RESEARCH ARTICLE

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Clinical impact of oncomirs 221 and 222 on breast cancer diagnosis

Elham Ali Ahmed [1], Sohair A. Abd El-bast[1], Mona A. Mohamed[2], Menha Swellam[3,4]

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Abstract

Background Dysregulation of miRNAs, non-coding RNAs of 18-25 (~22nt), is a hallmark of malignancies among them is breast cancer. The present study aimed to investigate the expression levels of circulating oncomiRNAs (miRNA-221and miRNA-222) as a minimally non-invasive method for early detection of breast cancer as compared to tumor markers (CEA, CA15.3).

Materials and methods MiRNA-221 and miRNA-222 expression levels were determined using quantitative real-time polymerase chain reaction (qPCR) in serum samples from three groups: primary breast cancer patients (n = 44), benign breast lesion patients (n = 25), and healthy individuals as control group (n = 19). Their diagnostic efficacy and relation with clinicopathological data were analyzed.

Results MiRNA-221 and miRNA-222 expression and tumor markers reported significant increase in their mean levels in breast cancer group as compared to the benign breast lesions or control individuals. Among clinicopathological factors, miRs reported significant relation with pathological types, clinical staging, histological grading and hormonal status, while CEA and CA15.3 did not revealed significance with these factors. The diagnostic efficacy for investigated miRNAs was superior to tumor markers especially for detection of early stages and low grade tumors.

Conclusion MiRNA-221 and miRNA-222 were superior over tumor markers for early detection of breast cancer especially those at high risk as primarybreast cancer patients with early stage or low grade tumors.

Key words Breast cancer; miRNAs; circulating molecular marker; diagnosis

1. Zoology Department, Faculty of Science (Girls), Al-Azhar University.
2. Biochemistry Division, Chemistry Department, Faculty of Science (Girls), Al-Azhar University.
3. Biochemistry Department, Genetic Engineering and Biotechnology Research Division, Al-Azhar University.
4. High Throughput Molecular and Genetic laboratory, Center for Excellences for Advanced Sciences, National Research Centre, Dokki, Giza, Egypt.
Correspondence: Professor Dr. Menha Swellam (High Throughput Molecular and Genetic laboratory, Center for Excellences for Advanced Sciences, Biochemistry Department, Genetic Engineering and Biotechnology Research Division, National Research Centre, El-BohouthStreet, Dokki, Giza, 12622, Egypt.; E-mail: menhamswellam@gmail.com).

Introduction

Breast cancer (BC) constitutes the second most prevalent cancer in Egypt [1]. It is the most commonly diagnosed cancer in females (i.e. about1 in 4 of all new cancer cases diagnosed in women worldwide are breast cancer), and this cancer is the most common in 154 of the 185 countries included in GLOBOCAN survey [2]. In Egypt, breast cancer is a well-documented highly prevalent cancer in females, according to National Cancer Registry Program; it represents 32% from total cancer cases in females [3]. A probable increase in the incident of cancer in Egypt may reach three times starting from 2013 to 2050 [4].

Early detection of breast cancer greatly improves the prognosis and treatment for patients, this critical for optimizing management strategies to this disease, but in spite of improvements in cancer screening techniques, major challenges remain [5, 6]. Cancer antigens (such as CEA and CA15.3) as well as other circulating protein molecules have been widely used as non-invasive biomarkers for monitoring patients with metastasis, but with insufficient sensitivities to diagnose primary breast cancer [7, 8]. Thus, great necessity needed to develop novel biomarkers for breast cancer detection with high sensitivity and specificity.

MicroRNAs (miRNAs) are a family of highly conserved noncoding single stranded RNA molecules of 21 to 25 nucleotides; It is can regulate one third of protein-coding genes and participate in the developmental and physiological processes of human body [9]. Aberrant expression of miRNAs has a link with breast tumorgenesis and their deregulation is reported in biological fluids of cancer patients [10]. MiRNAs have important regulatory roles in the cell; modulate their target genes through RNA interference pathways (RNAi). It can reversely modulate gene expression processes [11]. Circulating miRNAs are stable in body fluids, they have been expressed in many cancers among them is breast cancer, miRNAs are potential bio-markers in the diagnosis, prognosis, and prediction of response to treatment in breast cancer [12, 13].

