63.3 % specificity and 63.3 % sensitivity (Fig. 2c).

3.5 Correlation of MiR-21 Expression Level with Risk Factors

Elevated serum miR-21 levels were significantly associated with three risk factors, namely early menarche

at <12 years old, nullipara, and absent breast feeding. No associations were observed with any other types of risk factors (see Supplementary Table S5, ESM). In addition, we did not find any correlation with the number of risk factors existing in the same individual ($p = 0.551$).

3.6 Correlation of MiR-21 Expression Level with Disease Characteristics

Among BC patients, serum miR-21 was significantly associated with young age at diagnosis (<35 years) ($p = 0.016$) (see Supplementary Table S6, ESM). BC patients with high pathological grade, more nodal involvement, lympho-vascular invasion, distal metastasis, and advanced clinical stage had significantly higher miR-21 expression levels ($p < 0.01$) (Fig. 3). No significant association was observed between serum miR-21 levels and tumor size ($p = 0.232$) or site of metastasis whether visceral or bone ($p = 0.576$). Higher serum levels of miR-21 were also observed in patients with poor prognosis (for NPI: $p = 0.185$, and for IHPI: $p = 0.100$), and those with high recurrence risk >50 % ($p = 0.074$), but they did not reach significant difference (see Supplementary Table S7, ESM). Regarding the receptor status, miR-21 over-expression was significantly associated with $HER2^{+}$ tumors ($p = 0.015$). However, expression levels did not differ among patients with ER/PR negative disease ($p = 0.172$) or among various tumor intrinsic subtypes ($p = 0.060$) (see Supplementary Table S8, ESM).

4 Discussion

This study was conducted to examine the role of serum miR-21 as a biomarker for risk assessment, diagnosis and prognosis of BC. To the best of our knowledge, this is the first work examining circulating miR-21 expression profiles in an Egyptian population, and the first to report elevated miRNA levels in asymptomatic high-risk individuals susceptible to developing BC.

MiR-21 was quantified using an RT-PCR technique. This method was described by Schmittgen et al. as the most sensitive, specific, fast, and efficient method, which only needs small amounts of RNA for analysis, and could even differentiate between miRNAs that differ by only one or two base pairs [23]. We measured the active mature miR-21 in serum, rather than the inactive precursor, since it reflects the regulation of both miRNA processing and maturation, especially in various human disease where alterations in miRNA biogenesis produce levels of mature miRNA that are different from those of the pre-miRNA [22, 27]. RNU6B, 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 RNU6B as a normalizer in cancer [1, 7, 28, 29].

In our study groups, serum miR-21 was found to be over-expressed in the circulation of BC patients 12.72-fold compared with healthy controls, with greater up-regulation in patients with metastasis (stage IV; 26.5-fold) compared

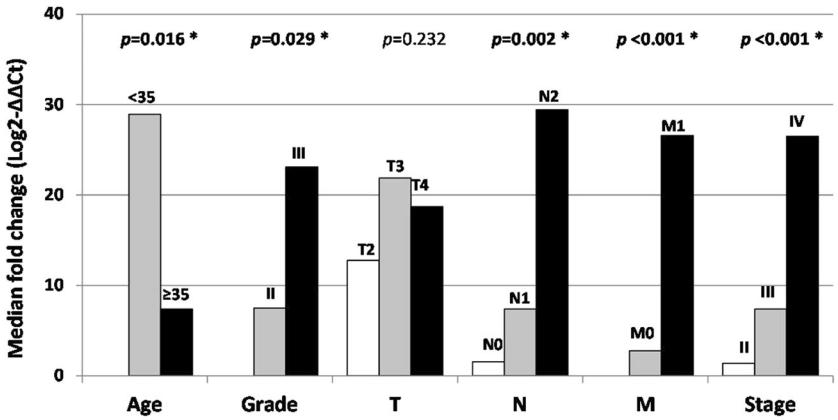

Fig. 3 Association of microRNA-21 expression level with disease characteristics in breast cancer patients ($n = 30$). MiR-21 was normalized by RNU6B using the delta–delta CT method compared to HC. Values are represented as medians. Mann–Whitney and Kruskal Wallis tests were used. Higher miR-21 was associated with cancer diagnosed at early age (<35 years), tumors with higher grade, more nodal involvement, metastasis, and advanced stage. No association was observed with tumor size. *M* metastasis, *N* lymph node status, *RNU6B* small nuclear RNA U6, *T* tumor size

