Diagnostic efficacy of circulating microRNAs-27a, -122 and -155 in detecting early-stage breast cancer

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Abstract. Circulating microRNAs (miRNAs/miRs) are found in numerous body fluids in healthy and disease conditions. In various types of malignancies, miRNAs are abnormally expressed and dysregulated. The present study determined and compared the circulating levels of miR-27a, -122 and -155 in patients with early-stage breast cancer, benign breast tumors and healthy controls. For this purpose, samples were collected from 56 newly-diagnosed patients with early-stage breast cancer, 56 patients with benign breast tumors and 56 age-matched healthy controls. Serum was separated and reverse transcription-quantitative PCR was used to quantify the expression levels of miRNAs. The diagnostic efficacy of individual miRNAs and the combined effect of all three miRNAs were compared in the three groups. The results revealed that the expression levels of the three miRNAs (miR-27a, miR-122 and miR-155) in serum were significantly increased in patients with early-stage breast cancer when compared to those with benign breast tumors patients and the healthy controls (P<0.001). Receiver-operating-characteristic curve (ROC) analysis indicated that the serum levels of the miRNAs, miR-27a, miR-122 and miR-155, exhibited sensitivity (80.4, 78.6 and 69.6%, respectively) and specificity (89.3, 98.2 and 71.4%, respectively) for the detection of early-stage breast cancer in patients compared to healthy controls. ROC curve analysis also revealed that the serum of the three miRNAs exhibited sensitivity (76.8, 78.6 and 69.6%%, respectively) and specificity (98.2, 87.5 and 78.6%, respectively) for the detection of early-stage breast cancer in patients compared to benign breast tumors. On the whole, the present study demonstrates a considerable diagnostic accuracy of these miRNAs for the detection of early-stage breast cancer. These miRNAs thus have potential for use as non-invasive biomarkers for the diagnosis and screening of breast cancer.

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Introduction

In 2020, breast cancer in females emerged as the global cancer burden, accounting for ~2.3 million newly diagnosed cases, which constituted 11.7% of all reported cancer cases. Worldwide, there were 685,000 deaths recorded due to breast cancer, ranking breast cancer 5th as regards cancer-related mortality globally (1). In 2020, in India, there were a total of 179,790 reported cases of breast cancer and this constituted 10% of all cancer cases in that year. In urban India, breast cancer among females is the most common malignancy. The National Program of Cancer Registries (NPCR) reported an annual percentage change (APC) of 0.68% for cancers at all sites and an APC of 2% for breast cancer during the 3-year period from 2011-2014 (2).

The risk factors for breast cancer are old age, late menopause, early menarche, obesity, smoking, alcohol, a family history of the disease, hormone-replacement-therapy, the use of oral contraceptives and nulliparity. A maternal age of >30 years at the first live birth is associated with a higher risk and <20 years with a lower risk of developing the disease. The most common genes involved are p53 and BRCA1; 85% of breast cancers are sporadic, while 10-15% are hereditary. Luminal-A breast cancer (estrogen receptor-positive, and progesterone receptor-positive and Her2neu-negative) is most common type and is associated with the optimal prognosis. Basal-triple-negative breast cancer (TNBC) is associated with outcomes and high chances of visceral metastasis (3). Mutations of BRCA predispose to hereditary breast and ovarian cancer syndrome (HBOC). HBOC syndrome requires that all primary relatives are counseled and tested for BRCA gene mutations (4).

The early diagnosis and prompt management of breast cancer will eventually greatly reduce the morbidity and mortality. Currently, the majority of cases are diagnosed

using the following techniques: Biopsy, mammography, magnetic resonance imaging, ultrasonography, computerized tomography and positron emission tomography (5).

Tissue gene biomarkers improve the diagnosis of breast cancer to a great extent; however, their applications are limited as sample collection is invasive, and there is a risk of hemorrhage and the unpleasant nature of diagnostic procedures. Thus, there is need for non-invasive marker or minimally-invasive markers which are highly sensitive and specific, and which can be used to detect early-stage breast cancer during screening, thus aiding in diagnosis and prognosis (6).

MicroRNAs (miRNAs/miRs) are short oligonucleotides (20-25 nucleotides in length), non-coding sequences. They regulate the expression of post-transcriptional target genes negatively by silencing mRNA translation and inducing mRNA degradation. miRNAs contain complementary sequences with their respective target gene promoter region and complementary sequences with mRNA transcript coding sequences. The RNA-induced silencing complex (RISC) is present in mRNA transcripts both on the 3' and 5'untranslated regions. miRNAs recruit RISC to complementary sites and regulates the target gene expression (7). On the target genes, the miRNAs have an increased control on amplification and decreased control on its deletion. Oncosuppressor miRNAs inhibit the genes which promote cell growth and onco-miRNAs increase cell proliferation by downregulating tumor suppressor genes and promoting apoptosis (8).

Of note, >1,200 miRNAs have been identified to be strongly associated with breast cancer; however, not one has been translated for use in clinical diagnostics due to a lack of validation and quality assessment (9). Several studies have been performed to assess the specificity and sensitivity of miRNAs as screening tools for the detection of early-stage breast cancer (10,11). miR-27a has been shown to be associated with tumor proliferation and metastasis, and its expression is elevated in the circulation in the presence of breast cancer, suggesting that it may serve as an early detection marker (12,13). miRNA-122 facilitates breast cancer metastasis by preparing fuel utilization in the premetastatic niche and has been tested as a screening biomarker for early-stage BC (14-16). Higher circulating levels of miR-155 in patients with breast cancer are attributed to its dysregulation of tumor suppressors and its involvement in the crosstalk of genetic and epigenetic messages for malignant transformations in BC (17,18).

