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Circulating miR-30b-5p Level in Plasma as a Potential Biomarker for Early Detection of Breast Cancer

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Abstract:

Purpose: We aimed to assess the rule of plasma miR-30b- 5P as a biomarker for early detection of breast cancer (BrC). **Patients and Methods:** This case control study was established on 50 participants, females, newly diagnosed with breast cancer before receiving any treatment. Subjects were categorized into two groups: Group I (patient group): 35 adults, their age was (53.6 ± 12.20) years, are newly diagnosed BrC before any treatment and Group II (control group): 15 subjects, their age was (47.7 ± 9.59) years, apparently healthy matching age and sex with patients. **Results:** There is statistically significant difference regarding presence of lesions by Mammogram when compare cases with control ($p < .001$). And there is a significant elevation in the expression of miR - 30 b - 5p when comparing cases with control group ($p < .001$). **Conclusion:** Plasma miR-30b-5P can be applied as a marker for early diagnosis of BrC.

Keywords: MiRNA-30b-5p; Biomarker; Breast Cancer; Mammography

Introduction

Breast cancer (BrC) is the most commonly detected malignancy around the world. It ranks second in the population in terms of cancer-related mortality with highest survival rate [1]. Histological sub classification based on human epidermal growth factor, progesterone receptor and estrogen receptor are part of pathological diagnosis. The four primary subtypes of tumors that are classified according to the Ki67 level are [luminal A, luminal B, HER2-positive (HER2+) and triple-negative (TN)]. This information aids physicians in making choices about treatment, progression, and prediction [2].

Twenty percent of patients develop metastasis despite advancements in detection and therapy, either as a result of treatment resistance or a late-stage diagnosis [3].

Mammography is the best available screening option but when combined with other diagnostic methods improve the

accuracy of diagnosis and survival rate of patients [4].

By-post transcriptional editing of target messenger (mRNAS) expression, miRNA implicated in tumor development, metastasis, or immune evasion, miRNA has been found to have a role in cancer biology [5]. Furthermore, a number of tumor-associated miRNA profiles were studied as potential biomarkers for tumor sub classification, diagnosis and response. Clinics are particularly interested in the improvement of early diagnostic tools as early detection is linked to an improved progression [6]. miRNAs were verified to be useful early diagnostic tools for numerous cancers, including BrC [7]. Numerous body fluids, including blood, have been demonstrated to contain miRNA, which are found there as call-free miRNAS. One benefit of using them in body fluids is their excellent stability, which allows for easy, affordable, rapid and non-invasive assays [8].

One of the miR-30b family, MIR-30b-5p, has been linked to the emergence of numerous cancer types [9].

This study intended to investigate the rule of miR-30b- 5P as a biomarker for early diagnosis of BrC in plasma.

Patients and Methods

This case control study involved 50 female participants. Following approval from the Benha University Hospitals' Ethical Committee, the study was conducted from August 2022 to February 2023 in Benha, Egypt [Approval code (M s 4-1-2022)]. The patients gave their informed written consent.

Patients newly diagnosed with breast cancer before receiving any treatment were included in this study. Exclusion criteria were patients with any other cancer or under any treatment for BrC.

Subjects were categorized into two groups: Group I (patient group): 35 adults, their age was (53.6 ± 12.20) years, are newly diagnosed BrC before any treatment and Group II (control group): 15 subjects, their age was (47.7 ± 9.59) years, apparently healthy females without breast cancer or having breast lesions other than cancer, matching age and sex with patients.

All subjects were subjected to history taking, clinical examinations, radiological investigation [mammogram], and laboratory investigations including [complete blood count (CBC), CA 15-3 and molecular assessment (level of miR – 30 b – 5 p was measured using real time PCR by sybr green technique).

Detection of miRNA 30b - 5p:

EDTA blood samples were subjected to total RNA extraction using GENE zol™ Reagent. Reverse transcriptase was used to form c DNA using TOP script™ cDNA Synthesis Kit Reverse Transcription Kits. Amplification and detection of mature miRNA were performed in two steps- Real Time PCR using SYBR Green with high Rox.

Total RNA extraction:

RNA extraction was carried out using GENE zol™ Reagent (cat.no. (GZR100)) supplied by Gene aid (Taiwan) according to manufacturer's instructions.

Principles:

GENE zol™ is a phenol and guanidine thiocyanate solution facilitate tissues' lysis, to inhibit RNases, and depends on organic extraction to eliminate cellular DNA and proteins.

cDNA Reverse Transcription:

The total RNA was subjected to reverse transcription using TOPscript™ RT DryMIX (dt18/dn6 plus), (cat. no. RT220)

enzynomics (South Korea), cDNA Synthesis Kit for the conversion of RNA to cDNA.