In the current study, miRNA-221 and miRNA-222 are homologous miRNAs located in tandem, within 1 kb from each other, on human X chromosome [14, 15]. Recent studies stated that microRNA-221 and miRNA-222 is aberrantly expressed in various malignancies [16] and was found to be overexpressed in triple-negative breast cancer patients [17]. MiRNA-221 and miRNA-222 are thought to serve as oncomiRs because they inhibit many tumor suppressors [18, 19, 20, 21].

The present study aimed to investigate the potential diagnostic role of circulating miRNA-221and miRNA-222 in blood samples from breast cancer patients as minimally invasive method and correlate their results with both tumor markers and clinicopathological data.

Materials and methods

Study population

After obtaining ethical approval from the Scientific Medical Ethical Committee (National Research Centre, number#15029), and between February 2018 and January 2019 a total of 88 females were enrolled in the current study and they were categorized into three groups based on their diagnostic criteria: primary breast cancer group (n=44), female patients with benign lesions (n=25) and healthy individuals (n=19) served as control group. All groups were of matched ages. The enrolled individuals who fulfilled the inclusion criteria (newly diagnosed breast cancer patients, and before they start any treatment strategies) signed their informed consent, while those with distant metastasis or who received radio-or- chemotherapy were excluded from the study. Clinicopathological data for enrolled individuals were collected from their clinical sheets, and breast cancer patients were diagnosed according to their staging and grading systems following the TNM classification [22] and modified Scarff-Bloom Richardson histologic grading system [23], respectively. The expression of hormonal receptors was examined using immunohistochemistry method as previously reported [24]. Both ER and PgR were consider positive if ≥10%nuclei was positively stained using 10 high-power field, andHER-2neu were considered positive if scored as +3 [25].

Sample collection and processing

Three milliliter blood samples were collected from all enrolled individuals in tubes with polymer gel and clot activator (Greiner bio-one, GmbH, Australia) then allowed to clot at room temperature for 30min, and all samples were centrifuged at10,000×g for 10min at 4°C (13-18KS, Sigma, Germany). Separated sera were aliquoted and stored at −80 °C for miRNA expression analysis.