With the research performed over the past two decades on breast cancer, circulating miRNA levels in blood serve as minimally invasive or non-invasive biomarkers (6). Recently, reverse transcription-quantitative PCR (RT-qPCR) was widely used in detecting circulating miRNAs. It is a highly sensitive and requires a minimal amount of RNA input (19). The present study aimed to investigate the diagnostic role of circulating miRNAs (miR-27a, -122 and 155) in detecting early-stage breast cancer and to compare the assayed levels with those found in women with benign breast tumors and healthy controls.

Materials and methods

Ethics approval and study participants. Ethical clearance obtained from the Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), Bhubaneswar, India (IEC/AIIMS BBSR/PG Thesis/2021-22/32). After obtaining written informed consent, blood samples were collected from the study participants (from August, 2021 to March, 2023). The study was conducted on individuals visiting the Surgery or Onco-Surgery Outpatient Department in our tertiary care hospital, All India Institute of Medical Sciences, Bhubaneswar, India. All patients resided in Eastern India, namely from the states of Odisha and West Bengal. They were all of South Asian Pacific ethnicity. The participants were classified into three groups as follows: Group 1, included cases with early-stage breast cancer; group 2, included cases with benign breast tumors; and group 3, included healthy age-matched females with no breast lumps or any other tumor or comorbidities.

The study sample size was calculated on the basis of the results of the study by Swellam *et al* (13), in which the presence of the miRNA marker was in 90% of the cases and in 10% of the controls. With an α error of 0.05 and 80%, the sample size calculated was 56 in each group, namely 56 healthy controls, 56 benign breast tumor cases and 56 early-stage breast cancer cases that had stage I, stage IIa and stage IIb disease.

According to the study design, the inclusion criteria were women within the age group of 20-60 years, women who had early-stage breast-cancer or benign breast tumors, confirmed by a FNAC/biopsy report, who had not received any intervention and had not reported any other malignancies. Blood samples were collected from all participants in the study from August, 2021 to March, 2023 and serum was separated. A total of 1 ml serum was used for biochemical analyses (liver and kidney function tests) on the AU 5800 auto analyzer (Beckman Coulter, Inc.), using reagents provided by the same vendor. Another 1 ml serum was stored in RNase free aliquots at -80˚C till the extraction of RNA was performed.

miRNA extraction. miRNA was extracted from the serum samples using the miRNeasy Serum/Plasma kit (cat. no. 217184, Qiagen, Inc.), according to manufacturer's instructions; all procedures were performed in RNase free conditions. Finally, elution with 14 μl RNase-free water resulted into a 12-μl eluate. The purity of the extracted miRNAs was detected using a Nanodrop spectrophotometer (Nano Bio-analytical Technologies Limited).

First-strand cDNA synthesis. This protocol involves the conversion of total miRNA into cDNA by reverse transcription. First-strand cDNA synthesis reactions were carried out using the miRCURY LNA miRNA PCR Starter kit (cat. no. 339320, Qiagen, Inc.) and miRCURY LNA RT kit (cat. no. 339340, Qiagen, Inc.). First the volume of template RNA was calculated using the following formula: [Template RNA (μl)=elution volume (μl)/original sample volume (μl)x16 (μl)]. Thus, for a 12-μl elution volume and a 100-μl original sample volume, the template RNA volume was 1.92 μl. As recommended in the manufacturer's instructions, the total volume of 10 μl reverse transcription reaction components were as follows: 2 μl 5X miRCURY SYBR®-Green RT Reaction Buffer, 1 μl 10X miRCURY RT Enzyme Mix, 0.5 μl UniSp6 RNA spike-in, 1.92 μl template miRNA and 4.58 μl RNase-free water. PCR tubes with the 10-μl total reaction volume were placed in thermal cycler (Eppendorf) and incubated for 60 min at 42˚C for the