with those without metastasis (stages II/III; 2.78-fold). This result was in agreement with a study conducted by Hafez et al., who reported a 10.69-fold increase in miR-21 expression compared with normal adjacent tissues among Egyptian women [30]. Similar over-expression levels were reported in the circulation of BC patients with different ethnic backgrounds [5, 13]. For example, a significant increase in miR-21 expression of 9.5-fold was also reported among an Italian population [31]. A study conducted in the US also showed circulatory miR-21 over-expression of 3.5-fold [32], and studies in Chinese populations revealed enhanced levels by 2- to 2.5-fold in the serum of BC patients [11, 29]. Circulatory miR-21 was also consistently elevated in other types of cancer: 6.8-fold in pancreatic cancer [33], 5.8-fold in gastric cancer [34], 1.9-fold in hepatocellular cancer [35], 7.7-fold in head and neck squamous cell carcinoma [36], and 2.56-fold in diffuse large B-cell lymphoma [37] compared with normal controls. Moreover, miR-21 was up-regulated by 4.8-fold in colorectal cancer tissues [38], 4.3-fold in papillary thyroid cancer tissues [39], and 4.4-fold in lung cancer tissues compared with normal adjacent tissues [40]. 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 pathways [41, 42].

The ROC curve analysis, in this study, revealed that serum miR-21 expression could differentiate BC patients from cancer-free individuals with 86.7 % specificity and 66.7 % sensitivity, and could even distinguish patients with AJCC stage IV from patients with earlier stages of BC, with 87.5 % specificity and 92.9 % sensitivity in identifying stage IV. This was consistent with multiple studies which reported a high diagnostic value of serum miR-21 for BC [14, 43–47]. Some meta-analysis studies highlighted its role as a tumor biomarker, especially in the diagnosis of early-stage BC [43, 44]. Others demonstrated a remarkable ability of circulating miR-21 levels to differentiate BC patients from healthy women with higher sensitivity and specificity than conventional serum biomarkers such as carbohydrate antigen 15–3 (CA15–3) and carcinoembryonic antigen (CEA), which exhibited low sensitivity and specificity, particularly in the context of early BC [45]. In addition, Asaga et al. revealed that a circulating miR-21 signature could be used to further distinguish BC patients with distant metastases from those with loco-regional disease [46]. Other investigators reported high sensitivity and specificity of circulatory miR-21 for detecting other types of cancer, including gastric, esophageal, colorectal, pancreatic, lung, prostate, and ovarian cancer [33, 34, 48–55]. Moreover, in a meta-analysis conducted on 73 studies with different cancers, the most identified cancer by miR-21 assay was BC, with 84 % specificity and 86 % sensitivity [56]. Taken together, these results held the point that miR-21 could serve as a potential broad-spectrum biomarker for cancer.

A unique finding in our study was that serum miR-21 levels were remarkably elevated in asymptomatic high-risk individuals (2.98-fold) relative to those of healthy controls. In addition, ROC curve analysis showed that serum miR-21 could predict high-risk individuals with 63.3 % specificity and 63.3 % sensitivity. The stepwise progression of circulatory miR-21 over-expression in the present study was previously observed in breast tissues; early elevation in the first transition, from normal breast to flat epithelial atypia and ductal carcinoma in situ (DCIS), and progression through multi-stage cancer [57–60]. This supports the speculation that the use of serum miR-21 expression profile in the general population could be used as a predictor for identifying individuals at risk in our population. Similar results were also reported by some investigators who observed significant changes in serum miRNA pattern in asymptomatic high-risk individuals susceptible to developing lung cancer, occurring 1–2 years before clinical presentation of cancer [61–63]. Also, a prospective study revealed that a panel of three serum miRNAs could distinguish gastric dysplasia from controls with 56.5 % sensitivity and 47.8 % specificity, and they also detected dramatic changes in the circulating miRNA profile at time points closer to gastric cancer diagnosis [28]. Moreover, in 2013, Madhavan et al. raised the possibility of using serum miRNAs as a preliminary screening method in the general population at high risk of cancer [64]. These findings, along with our observation that asymptomatic high-risk BC individuals have higher circulating levels of miR-21 compared with the non-risk group, suggests that following such individuals in a prospective study design, with series measurements over a period of time, could be a valuable method to test the merit of serum miR-21 as an early BC biomarker.