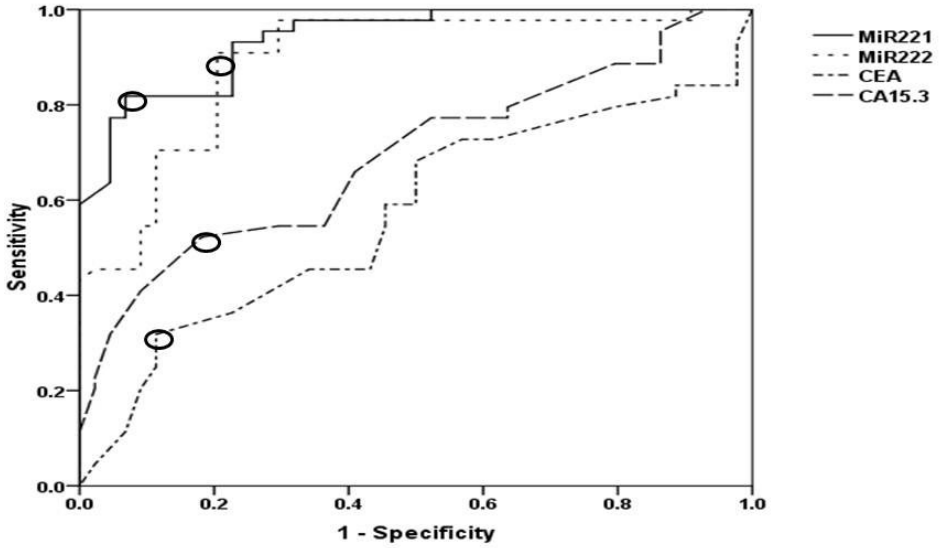
Assessment of Tumor markers

By using enzyme-linked immunosorbent assay (ELISA) both tumor markers, CEA and CA15.3 were detected in serum samples using available commercial ELISA kit (Immunospec Corporation, Canoga Park, CA, USA). According to instructions in the manual manufacturer's protocol, their concentration was detected using GloMax®-Multi detection system (Promega, Fitchburg, WI, USA).

MiRNA extraction

Circulating miRNA was extracted from the serum samples using miRNeasy Mini kit (Catalogue # 217004, Qiagen, USA), as recommended by the manufacturer's instructions, briefly: QIAzol Lysis Reagent (RNA extraction reagent) was added to serum samples (with volume ratio 5:1) and then vortex was applied. After incubation for 5 min at room temperature (25 °C) Lysates were left to promote dissociation of nucleoprotein complexes. Phase separation step was carried out by adding an equal volume of chloroform to the tube containing the lysate, and vortex followed by centrifugation for 15 min at 12,000 xg at 4 °C using cooling centrifuge (13-18KS, Sigma, Germany). The upper aqueous phase was transferred to a new collection tube. Then 1.5 volumes of 100% ethanol were added to the aqueous phase followed by pipetting up and down several times. Up to 700 µl of the sample was transferred into an RNeasy Minispin column (Qiagen) in a 2 ml collection tube and centrifuged for 15 s at ≥ 8000×g for 15 min at room temperature. The RWT buffer (700 µl) was added to the RNeasy Mini spin column the centrifugation was applied and the flow-through was discarded. Afterwards, 500 µl of RPE buffer was added then the column was centrifuged and flow-through was discarded, this step was repeated. The RNeasy Mini spin column was placed into a new 2 ml collection tube and centrifuged at full speed for 2 min. After washing steps; the RNeasy Mini spin column was placed into a new 2 ml collection tube and centrifuged at full speed for 2 min. Finally, the RNeasy Mini spin column was transferred to a new 1.5 ml collection tube. The RNA was eluted using RNase-free water in a final volume (30 µl) was added directly onto the RNeasy Mini-spin column membrane and centrifugation was done for 1min at≥8000×g. The purity and the concentration of the purified miRNA was detected using nano-drop spectrophotometer (Quawell, Q-500, Scribner, USA) and stored at −80 °C till further assessments.

Reverse transcription and cDNA preparation



Marker	AUC	SE	95% CI
CEA	569	0.0612	0.459 - 0.674
CA15.3	0.692	0.0562	0.585 - 0.786
MiR-221	0.939	0.023	0.893 - 0.984
MiR-222	0.891	0.035	0.822 - 0.96

Figure 1. Receiver operating characteristic curve (ROC) for investigated miRNAs and tumor markers. The open circle donates the best cutoff point for investigated markers; for miR-221 was 32.4 fold change with AUC (95%CI, SE) 0.939 (0.893-0.894, 0.023) at P<0.001, for miR-222 was 21.6 fold change with AUC 0.891 (0.822-0.96, 0.035) at P<0.001, for CEA 15.3 ng/ml with AUC 0.569 (0.459 - 0.674, 0.0612) at P=0.018, and for CA15.3 was 21 ng/ml with AUC 0.692 (0.585 - 0.786,0.0562) at P<0.0001.

Reverse transcription of miRNA was performed MiScript II reverse transcription kit (Cat number # 218160, Qiagen, USA). As recommended in the manufacturer's instructions by using a total volume of 20 µl of reverse transcription reaction components as follows: 4 µl MiScript HiFlex buffer, 2 µl nucleic mixture, 2 µl MiScript RT mixture, and RNase-free water (variable depending on the volume of the added template miRNA) and template of purified miRNA with adjusted concentration 100 ng/ml. The polymerase chain reaction (PCR) tubes were then placed in thermal cycler (Sure Cycler 8800, Agilent, USA) and the transcription profile was adjusted for 60min at 37 °C. Complementary DNA concentration and purity were detected using nano-drop spectrophotometer (Quawell, Q-500, Scribner, USA) and stored at −20 °C till performing qPCR.