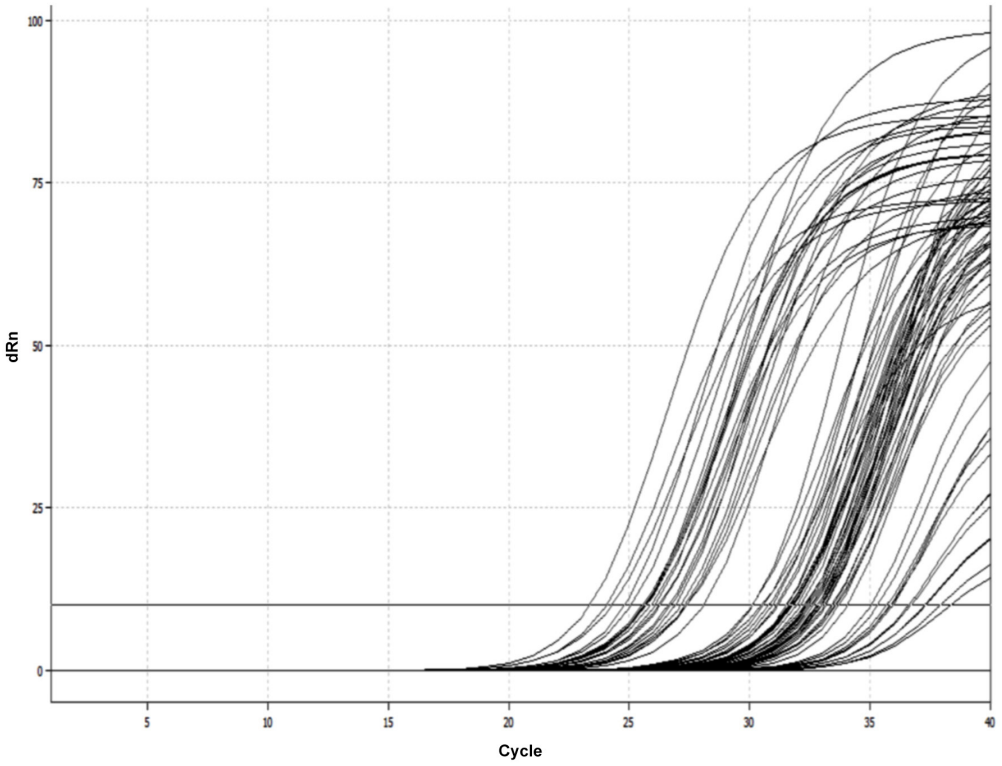

Figure 1. Amplification plot representing microRNAs-16, -27a, -122, -155 gene expression.

reverse transcription step, for 5 min at 95˚C to inactivate reverse transcriptase and finally cooled to 4˚C immediately. PCR tubes were stored at 4˚C until quantitative PCR was performed.

Quantitative PCR. Quantitative PCR was carried using the miRCURY LNA miRNA PCR Starter kit (cat. no. 339320, Qiagen, Inc.) and the miRCURY LNA SYBR®-Green PCR kit (cat. no, 339346, Qiagen, Inc.) on a real-time thermal cycler qTOWER^3 (Analytik Jena India Private Limited). First, the cDNA volume (10 μl total RT reaction volume) was diluted to 1:30 by the addition of 290 μl RNase-free water to a 10-μl RT reaction prior to use. The primers of the miRCURY LNA miRNA PCR assays used were for miR-16, miR-27a, miR-122 and miR-155. All reactions were carried out in duplicate. The reaction mixture and real-time cycler program were set up as per the instructions of the manufacturer. The florescence was detected and cycle-threshold (Cq) values were obtained. In the present study, miR-16 was used as an endogenous control to normalize the expression levels of miR-27a, miR-122 and miR155 (20). Data were analyzed using the comparative ΔΔCq method ($2^{-\Delta\Delta Cq}$) (21). The data of miR-27a, miR-122 and miR155 expression levels were calculated as follows: ΔΔCq=Cq (target miR)-[Cq (target miR)-Cq (miR-16)] mean of controls. The relative quantification was calculated using the $2^{-\Delta\Delta Cq}$ method for all miRNAs (miR-27a, miR-122 and miR-155) (Fig. 1).

Statistical analysis. Data analysis was performed using SPSS software (SPSS software version 29.0.1.0; IBM Corp.). The general characteristics of the cases and controls were compared using the t-test; the t-test was used for the comparison of continuous variables and the Chi-squared was used for categorical data where the data could be placed in a 2x2 table representation. The Kruskal-Wallis-H test was used to compare differences in the serum levels of miRNAs among the three groups (patients with early-stage breast cancer, benign breast tumors and healthy controls). Dunn's post hoc test was used following the Kruskal- Wallis test. The Mann-Whitney test was used for those continuous variables which did not have normal distribution of data as demonstrated by the Shapiro-Wilk test. In all analyses, a value of $P<0.05$ was considered to indicate a statistically significant difference. For assessment of the diagnostic potential of miRNAs, receiver operating characteristic (ROC) curve analysis was performed and the area under the ROC curve (AUC) was calculated. The sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and likelihood ratios (LRs) for differentiating between early-stage breast cancer, benign breast tumors and healthy control were also calculated.

Results

Expression levels of miRNA-27a, -122 and -155 in the study samples. The present study included 112 cases (56 patients with early-stage breast cancer and 56 patients with benign breast tumors) and 56 healthy controls to determine the circulating levels of miRNA-27a, -122 and -155.

The comparison of the age, parity, menstrual status and body mass index revealed that there were no statistically significant differences between the cases and healthy controls (P-value >0.05); however, statistically significant differences were found in family history and personal habits (tobacco intake) between the cases and healthy controls (P-value <0.05). Biochemical parameters, such as alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, hemoglobin (Hb), total white blood cell (WBC)

Table I. General examination parameters and biochemical parameters.