Real-Time qPCR for quantification and detection of miRNA -30b-5p:

Real time PCR was carried by TOPreal™ qPCR 2X Pre-MIX (SYBR Green with high ROX), enzynomics (South Korea), (cat.no. RTS01S), which is designed to have improved specificity and minimize nonspecific noise signal resulting from primer dimer formation or nonspecific product creation. This is done using Applied Biosystem (Step One Real –Time PCR System) USA, serial number 271003648.

The test primers:

The primers were lyophilized and prepared as directed by the manufacturer by adding to the vials the necessary amounts of nuclease-free water (which was included with the master mix) so that each one microliter had 25 pmoles of the primer. Once prepared, it was kept in storage at -20°c. Before opening, spin the priming vials for five minutes at a speed of 1000 rpm.

Primer sequences and their reconstitution:

Endogenous control:

Forward 5' – 3'
(GGAAATCGTGCGTGACATTA)
Reverse 3'- 5'
(AGGAAGGAAGGCTGGAAGAG)

Primer miR- 30b-5p:

Forward 5' – 3'
(CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCTGAGTT)
Reverse 3' – 5'
(ACACTCCAGCTGGGTGTAAACATCCTACAC)

Relative gene expression determination:

The Ct was obtained for the target gene (Ct_{target}) for miR 30b-5p and for the reference gene (GAPDH) (Ct_{ref}) for each well of the PCR run. ΔCt was calculated for patients and controls samples by subtracting the Ct_{target} from Ct ref that were calculated for each well in the run. The average of them was calculated ΔCt avgcont, then the ΔΔCt was calculated for each sample by subtracting the $\Delta Ct_{patient}$ from ΔCt avgcont for this sample. Relative quantitation (RQ) of mRNAs expression were deduced for each patient or control samples by calculating of the fold change ($2^{-\Delta\Delta Ct}$) of expression of each gene after normalization to an endogenous reference and related to the expression of the same gene in healthy control samples as above.

Statistical analysis

Data was statistically analyzed by SPSS version 26 (IBM Inc., Chicago, IL, USA). To assess if the data distribution was normally distributed, the Shapiro-Wilks test and histograms were employed. The mean ± standard deviation (SD) of quantitative parametric variables were reported, and the unpaired Student's t-test was used to compare the two groups. The Mann Whitney-test was used to evaluate quantitative non-parametric data, which were reported as the median and interquartile range (IQR). When appropriate, the Fisher's exact test or the Chi-square test were used to examine the frequency and percentage (%) of the qualitative variables. The Pearson moment correlation equation was used to perform a correlation between a number of variables. The Roc curve was employed to assess the sensitivity and specificity of the diagnostic performance. A statistically significant result was defined as a two-tailed P value less than 0.05.

Results:

Regarding age, hormonal therapy, family history, history of exposure to radiation, obesity, menstrual and obstetric history were insignificantly different between cases and control group. No patient was smoker or drinking alcohol in both groups. Table 1

There was statistically significant difference between cases and control group regarding Lumps at the breasts and presence of enlarged L.N near the breasts (P <0.001). But there is no statistically significant difference between cases and control group regarding Skin covering breast for any rash or dimpling and Any fluids from nipples. Table 2

There was significantly variation between the patients and controls, regarding CA 15 - 3, presence of lesions by Mammogram and expression of miR - 30 b - 5p ($p < .001$), while there was insignificantly different between Hb level, platelet count and TLC. The expression of miR - 30 b - 5p was significantly elevate when comparing subjects with enlarged L.N and subjects without enlarged L, N ($P < .001$). Table 3

There was statistically significant positive correlation between miR - 30 b - 5p and age. There was no significant correlation between miR - 30 b - 5p and [Hb, PLT (x 10^3), TLC (x 10^3) and breast tumor markers (cA 15-3) (< 30 u lmL)]. Table 4

miR - 30 b - 5p expression showed excellent (AUC=0.943), at cutt of value of > 2.17, sensitivity was 97.1%, specificity was (80 %), PPV was 91.9%, NPV (92.3%) and accuracy was 92.0 %. Figure 1

Table 1: Sociodemographic data, hormonal therapy, history, menstrual and obstetric history of the participants