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was carried out using miScript primer assay (Cat number 218300, Qiagen, USA) for miRNA-221 (Hs_miR_221_2miScript Primer Assay, MS00009079), miRNA-222 (Hs_miR_222_2miScript Primer Assay, MS0007609), the reaction was performed using MiScript SYBR Green PCR kit (Cat number 218073, Qiagen, USA).Also, RNU6–2 (Hs_RNU6-2_11 miScript Primer Assay, MS00033740) was used as an endogenous control to normalize the expression levels of the investigated miRNAs. The reaction for miScript primer assays were carried out by using cDNA with concentration adjusted to 2 ng/ml and atotal volume of 20 µl, whereas thermal reaction conditions were as follows: 95 °C for 15min followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 34 s, in which fluorescence was acquired and detected by Strata gene Real-time PCR system (Max3005P QPCR system, Strata gene, Agilent biotechnology, USA). The expression levels of the investigated miRNAs were evaluated using the ΔCt method 26. The cycle threshold (Ct) value is the number of qPCR cycles required for the fluorescent signal to cross a specified threshold. ΔCt was calculated by subtracting the Ct values of RNU6–2 from those of investigated miRNAs. ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the cancer samples.

Statistical analysis

The fold change values in investigated miRNAs was calculated using the equation of 2ΔΔCT. The association between the clinicopathological and demographic factors with investigated miRNAs was determined by ANOVA analysis. The optimal cutoff point was determined using Receiver Operating Characteristic (ROC) Curve which was plotted by calculating the true-positive fraction (sensitivity [%]) and false-positive fraction (100-specificity [%]) and the best cutoff point was detected as the point that maximize the sum of the sensitivity and the specificity and area under curve (AUC) approach to 1 (range 0.5-1). Accordingly ROC curbves for investigated items were blotted to detect the sensitivities and the specificities for the miRNAs and tumor markers with their diagnostic efficiencies 27. Correlations between investigated miRNAs were analyzed using the Spearman's correlation coefficient (CC). SPSS (version 10 SPSS, Inc., Chicago USA) was used in data analyzing and P-value were two-tailed and considered significant if < 0.05.