Parameter	Cases (n=112)	Controls (n=56)	P-value
Age group			P=0.860[c]
<46 years	78 (69.6%)	38 (67.9%)	
≥46 years	34 (30.4%)	18 (32.1%)	
Age (years)	37.43±12.51	40.5±10.6	P=0.099[c]
Parity			P=0.589
Nullipara	33 (29.5%)	14 (25%)	
Para	79 (70.5%)	42 (75%)	
Menstrual status			P=0.721
Premenopausal	32 (28.6%)	18 (32.1%)	
Postmenopausal	80 (71.4%)	38 (67.9%)	
BMI	23.28±4.15	22.83±2.91	P=0.416[c]
Family history			P=0.032[c]
Negative	102 (91%)	56 (100%)	
Positive	10 (9%)	0	
Personal habits: Tobacco intake			P=0.03[c]
No	103 (92%)	56 (100%)	
Yes	9 (8%)	0	
ALT (U/l) mean ± 2SD	26±11.25	27.1±8.5	P=0.514[t]
AST (U/l) mean ± 2SD	23.82±14.68	27.29±10.39	P=0.08[t]
ALP (U/l) mean ± 2SD	83.7±19.32	80.8±18.13	P=0.342[t]
Urea(mg/dl) mean ± 2SD	20.19±5.98	20.77±5.95	P=0.557[t]
Creatinine (mg/dl) mean ± 2SD	0.79±0.13	0.83±0.114	P=0.108[t]
Hb (g/dl) mean ± 2SD	11.74±14.68	11.79±14.68	P=0.735[t]
WBC ($x10^3/\mu l$) mean ± 2SD	8.14±2.26	7.93±1.91	P=0.548[t]
Platelets ($x10^3/\mu l$) mean ± 2SD	268.38±79.5	262.29±66.3	P=0.601[t]

Categorical data were analyzed using the Chi-squared test (c), and continuous data were analyzed using an independent samples t-test (t). A value of P>0.05 was considered to indicate a statistically significant difference. ALT, alanine transaminase; ALP, alkaline phosphatase; Hb, hemoglobin; WBC, white blood cell.

and platelet count exhibited no statistically significant differences between the cases and healthy controls (P-value >0.05) (Table I).

The results revealed that the expression levels of the three miRNAs (miR-27a, miR-122 and miR-155) in serum were significantly increased in patients with early-stage breast cancer when compared to those of patients with benign breast tumors and healthy controls (P<0.001). There was a significant difference in the serum levels of miR-27a (P=0.015) and miR-122 (P=0.028), between the patients with benign breast tumors and healthy controls; the serum levels of these two miRNAs were upregulated in patients with benign breast tumors when compared to those of the healthy controls. However, there was no significant difference in the serum levels of miR-155 (P=0.935) between the healthy controls and patients with benign breast tumors (Table II and Figs. 2-4). It was also found that the miRNA-16 expression levels were relatively constant (mean, 1.4586; SD, 0.08; data not shown).

To examine the effect of aging and menstrual status on the serum levels of miR-27a, miR-122 and miR-155, the samples were classified into different groups. In the group aged <46 years and in the premenopausal group, the serum levels of miR-27a and miR-122 were significantly increased in the patients with early-stage breast cancer when compared to those with benign breast tumors and the healthy controls (P<0.01); however, there was no significant difference between the patients with benign breast tumors and the healthy controls (miR-27a, P>0.05; and miR-122, P>0.05). The serum levels of miR-155 exhibited no significance difference among all three groups (P>0.05). In the group aged ≥46 years and in the post-menopausal group, the serum levels of all three miRNAs were significantly increased in the patients with early-stage breast cancer when compared to those with benign breast tumors and the healthy controls (P<0.01); however, there was a no significant difference between the patients with benign breast tumors and the healthy controls (P>0.05) (Table II).

The serum levels of miR-27a, miR-122 and miR-155 exhibited a statistically significant difference between the groups as regards clinicopathological features, such as Breast Imaging Reporting and Data System (BI-RADS) scoring and pathological types; however, there were no statistically significant differences (P>0.05) among the groups in parameters, such as hormone receptors (molecular subtypes: Luminal-A, Luminal-B, Her2-enriched and TNBC), Ki67

Table II. Serum levels of miR-27a, -122 and -155 in patients with early-stage breast cancer, patients with benign breast tumors and healthy controls.

A, Based on the total study population (n=168)

Variables	miR-27a	P-value	miR-122	P-value	miR-155	P-value
Early-stage breast cancer (n=56)	3.75±1.98, 3.3995 (1.19-8.07)	<0.001[a,b,c]	4.94±2.07, 4.8356 (1.37-8.71)	<0.001[a,b,c]	2.22±1.77, 1.6279 (0.22-7.23)	<0.001[a,b]
Benign breast tumors (n=56)	1.52±0.49, 1.6566 (0.38-2.56)		1.97±1.19, 1.7683 (2.23-5.95)		0.95±0.77, 0.7364 (0.15-3.24)	
Healthy controls (n=56)	1.22±0.69, 1.1175 (0.08-2.92)		1.36±0.87, 1.2031 (0.05-3.29)		0.91±0.67, 0.6949 (0.02-2.78)	

B, Based on age status (years)