	Control (n=15)	Cases (n=35)	*P*
Age (years)	47.7±9.59	53.6±12.20	0.103
Sex Female	15 (100.0%)	35 (100.0%)	
Smoking or alcohol	0 (0.0%)	0 (0.0%)	--
Hormonal therapy	1(6.7%)	1(2.9%)	1.00
Family history of mother, sister or daughter BrC particularly at a young age	1(6.7%)	3(8.6%)	1.00
History of exposure to radiation	15(100.0%)	35(100.0%)	--
Obesity	4(26.7%)	11(31.4%)	1.00
Menstrual and obstetric history			
Beginning period at young age before age 12	1(6.7%)	4(11.4%)	1.00
Beginning menopause at an older age after 55	0(0.0%)	2(5.7%)	0.571
Having never been pregnant	1(6.7%)	1(2.9%)	1.00

Data are expressed as mean ± SD or frequency (%). BrC: breast cancer. * Significant *p* value <.05

Table 2: Clinical presentation of the participants.

Clinical presentation	Control (n=15)	Cases (n=35)	*P*
Skin covering breast for any rash or dimpling	0 (0.0%)	3 (8.6%)	0.545
Any fluids from nipples	1 (6.7%)	2 (5.7%)	1.00
Any lumps at the breasts	0 (0.0%)	35 (100.0%)	**<0.001***
Any enlarged lymph nodes near the breast	1 (6.7%)	32 (91.4%)	**<0.001***

Data are expressed as frequency (%). * Significant p value <.05.

Table 3: CBC, mammogram, and level of miR - 30 b - 5p expression of the participants and according to presence of L.N

		Control (n=15)	Cases (n=35)	*P*
Hb (g/dl)		11.4 ± 1.13	11.6 ± 0.74	0.435
Platelet (x 10^3)		256.1 ± 54.33	260.7 ± 64.47	0.687
TLC (x 10^3)		6.8 ± 1.16	6.7 ± 1.74	0.992
Breast tumor markers (cA 15-13) (<30 u lmL)		14.5 (11.5 – 16.0)	16.7 (14.0 – 21.5)	**0.031***
Mammogram	**No lesions**	15(100.0%)	0(0.0%)	**<0.001***
	Lesions	0(0.0%)	35(100.0%)	
Level of miR - 30 b - 5p expression		1.3 (0.99 – 1.97)	9.14 (4.71 – 20.84)	**<0.001***
level of miR - 30 b - 5p expression according to presence of L. N				
		Not enlarged(n=17)	**Enlarged (n=33)**	
Level of miR - 30 b - 5p expression		1.41 (1.03– 3.57)	9.14 (5.15 – 21.51)	**<0.001***

Data are expressed as mean ± SD or frequency (%) or median (IQR). * Significant *p* value <.05, CBC: complete blood count, TLC: total leucocytic count. L.N: Lymph node.

Table 4: Correlation between miR – 30 b – 5p expression and other parameters for all participants.

	r	*p*
Age (years)	0.304	**0.032***
Hb (g/dl)	0.156	0.279
PLT (x 10^3)	0.085	0.557
TLC (x 10^3)	-0.121	0.403
Breast tumor markers (cA 15-3) (< 30 u lmL)	0.177	0.219

Rs: Spearman coefficient, * Significant *p* value <.05, CBC: complete blood count, TLC: total leucocytic count.

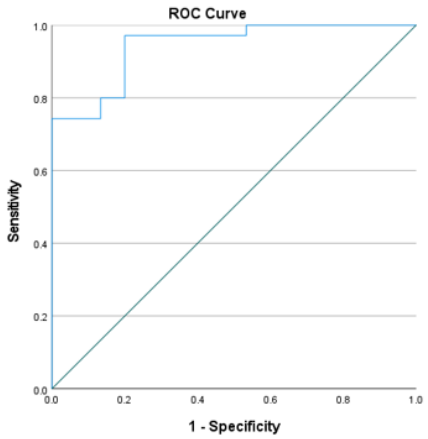

Figure 1: ROC curve for miR - 30 b - 5p expression in diagnosis of cancer breast.

Discussion

Breast cancer is the most predominant malignancy in females and the primary cause of cancer-related mortality in them, despite advancements in early detection and treatment. This is mostly because of

the emergence of recurrence and/or metastasis. Only about 5% of patients have

distant metastases upon diagnosis, and within the first three years, up to 10-15% of patients get distant metastases [10, 11].

MicroRNAs' innate stability and durability in body fluids and tissues make them promise as noninvasive cancer indicators. There is mounting evidence that certain microRNAs operate as signaling mediators to facilitate the colonization of a particular organ at various stages of the metastatic cascade [12].

The current data showed no statistically significant difference regarding the beginning period at a young age before age 12, beginning menopause at an older age, and having never been pregnant between both groups. Also, no statistically significant difference regarding hormonal therapy, family history of mother, sister, or daughter BrC particularly at a young age, history of radiation exposure, and obesity between cases and control group was detected.