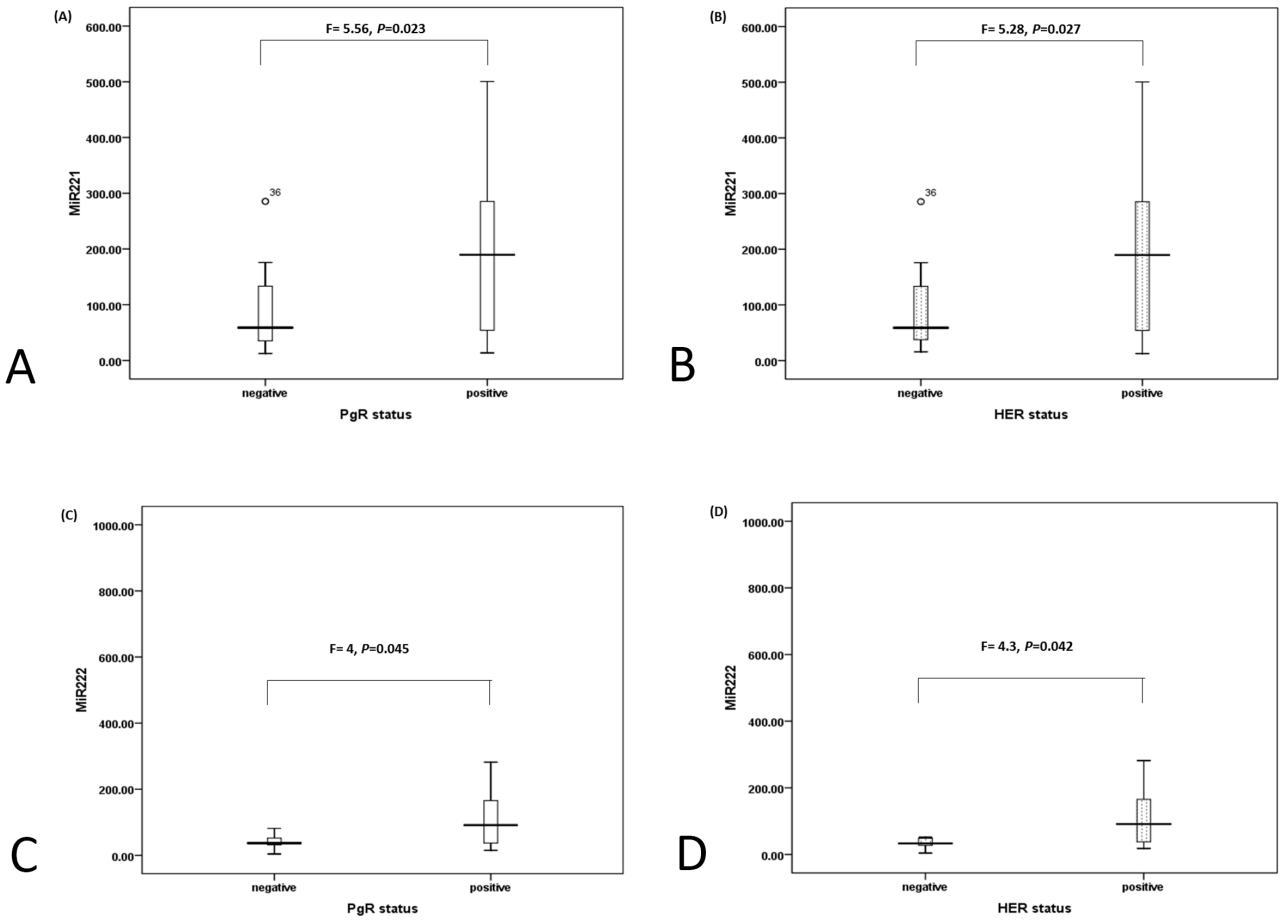


Figure 2. Box-Plot represents the relation between hormonal status and miR-221 and miR - 222. (A) Relation between miR-221 and PgR as mean level± SEM is 95 ± 23 for negative PgR and 191 ± 26.6 for positive PgR. (B) Relation between miR-221 and HER-2/neu as mean level ± SEM is 96 ±23 for negative HER-2 and 190 ± 26.7 for positive HER-2. (C) Relation between miR-222 and PgR as mean level ± SEM is 46.7± 7.7 for negative PgR and 128.3 ± 29.3 for positive PgR. (D) Relation between miR-222 and HER-2/neu as mean level ± SEM is 44.2 ±8 for negative HER-2 and 129 ± 29 for positive HER-2.

Results

A total of 88 patients were enrolled in the current study they were divided according to their clinical criteria into 44 patients with breast cancer and the remaining (n=25) were diagnosed with benign breast lesion, a group of 19 healthy individuals were recruited as control group. All individuals were of matched ages as no significant difference (F=0.202, P=0.818) was reported between them regarding their ages, premenopausal status was reported in 57 cases and 31 were reported as postmenopausal with no significant difference between studied groups (F=2.54, P=0.28).