Variables	miR-27a	P-value	miR-122	P-value	miR-155	P-value
<46 years						
Early-stage breast cancer (n=32)	3.10±1.92, 2.5137 (1.19-8.00)	<0.001[a,b]	4.26±1.95, 4.0168 (1.37-8.71)	<0.001[a,b,c]	1.43±1.21, 1.1016 (0.22-4.87)	0.172
Benign breast tumors (n=46)	1.53±0.49, 1.6567 (0.38-2.56)		1.93±1.19, 1.6457 (0.23-5.95)		1.02±0.82, 0.7364 (0.15-3.24)	
Healthy controls (n=56)	1.32±0.69, 1.2012 (0.14-2.92)		1.52±0.73, 1.3465 (0.32-2.94)		1.07±0.72, 0.7789 (0.21-2.78)	
≥46						
Early-stage breast cancer (n=24)	4.62±1.75, 3.9969 (1.54-8.07)	<0.001[a,b,c]	5.85±1.89, 6.5157 (1.39-8.15)	<0.001[a,b,c]	3.26±1.86, 2.6926 (0.55-7.24)	<0.001[a,b,c]
Benign breast tumors (n=10)	1.47±0.55, 1.7148 (0.51-1.91)		2.19±2.21, 2.0998 (0.36-4.09)		0.63±0.35, 0.6560 (0.22-1.38)	
Healthy controls (n=18)	1.00±0.64, 1.0235 (0.08-2.56)		1.01±1.05, 0.5133 (0.05-3.29)		0.58±0.42, 0.5117 (0.02-1.39)	

C, Based on menstrual status

Variables	miR-27a	P-value	miR-122	P-value	miR-155	P-value
Premenopausal						
Early-stage breast cancer (n=29)	2.74±1.51, 2.3347 (1.19-6.99)	<0.001[a,b,c]	3.99±1.82,3 .5368 (1.37-8.71)	<0.001[a,b,c]	1.16±0.89, 0.8317 (0.22-4.82)	<0.412

Table II. Continued.

C, Based on menstrual status

Variables	miR-27a	P-value	miR-122	P-value	miR-155	P-value
Benign breast tumors (n=51)	1.54±0.48, 1.6662 (0.38-2.56)		2.01±1.19, 1.8169 (0.23-5.95)		0.98±0.71, 0.7406 (0.15-3.24)	
Healthy controls (n=38)	1.35±0.67, 1.2012 (0.41-2.92)		1.51±0.71, 1.3465 (0.32-2.94)		1.07±0.70, 0.8378 (0.21-2.78)	
Early-stage breast cancer (n=27)	4.84±1.87, 4.0706 (1.54-8.07)	<0.001[a,b,c]	5.95±1.84, 6.5415 (1.39-8.25)	<0.001[a,b,c]	3.36±1.77, 3.0635 (1.24-7.24)	<0.001[a,b]
Benign breast tumors (n=5)	1.30±0.66, 1.6375 (0.51-1.90)		1.62±1.29, 1.7196 (0.36-3.48)		0.64±0.47, 0.5599 (0.22-1.38)	
Healthy controls (n=18)	0.95±0.67, 0.8877 (0.08-2.56)		1.03±1.08, 0.5133 (0.05-3.29)		0.58±0.46, 0.4451 (0.02-1.75)	

Data are presented as the mean ± 2SD, median (min-max) and were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. [a]Comparison between the early-stage breast cancer and benign breast tumor groups; [b]comparison between the early-stage breast cancer and healthy control groups; [c]comparison- between the benign breast tumor and healthy control groups.

Figure 2. miR-27a fold change values in patients with early-stage breast cancer, benign breast tumors and healthy controls. The circles on top of the box plots denote the outliers. FC, fold change; BC, breast cancer; HC, healthy controls.

Figure 3. miR-122 fold change values in patients with early-stage breast cancer, benign breast tumors and healthy controls. The circles on top of the box plots denote the outliers. FC, fold change; BC, breast cancer; HC, healthy controls.

Figure 4. miR-155 fold change values in patients with early-stage breast cancer, benign breast tumors and healthy controls. The circles on top of the box plots denote the outliers. *P<0-05. FC, fold change; BC, breast cancer; HC, healthy controls.

proliferation-index (<14 group and ≥14 group) and the staging of early-stage breast cancer (stage I, IIA and IIB) (Table III).

Diagnostic efficacy of miR-27a, miR-122 and miR-155 evaluated by ROC curve analysis. The diagnostic efficacy of the three miRNAs (miR-27a, miR-122 and miR-155) in differentiating between patients with early-stage breast cancer and between patients with benign breast tumors and healthy

Table III. Comparison of clinicopathological characteristics of the patients and the serum levels of the three miRNAs.