Breast development and hormonal changes at puberty might affect BrC risk, but epidemiological analyses have focused

largely on age at menarche and not at other pubertal stages [13].

Also, Fang et al. [14] have informed that tumor immunomarkers that may be used to screen patients and help identify which of them will benefit from BrC immunotherapy are desperately needed. Currently, no one tumor marker can accurately predict the outcome of treatment. To more precisely identify individuals who will respond favorably to immune checkpoint blockade medication, several markers may be combined.

In this study, the presence of enlarged LN near the breasts and lumps at the breasts was significantly different between the control group and the patients. However, regarding any rash or dimpling on the skin surrounding the breast and any nipple fluid, there was no significant variation between the two groups. Additionally, Kumari et al. [15] demonstrated that in 50% of patients, the tumor was found to be most typically engaged in the upper outer quadrant of the breast.

Regarding Hb level, platelet count, and total leucocytic count, no substantial change between the patients and the controls in the current study was observed. In terms of CA 15-3, there was a statistically significant difference between the patients and the control group. This was consistent with what Kabel et al. [16] who shown that although many breast tumor markers are unique to a particular cancer type, others are present in multiple cancer types. Typically, these markers are used to track the presence of metastasis or recurrences or to assess how well the patient is responding to treatment. BrC is one of the most common malignancies in females worldwide.

Our findings revealed a statistically significant difference regarding the presence of lesions by mammogram when comparing cases with control. Also, the miR - 30 b - 5p expression was significantly elevated in the patients than the controls. In the same context Wu et al. [17] found that miR-30b-5p expression elevated in BrC tissues than in adjacent noncancer tissues.

We found that, the expression of miR - 30 b - 5p was higher when compared to subjects with enlarged L.N and subjects without enlarged L.N. Mir - 30 b - 5p expression showed excellent AUC (=0.943), at best cut of value of > 2.17, sensitivity was 97.1%, specificity was (80%) , PPV was 91.9%, NPV (92.3%) and accuracy was 92.0 %.

MiR-30b-5p has been linked to enhanced apoptotic resistance and cell viability, which may support the propagation and survival of cancer cells. Additionally, miR-30b-5p has been shown to inhibit the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which is involved in the survival and proliferation of cells [12, 18].

Adam-Artigues et al. [19] reported that BrC patients had significantly higher levels of circulating miR-30b-5p than healthy contributors. Additionally, both de novo metastatic patients and patients with positive axillary lymph nodes had circulating miR-30b-5p levels that were considerably greater. As a result, this study emphasized the possible use of miR-30b-5p as a non-invasive, quick, repeatable, and affordable diagnostic biomarker of BrC. The ROC curve analysis showed that miR-30b-5p has a great diagnostic capability for identifying BrC, even at an early stage of the illness.

We observed a significant positive correlation between miR - 30 b - 5p and age, presence of lumps at the breasts, presence of enlarged L.N near the breasts, and mammogram. However, there was a significant negative correlation between miR - 30 b - 5p and hormonal therapy. This agreed with Zhang et al. [20] who reported that the dysregulated miRNAs and the target genes were correlated with the survival of BrC cases.

Similarily, miR-30b-5p and miR-148a-3p were reported to be linked with lymph node invasion by Li et al. [21] All of the sex hormone receptor, clinical stage, lymph node metastasis, and the levels of miR-30b-5p, miR-130a-5p, miR-144-3p, miR-148a-3p and miR-152-3p expression were increased in BrC patients than in normal individuals, suggesting the utility of these miRNAs in the diagnosis.

Additionally, there are certain restrictions on the sensitivity and specificity of a single miRNA as a diagnostic biomarker. Thus, it has been claimed that miRNA panels or signatures, as indicated by Adam-Artigues et al. [19] might improve the quality of proposed diagnostic biomarkers. Therefore, the creation of a circulating miRNA signature signature that is both accurate and reliable for BrC identification is still ongoing. These difficulties show that before miRNA measurement in liquid biopsy is applied in clinical settings, it must be validated and standardized.

The restriction of this study is the relatively small sample size, as the study was in a single center.

Conclusions:

MiR-30b-5p was statistically increased in breast cancer patients in comparison with the controls and there is a positive correlation between miR - 30 b - 5p and age, presence of lumps at the breasts, presence of enlarged L.N near the breasts, and mammogram. However, there is a negative correlation between miR - 30 b - 5p and hormonal therapy. miR-30b-5P can be used as a plasma biomarker for early detection of BrC.

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