Diagnostic Efficacy of circulating miRNA-221 and miRNA-222

Mean levels of investigated miRNAs reported significant difference among studied groups as the miRNA-221 level was higher in breast cancer group as compared to the other two enrolled groups (benign and healthy control groups) as reported in **Table 1**, the median level was higher 9.2 folds in breast cancer (median119) compared to benign lesion (median 12.9) and 25.3 folds than healthy control (median 4.7) at P<0.0001, and the benign. Similarly miRNA-222 the mean levels were superior in breast cancer group as compared to benign and control ones as reported in **Table 1**. The increment in the median levels was 2.7 folds higher in cancer patients (median 52.8) as compared to benign ones (median 19.5) while it was 9.8 folds as compared to control group (median 5.4). Tumor markers CEA and CA15.3 also reported significant increase in breast cancer patients as compared to the other two studied groups as shown in **Table 1**.

The positivity level was considered as values above the cutoff points which have been reported from the ROC curves as shown in **Figure 1** as 32.4 fold change for miRNA-221, 21.6 fold change for miRNA-222, 15.3 ng/ml for CEA and 21 ng/ml for CA15.3. Considering these cutoff points the positivity rates were significantly increased in cancer as compared to benign or control group as reported in **Table 1**.

Table 1. Level and Positivity rates of investigated markers among the three studied groups.

	MiR-221		MiR-222		CEA		CA15.3	
	Mean± SEM	Positivity	Mean ± SEM	Positivity	Mean ± SEM	Positivity	Mean ± SEM	Positivity
Control	8.18 ± 1.7	0%	5.6 ± 0.28	0%	8.86 ± 0.84	0%	12.4 ± 1.3	0%
Benign lesion	21.5 ± 3.3	7.7%	26.6 ± 5	18.4%	12.35± 0.85	20.0%	16.4 ± 1.4	32.0%
Breast cancer	158 ± 2	92.3%	100 ± 2	81.6%	13.1 ± 1.1	31.8%	19.7 ± 1.4	52.3%
	F=24, P<0.0001	X^2= 50, P<0.0001	F=8.3, P<0.0001	X^2= 49, P<0.0001	F=3.6, P=0.03	X^2= 7.98, P=0.018	F=5.5, P=0.006	X^2= 16, P<0.0001

Relation between miRNAs , tumor markers and clinicopatholical factors

MiRNA-221 expression level reported significant difference (F=5.9, P=0.019) with pathological types as its level was increased among breast cancer patients with invasive duct carcinoma (IDC) (mean ± SEM) (195.7 ± 26) as compared to those with duct carcinoma in situ (DCI) (99 ± 25). Also miRNA-221 expression level was increased with positive lymph nodes (203.8 ± 29) as compared to its expression (112.7 ± 24) in negative lymph nodes at (F=5.56, P=0.023). Clinical stages reported significant difference with miRNA-221 as for early stage it was (104.5 ± 24), while for late stage it was (195.5 ± 28) at (F=5.34, P=0.026). Histological grading was significantly related with miRNA-221 expression as increased expression was observed with high grade breast cancer (190 ± 26) as compared with low grade (102 ± 26) at (F=4.6, P=0.035). Among hormonal status, ER- status did not report significant level while significant difference was reported between PgR and HER-2/neu positive levels with miRNA-221 as shown in **Figure (2 A-B)**. MiRNA-222 expression level showed significant relation (F=4.9, P=0.032) with pathological type since its mean level (mean ± SEM) was increased among patients with IDC (134 ±30) as compared to those with DCI (46.2 ±10). For clinical staging the expression of miRNA-222 was significantly (F=4.08, P=0.05) as in early stage it was (52.3 ±11) and for late stage (133.5 ±32). Also its expression was increased significantly (F=6.1, P=0.017) with high grade breast tumor (136.4 ±29) as compared to low grade ones (37.6 ±6.5). Regarding hormonal status, significant difference (P<0.05) was reported between miRNA-222 with PgR and HER-2/neu as shown in **Figure (2C-D)**.

Both CEA and CA15.3 reported no significant relation with clinicopathological factors.

Concordance between investigated markers and enrolled groups

Assessment of investigated markers; miRNAs and tumor marker regarding enrolled individuals (n=88) was reported in **Table 2**, while among breast cancer group (n=44), significant relation between both miRNAs (R= 0.433, P=0.003) was detected.