Variables	No. of cases	miR-27a		miR-122		miR-155	
		Mean ± 2SD, median (min-max)	P-value	Mean ± 2SD, median (min-max)	P-value	Mean ± 2SD, median (min-max)	P-value
BI-RADS scoring							
Group 1: 0-2 (0,1,2)	27	1.67±0.37, 1.7119 (0.49-2.56)	<0.001[b,c]	1.94±1.25, 1.6945 (0.45-5.95)	<0.001[a,b,c]	0.77±0.63, 0.5760 (0.15-2.75)	<0.001[b,c]
Group 2: 3-5 (3,4,5)	29	1.39±0.56, 1.5369 (0.38-2.17)		2.00±1.16, 1.9790 (0.23-4.45)		1.11±0.85, 0.8201 (0.15-3.24)	
Group 3: 6	56	3.75±1.98, 3.3995 (1.19-8.07)		4.94±2.07, 4.8356 (1.37-8.71)		2.22±1.77, 1.6279 (0.22-7.24)	
Pathological types							
Fibroadenoma (benign breast tumor)	56	1.52±0.49, 1.6567 (0.38-2.56)	<0.001[a,b]	1.97±1.19, 1.7683 (0.23-5.95)	<0.001[a,b,c]	0.95±0.77, 0.7364 (0.15-3.24)	<0.001[a,b,c]
Group 1: IDC	49	3.73±2.02, 3.2804 (1.25-8.07)		4.85±2.13, 4.6807 (1.37-8.71)		2.19±1.77, 1.6275 (0.22-7.24)	
Group 2: ILC	7	3.91±1.80, 1.0706 (1.19-7.05)		5.54±1.51, 5.4832 (3.52-7.51)		2.44±1.86, 1.6283 (0.71-5.65)	
Hormone receptors							
Group 1: Luminal A	26	3.23±1.78, 2.9518 (1.19-7.02)	0.119	4.59±2.05, 4.3141 (1.37-7.93)	0.139	2.19±1.85, 1.4834 (0.41-6.67)	0.931
Group 2: Luminal B	13	3.84±1.64, 3.9466 (1.34-7.05)		5.86±1.79, 6.0528 (2.79-8.71)		2.07±1.35, 1.8185 (0.51-4.96)	
Group 2: Her2-enriched	9	4.14±2.56, 3.2271 (1.31-8.07)		4.14±2.01, 4.2586 (1.46-7.34)		2.54±2.33, 1.5536 (0.22-7.24)	
Group 4: TNBC	8	4.89±2.18, 4.0526 (1.96-8.00)		5.46±2.30, 5.9865 (1.89-8.25)		2.19±1.67, 1.5673 (0.59-4.87)	
Ki67							
Group 1: <14	28	3.54±1.93, 3.4505 (1.19-8.07)	0.523	5.04±1.88, 4.8381 (1.92-8.71)	0.819	2.12±1.65, 1.7569 (0.51-6.67)	0.819
Group 2: ≥14	28	3.96±2.05, 3.3030 (1.30-8.00)		4.83±2.27, 4.8355 (1.37-8.25)		2.32±1.90, 1.5673 (0.22-7.24)	
Tumor staging							
Group 1: I	20	3.70±1.91, 3.4505 (1.25-8.07)	0.997	4.49±2.01, 4.2114 (1.39-7.51)	0.104	2.53±1.97, 1.8038 (0.22-7.24)	0.663
Group 2: IIA	18	3.83±2.20, 3.2149 (1.19-8.00)		4.58±2.21, 4.6483 (1.37-8.25)		2.09±1.83, 1.3606 (0.41-6.67)	
Group 3: IIB	18	3.73±1.94, 3.5885 (1.31-7.23)		5.79±1.81, 5.9668 (1.77-8.71)		1.99±1.48, 1.5604 (0.49-4.82)	

Data were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test or the [u]Mann-Whitney U test (for two-group comparisons). [a-c]P<0.05, significant difference between Groups 1 and 2, Groups 2 and 3, and between Groups 1 and 3, respectively. IDC, intraductal carcinoma; ILC, intraluminal carcinoma.

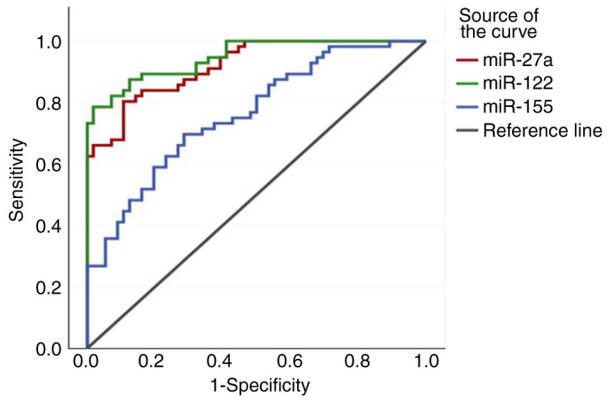

Figure 5. Receiver operating characteristic curve analysis of the three miRNAs for differentiating between patients with early-stage breast cancer and healthy controls.

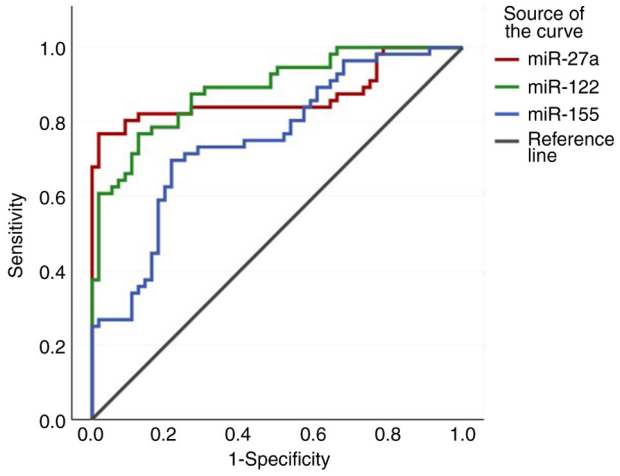

Figure 6. Receiver operating characteristic curve analysis of the three miRNAs for differentiating between patients with early-stage breast cancer and those with benign breast tumors.