Assessment of miR-21 expression before and after surgical tumor removal in our BC patients revealed that serum miR-21 levels declined immediately following tumor resection. This was similar to several studies that reported the normalization of circulating miRNA levels within 1–4 weeks in different tumor types such as breast, gastric, colorectal, oral, lung, head and neck squamous cell carcinoma, and papillary thyroid cancer, not only after surgical removal but also following tumor eradication by other therapeutic modalities such as chemotherapies and radiotherapy [22, 26, 36, 47, 54, 65–67]. Thus, normalization of all samples in our study suggests the probable role of serum miR-21 as a potential biomarker for monitoring disease recurrence during the follow-up period after mastectomy, especially at an earlier time than current imaging technologies. In addition, it further emphasizes that miRNAs originate specifically from the primary tumor tissue.

Extracellular miRNAs existing in the circulation of cancer patients may be present as a result of the fast turnover of tumor cells resulting in cell death and lysis [68], or through the tumor cells releasing microvesicles, exosomes, or protein/lipoprotein complexes that contain miRNAs into the tumor micro-environment, where they make their way into the circulation [63, 69]. Alternatively, these extracellular miRNAs might be derived from the tumor tissue as a result of increased cellular expression levels due to genomic alterations [10, 65, 66].

One of the main challenges in developing tumor biomarkers for routine clinical use is defining the potential variables which may interfere with the biomarker behavior and expression. Thus, in our population, we quantitatively and qualitatively assessed several factors that might alter the biomarker status in controls and cancer patients to be taken into consideration when applying this biomarker in the clinic. Surprisingly, for the first time we observed elevated expression levels of serum miR-21 in women with no history of pregnancy or breast feeding. Pregnancy and lactation are known to promote terminal differentiation of lobulo-acinar structures in the breast [70], and to induce permanent genomic changes in the breast epithelium that confer genomic stability and reduce the susceptibility to BC [71]. Differential expression of miRNA during pregnancy and lactation was previously reported, which might occur as part of the mammary gland structural and functional reorganization [72]. In addition to nulliparity and the absence of lactation, we have identified early menarche, in both the BC and the RC groups, as a third risk factor for BC that has been associated with serum miR-21 over-expression. These three risk factors have long been recognized to predispose females to a longer duration of estrogen exposure throughout life [71]. Estrogen is known to have a carcinogenic role in mammary tumors [73]. This correlation is supported by the detection of estradiol (E_2)-induced miR-21 over-expression in breast cells [74]. However, contradictory results were reported by another study where the investigators found that E_2 inhibits miR-21 expression and increases the expression of miR-21 target genes in BC cell lines [75]. This discrepancy in data reported in the last two studies may be explained by the different experimental conditions and cell lines used. Understanding the aberrant regulation of miR-21 by E_2 in BC cells is of great significance, since these signaling pathways are heavily involved in the development and progression of breast tumors and importantly govern the responsiveness of BC patients to endocrine therapies.

Regarding the influence of disease-related characteristics in our BC patients on the biomarker, serum miR-21 levels were significantly correlated with younger age at diagnosis, higher pathological grade, more nodal involvement, distal metastasis, lympho-vascular invasion, and advanced clinical stage. Thus, a high miR-21 expression profile indicated a more aggressive phenotype among Egyptian women and could be a useful tool to stratify patients into prognostic groups. In particular, serum miR-21 levels could be used to identify those patients who already have distant micro-metastases that are too small to be diagnosed with high specificity and sensitivity. Meanwhile, no significant association was found between expression of miR-21 and tumor location, histological type, or size. Our results were in agreement with other prior studies which reported that miR-21 over-expression was associated with tumor grades, higher proliferation index, and lymph node metastasis in BC [5, 13, 30, 76]. Other previous studies showed that miR-21 levels are positively correlated with vascular invasion, visceral metastasis, and advanced clinical stage [13, 46, 77], and associated with poor response to therapy, and worse survival in BC [49, 78–81]. Moreover, miR-21 deregulation represented a global cancer phenomenon; associated with poor prognosis and survival in other cancer types including lung cancer [42, 82], colorectal carcinoma [54, 83], gastric cancer [84], pancreatic cancer [33], diffuse large B-cell lymphoma [36, 37]. These results were consistent with the oncogenic role of miR-21 in our in silico analysis. Hundreds of genes were predicted as targets for miR-21, many of which were specifically involved in critical pathways of BC formation and progression, including *CDKN1A*, *RECK*, *TMP3*, *Bcl2*, *FAS*, *BACH1*, *VHL*, *ATM*, *PDCD4*, *PTCH1*, *ITGA2*, *CASP8*, *TNFBR1*, *DNMT3B*, and others. Some of the genes had been functionally validated in previous experiments. Defining this functional network connecting miR-21 and its targets is mandatory, as it will offer new insights into pathological mechanisms.