Overall sensitivities, specificities, PPVs, NPVs and accuracies of miRNAs and tumor markers among breast cancer diagnosis and high risk groups

As reported in **Table 3**, the sensitivity and PPV of both miRNA-221 and miRNA-222 was superior over the other investigated markers (CEA and CA15.3) for early detection of breast cancer especially those at high risk as early stage and low grade tumors.

Discussion

Circulating miRNAs are attractive molecules as non-invasive cancer biomarkers due to their surprising degree of stability in biological fluids. Several recent studies have demonstrated that miRNAs are stably detectable in plasma/serum [28, 29, 30]. MiRNA-221 and miRNA-222 are overexpressed in different types of malignant neoplasms including ovarian cancer 31, hepatocellular cancer [32], glioblastomas [33] and breast cancer [10, 13, 34]. Both miRNAs were shown to promote cell growth, cell cycle progression and invasion in these tumore types in vitro and in vivo [32, 33]. Thus, they act as so-called “oncomirs”. These effects are mediated by the direct inhibition of the tumor suppressors PTEN [35] and CDKN1B [31], previously it has been

Table 2. Correlation between investigated markers and enrolled individuals (n=88).

Markers	MiR-221	MiR-222	CEA	CA15.3
	R, *P*	R, *P*	R, *P*	R, *P*
MiR-221	-	0.371*, 0.0001	0.364*, 0.01	0.143, 0.184
MiR-222	0.371*, 0.0001	-	0.159, 0.139	0.104, 0.333
CEA	0.364*, 0.0001	0.159, 0.139	-	0.040, 0.710

Table 3. Overall sensitivities, specificities, PPVs, NPVs and accuracies of miRs and tumor markers among breast cancer diagnosis and high risk groups.

	Breast cancer diagnosis	Early stage	Low grade
MiR-221			
Sen.%	92.3	77.8	81.2
Spec%	83.7	93.2	93.2
PPV	81.8	82.4	81.2
NPV	93.2	91.1	93.2
Acc.	87.5	88.7	90
MiR-222			
Sen.%	81.6	77.8	75
Spec%	89.7	79.5	79.5
PPV	90.9	60.9	57.1
NPV	79.5	89.7	89.7
Acc.	85.2	79	78.3
CEA			
Sen.%	31.8	22.2	18.8
Spec%	88.6	88.6	88.6
PPV	73.7	44.4	37.5
NPV	56.5	73.6	75
Acc.	60.2	69.4	70
CA15.3			
Sen.%	52.3	55.6	56.2
Spec%	81.8	81.8	81.8
PPV	74.2	55.6	52.9
NPV	63.2	81.8	83.7
Acc.	67	74.2	75

reported that the critical role of miRNA-221and miRNA-222 as oncomiRs in breast cancer carried out by adversely regulating growth arrest specific transcript 5 (GAS5), a tumor-suppressor gene involved in regulating the apoptosis of tumors, as a direct target gene of miRNA-221 and miRNA-222 [36]. Also Roscigno and his team [37] have shown that overexpression of miRNA-221 and miRNA-222 might initiate breast tumor formation through repressing translation of DNMT3b (DNA methyltransferase3b), where loss of DNMT3b leads to decreased differentiation of cells, and enhances stem-ness potential, resulting in the genesis of breast tumor.

In the present study, expression level of MiRNA-221and miRNA-222 were detected in serum samples from a total of 88 individuals grouped according to their clinical diagnosis into