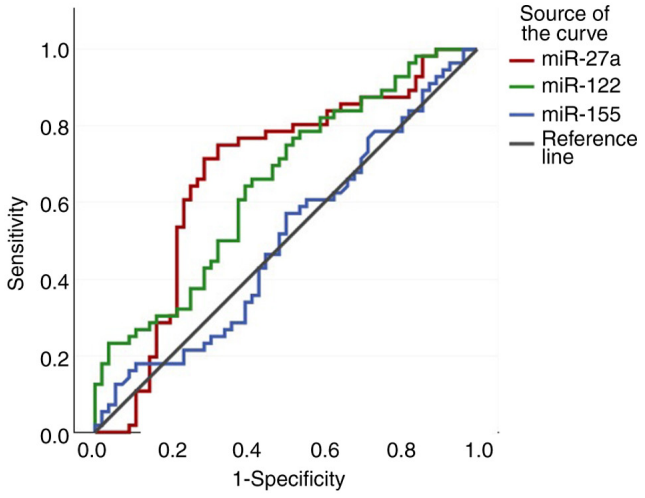

Figure 7. Receiver operating characteristic curve analysis of the three miRNAs for differentiating between patients with benign breast tumors and healthy controls.

Table IV. Sensitivity, specificity, NPV, PPV and LRs of the three miRNAs.

Compared Groups	miRNAs	P-value	Cut-off FC value	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	LR+	LR-
Early stage breast cancer (BC)-healthy controls	miR-27a	<0.001	2.0651	0.920	80.4	89.3	88.2	81.9	7.51	0.22
	miR-122	<0.001	3.0163	0.947	78.6	98.2	97.7	82	43.6	0.22
	miR-155	<0.001	1.1272	0.762	69.6	71.4	70.9	29.8	2.43	0.42
Early stage breast cancer-benign tumour	miR-27a	<0.001	2.2016	0.870	76.8	98.2	97.7	80.8	42.66	0.24
	miR-122	<0.001	3.1888	0.888	78.6	87.5	86	79	6.14	0.26
	miR-155	<0.001	1.1336	0.753	69.6	78.6	76.4	72.1	3.25	0.39
Benign tumour-healthy controls	miR-27a	0.015	1.3492	0.675	75	67.9	70	73	2.33	0.37
	miR-122	0.028	0.9302	0.651	82.1	58.9	58.2	69.6	1.39	0.43
	miR-155	0.935	0.6101	0.502	60.7	44.6	52	53.1	1.09	0.88

FC, fold change; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio.

Table V. ROC curve analysis of the three miRNAs for differentiating between patients with early-stage breast cancer and healthy controls (corresponding to Fig. 5).

miRNA	Test	Early-stage breast cancer	Healthy controls	Total	P-value from χ^2 test
miR-27a cut-off fold change=2.06519	Test+	45	6	51	0.001
	Test-	11	50	61	
Total		56	56	112	
miR-122 cut-off fold change=3.016371	Test+	44	1	45	0.001
	Test-	12	55	67	
Total		56	56	112	
miR-155 cut-off fold change=1.12727	Test+	39	16	55	0.001
	Test-	17	40	57	
Total		56	56	112	

Table VI. ROC curve analysis of three miRNAs for differentiating between patients with early-stage breast cancer and patients with benign breast tumors (corresponding to Fig. 6).

miRNA	Test	Early-stage breast cancer	Healthy controls	Total	P-value from χ^2 test
miR-27a cut-off fold change=2.20164	Test+	43	1	44	0.001
	Test-	13	55	68	
Total		56	56	112	
miR-122 cut-off fold change=3.18884	Test+	43	7	60	0.001
	Test-	13	49	62	
Total		56	56	112	
miR-155 cut-off fold change=1.13369	Test+	39	12	51	0.001
	Test-	17	44	61	
Total		56	56	112	

Table VII. ROC curve analysis of the three miRNAs for differentiating between patients with benign breast tumors and healthy controls (corresponding to Fig. 7).

miRNA	Test	Early-stage breast cancer	Healthy controls	Total	P-value from χ^2 test
miR-27a cut-off fold change=1.34919	Test+	42	18	60	0.001
	Test-	14	38	52	
Total		56	56	112	
miR-122 cut-off fold change=0.93025	Test+	46	33	79	0.001
	Test-	10	23	33	
Total		56	56	112	
miR-155 cut-off fold change=0.61011	Test+	34	31	65	0.001
	Test-	22	25	47	
Total		56	56	112	

controls was then determined. Cut-off fold change values, P-values, PPV and NPV (Table IV) were obtained. The results revealed that miR-27a and miR-122 exhibited significant efficacy in differentiating all groups; however, miR-155 did not exhibit significant efficacy in differentiating between benign tumors and healthy controls. The corresponding AUC (95% CI), sensitivity and specificity of the ROC curves are depicted in Figs. 5-7 and Tables V-VII.

Discussion

Tissue gene biomarkers have improved the diagnosis of breast cancer to a great extent; however, there are limitations to their applications due to invasiveness, risk of hemorrhage and the unpleasant nature of the diagnostic procedures. The estimation of non-invasive or minimally invasive biomarkers, such as miRNA expression levels in blood and serum, is a highly sensitive and specific method which can benefit both clinicians and patients in the screening, diagnosis and prognosis of patients with early-stage breast cancer (6). Although there has been substantial success in the discovery of serum biomarkers, it is imperative to identify biomarkers that are useful for the early diagnosis of affected patients.

Of note, in the present study, immunohistochemistry was performed as routine diagnostic workup for sub-classifying the patients with breast cancer. In the present study, the expression levels of the three miRNAs (miR-27a, miR-122 and miR-155) were significantly upregulated in the serum of patients with early-stage breast cancer compared to those in patients with benign breast tumors and healthy controls. These findings indicate that these three miRNA are more likely related to early-stage breast cancer. These findings also indicate these three miRNAs may be beneficial biomarkers for the diagnosis of early-stage breast cancer.