Another important finding in the current study was the significant high miR-21 expression levels in $HER2^+$ positive tumors as receptor status represents a valuable prognostic and predictive marker in BC. Elevated expression of miR-21 in $HER2^+$ BC tissues was previously reported [81]. Functional studies revealing this mechanistic interaction showed strong correlation between miR-21 expression level and $HER2^+$ status in BC cells, and demonstrated that miR-21 expression is induced by $HER2^+$ signaling via the mitogen-activated protein kinase (MAPK) pathway [70, 77, 85]. Another proposed mechanism for this association can be through the linkage of *HER2* and miR-21 by chromosomal region amplification. The *HER2* gene is located in the chromosomal region (17q12-21) and is amplified in certain types of BC. Interestingly, the miR-21 gene also maps very close to this region (17q23), which may explain the over-expression of both genes.

Although there is a large amount of evidence indicating that elevated miR-21 expression is associated with cancer development and progression, several issues need to be

addressed when interpreting our results. First, our study lacked premalignant and in situ BC patients and thus could not audit the miR-21 profile in the intermediate tumorigenesis pathway. Second, sample size in our study was not large enough to fully explore the synergistic relationship between a miR-21 expression profile and different combinations of confounding factors that may affect its behavior. Further evaluation of circulating miR-21 by a prospective study in larger control and cancer cohorts with well annotated clinicopathological characteristics is necessary to validate our findings, explore potential factors and environmental variations that may influence diagnostic precision, and determine what the normal range is in the circulation of both genders. Third, we evaluated a single microRNA in our study; using a panel of microRNA might achieve better diagnostic accuracy for BC detection than using an individual marker. Moreover, BC is a heterogeneous disease at the phenotypic and molecular levels, even within the same patient, that could be derived from multiple combinations of mutant cancer-causing genes leading to various altered signaling pathways. Functional studies correlating miR-21 with their predicted target genes known to be involved in BC are warranted to address this networking. Furthermore, as circulating miR-21 was found to be correlated with the presence and severity of BC in the current study similar to other publications, and yet is also correlated with the presence of other types of cancer and non-neoplastic conditions such as inflammation, the specificity of elevated miR-21 expression for particular cancers needs to be examined in more detail. In addition, a standardized protocol and normalization strategies for the analysis of circulating miRNAs should be established to be consistent across various sample types and disease conditions. Only then it will be possible to truly establish the levels of systemic miRNA in both healthy and diseased individuals and thus allow the use of expression level for diagnosis of pathological state(s).

Despite these limitations and challenges to achieve translational application of miRNAs as a routine diagnostic tool, our study provided initial data that miR-21 could identify asymptomatic women with high risk for developing BC, differentiate BC patients from healthy controls, and distinguish patients with metastasis from those with earlier stages of BC with remarkable specificity and sensitivity. Our preliminary findings also showed, for the first time, differential expression of miR-21 in women with history of early menarche, nulliparity, and absent lactation. Moreover, elevated miR-21 expression could be used as a prognostic indicator for poor outcome in Egyptian BC patients. In addition, our findings indicated that serum miR-21 could be a useful biomarker for disease monitoring after curative tumor resection.

5 Conclusion

The results found in this study, taken in the context of the role of miR-21 in cancer biology, suggest that serum miR-21 profiling can potentially serve as a promising non-invasive biomarker for risk assessment, diagnosis, and prognosis in breast cancer.

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Conflict of interest ET, EM, SF, NW, and SH declare no conflicts of interest.

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Author contributions ET, EM, and SH conceived and designed the experiments. ET and SF collected samples and clinical data. ET performed the laboratory experiments. ET, EM and SH analyzed the clinical and molecular data. ET contributed reagents/materials/analysis tools. ET, EM, and SH wrote the paper. ET, EM, SF, NW and SH critically revised and gave final approval of the manuscript. ET is responsible for the overall content.

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