patients with primary breast cancer, patients with benign breast lesions, and healthy individuals served as control, the results compared to other commonly used protein-based markers (CEA and CA15-3) to assess the role of miRNAs as diagnostic markers for breast cancer. Currently, both MiRNA-221and miRNA-222 were up-regulated in breast cancer group with high median levels (119) for miRNA-221 and (52.8) for miRNA-222 compared to other groups (benign and healthy control), a result in harmony with Kim et al.38 and in line with Swellam et al 10, 13 for miRNA-222. An in vitro study by Falkenberg and his colleagues 39 stated that overexpression of miRNA-221and miRNA-222 induced cell proliferation and invasion. The diagnostic efficacy was detected using ROC curve and revealed high AUC (0.939) and (0.891) for miRNA-221 and miRNA-222, respectively compared to routine tumor markers. These results indicated the oncogenic properties of miRNA-221 and miRNA-222 that may be due to their role as promoting malignant cell proliferation through suppressing cyclin-dependent kinase inhibitors expression regulators of cell cycle progression [40]. The present results in concordance with a study carried using breast tissue samples by Zong and his colleagues [36] whose stated that the expression level of miRNA-221and miRNA-222 increased in breast cancer tissues compared with non-cancerous tissues.

The relation between investigated markers with clinicopathological factors revealed that miRNA-221and miRNA-222 expressions reported statistically significant increase in late stages (III-IV) and high-grade tumors (III). Moreover their expression increased significantly with lymph nodes involvement, which are in concordance of previous studies [39, 41] that emphasize their involvement in breast cancer progression and metastasis, which is one of the critical causes of breast cancer patient's death. The spread of primary breast tumor cells to the lymph nodes is at the forefront of symbolizing the first signs of metastatic expansion [42] thus, aberrant expression of molecular markers in BC tissues with lymph node metastases may represent early biomarkers of the risk for developing distant metastases.

Overexpression of miRNA-221and miRNA-222 significantly increased in breast cancer patients with IDC compared to non-IDC which indicates that it can be used to discriminate between different pathological types; this may be due to its negative correlation with inhibitor matrix metalloproteinase −3 (TIMP-3) which have anti-invasive [43] and anti-angiogenic features [44].

In breast cancer, miRNA-221and miRNA-222 was more abundant in basal-like tumors than in ER/PR–positive tumors 44 miRNA-221and miRNA-222 directly target ER-alpha gene transcription, which is implicated in breast tumorgenesis, especially more aggressive basal-like breast cancer [46, 47], hence the ER is inhibited upon overexpression of miRNA-221and miRNA-222 and cancer cells grow in an estrogen independent mode. Current study revealed non-significant difference between hormonal ER- status and miRNA-221and miRNA-222, which may postulate that this group of breast cancer patients may not benefit from tamoxifen, a traditional anti-estrogen that competes with ER for ER-α [48]. On the other hand this study reported significant difference between PgR and HER-2/neu positive levels with miRNA-221 and miRNA-222, which may suggest their possible role as predictive prognostic marker for this type of cancer.

Currently, significant positive correlation was reported between miRNA-221 and miRNA-222 and this may be due to the fact that they are homologues miRs in human DNA, chromosome Xp11.3 is the miRNA-221and miRNA-222 gene cluster site [49] and hence they both may contribute in breast cancer aggressiveness.

As reported in **Table 3**, the sensitivities and PPV values for miRNA-221 and miRNA-222 were superior to CEA and CA15.3 for detection of breast cancer as well as for identification of breast cancer patients with early stages and low grades. These finding indicate the prospective role of using miRNA-221 and miRNA-222 as early diagnostic molecular markers in breast cancer.

Upon detection of breast cancer patients, our results emphasize high significant expression of miRNA-221 and miRNA-222 in serum samples of breast patients as compared to routinely used blood-based tumor markers, CEA and CA15.3, these results point out the value of miRNA-221 and miRNA-222 as noninvasive markers for early detection of breast cancer patients especially those at high-risk groups.

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Author contributions

Study conception and design: by MS, EAA, SAA and MAM; Acquisition of data: EAA and MAM; Analysis and interpretation of data: MS and EAA; Drafting of manuscript: MS, EAA, SAA and MAM; Critical revision: MS, EAA, SAA and MAM.

Competing interests

The authors declare no conflict of interest with the work.

Ethical statement

Ethical approval from the Scientific Medical Ethical Committee (National Research Centre, number#15029) was obtained for the current study.

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