In their study, Swellam *et al* (13) examined the expression levels of miR-27a and tumor markers (CEA and CA15.3) among three investigated groups; patients with primary breast cancer, patients with benign breast tumors and healthy controls; they demonstrated that miRNA-27a was superior to the two tumor markers in specificity and accuracy for the detection of early-stage breast cancer. The expression levels of miR-27a in serum were significantly increased in patients with primary breast cancer when compared to those in patients with benign breast tumors followed by the healthy controls who had the lowest serum level (13). The results of their study are comparable to those of the present study as regards the serum levels of miR-27a. Previous studies performed by Wu *et al* (22) and Ding *et al* (23) reported the oncogenic role of miR-27a among several other types of cancer. It was observed that, the upregulation of miR-27a targeted the FOXO1 gene, which was responsible for cell cycle regulation, thus promoting evasion from apoptosis and cell proliferation in breast cancer (24).

Saleh *et al* (25) demonstrated that miR-122 exhibited an improved specificity and sensitivity than tumor markers (CEA and CA15-3) in the diagnosis of breast cancer and in predicting breast cancer metastasis. In a cohort study, Wu *et al* (26) demonstrated that circulating levels of miRNA-122 had a good specificity and sensitivity in predicting metastasis. It was also found that the miR-122 level was increased in patients who had relapsed after recovery and was able to determine relapse in patients with stage IIa, IIb and III breast cancer (26). On the other hand, Wang *et al* (27) demonstrated that miR-122 functioned as a tumor-suppressor in breast cancer cells by suppressing the IGF1R-mediated downstream Akt/mTOR/p70S6K signaling pathway and inhibiting breast cancer cell growth. They demonstrated that the miR-122 expression levels decreased in breast cancer cells when compared to normal breast tissue (27). This discrepancy in the results of miR-122 levels, between studies performed on breast tissue samples and blood samples can be explained in the study by Fong *et al* (28). Fong *et al* (28) demonstrated that breast cancer cells secrete large amounts of miR-122-secreting vesicles, which leads to a decrease in miR-122 levels intracellularly. This secreted miR-122 affects non-cancer cells and decreases their uptake of glucose by downregulating glycolytic enzyme pyruvate kinase. In this manner, miR-122 supports cancer cell viability and metastasis by increasing nutrient supply (28). Thus, the majority of breast cancer cells have decreased levels of miR-122. In the present study, miR-122 expression levels in serum were increased in patients with early-stage breast cancer compared to those with benign breast tumors and healthy controls. miR-122 also exhibited improved sensitivity and specificity in detecting early-stage breast cancer.

In the study by Hosseini Mojahed *et al* (29), it was demonstrated that the miR-155 expression levels in serum were significantly higher in patients with breast cancer when compared with healthy controls. The combination of three miRNAs, miR 17-5p, miR-155 and miR-222 levels, when compared with tumour markers (CEA and CA15.3) for breast cancer screening, has revealed that the three miRNAs were more effective than tumour markers for the early diagnosis of breast cancer, particularly in high-risk groups (13,30). In another study by Bašová *et al* (31), the serum levels of miR-155 were highly predictive in determining relapse in early-stage breast cancer. Another study demonstrated that the upregulation of miR-155 inhibited the translation of mRNAs, such as RhoA, FOXO3A, and SOCS1. which led to the evasion of apoptosis and increased cell proliferation (32). The decreased levels of BRCA1 due to mutation upregulate the levels of miR-155 in serum. This occurs since BRCA1 regulates miR-155 by binding to the miR-155 promotor and recruiting histone deacetylases (33). In the present study, the levels of miR-155 were significantly increased in patients with early-stage breast cancer, when compared to healthy controls and have good sensitivity and specificity were observed.

In the present study, ROC analysis revealed that the serum levels of miR-27a, miR-122 and miR-155 exhibited good accuracy with AUC values of 0.920, 0.947 and 0.762, respectively for differentiating between early-stage breast cancer and healthy controls. These three miRNAs also exhibited fair accuracy with AUC values of 0.870, 0.888 and 0.753, respectively for differentiating between early-stage breast cancer and benign breast tumors.

In conclusion, the results of the present study indicate that the levels of miR-27a, miR-122 and miR-155 are increased in patients with breast cancer. Their serum levels have reliable sensitivity and specificity in detecting early-stage breast cancer. These three miRNAs may thus potential for use as non-invasive biomarkers in the diagnosis and screening of breast cancer. However, their clinical applicability needs to be evaluated in multiple centers in order to confirm their diagnostic efficacy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SSr and SSa wrote the manuscript and performed the experiments and generated the figures. DKM and TSM collected the clinical data. PM and SM provided the data of the recruited patients from the Departments of Pathology and Radiodiagnosis, All India Institute of Medical Sciences (Bhubaneswar, India), respectively. All authors made critical revisions, and all authors have read and approved the final manuscript. All authors (SSr, SSa, DKM, TSM, PM and SM) confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical clearance obtained from the Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), Bhubaneswar, India (IEC/AIIMS BBSR/PG Thesis/2021-22/32). Each patient was explained the nature of study and after obtaining their written consent, they were inducted into the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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