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Branched rolling circle amplification method for measuring serum circulating microRNA levels for early breast cancer detection

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Abstract

Serum circulating microRNAs (c-miRNAs) are serving as useful biomarkers for cancer diagnosis. Here, we describe the development of a one-step branched rolling circle amplification (BRCA) method to measure serum c-miRNAs levels for early diagnosis of breast cancer. Four c-miRNAs, c-miRNA16 (c-miR-16), c-miRNA21 (c-miR-21), c-miRNA155 (c-miR-155), and c-miRNA195 (c-miR-195) were isolated from the serum of 49 breast cancer patients (stages I-IV) and 19 healthy controls, and analyzed using one-step BRCA. The serum levels of c-miR16, c-miR21, c-miR155, and c-miR195 were higher ($P < 0.0001$) in stage I breast cancer patients than healthy controls. These levels were also higher in several breast cancer molecular subtypes (HER-2 overexpression, Luminal A, Luminal B, and triple negative breast cancer) than in healthy control subjects. The diagnostic accuracy of c-miR16, c-miR21, c-miR155, and c-miR195 for early diagnosis of breast cancer was confirmed by receiver operating characteristic (ROC) curve assay. These results show that the BRCA method can be used to measure serum c-miRNAs levels, and that this method has high accuracy, sensitivity, and specificity. Moreover, both BRCA approach and quantitative real-time PCR (qRT-PCR) method show that the serum levels of c-miR16, c-miR21, c-miR155, and c-miR195 could be used as biomarkers to improve the early diagnosis of breast cancer, and distinguish different breast cancer molecular subtypes.

KEYWORDS
biomarkers, branched rolling circle amplification, breast cancer early detection, breast cancer screening, circulating microRNAs

1 | INTRODUCTION

All over the world, there are more than 1.3 million women diagnosed with breast cancer every year, which makes it the second most common cancer.[1-3] Although breast cancer diagnostic techniques have greatly improved in recent years, the lack of specific biomarkers limits the early diagnosis of breast cancer.[4,5] Most importantly, early diagnosis of breast cancer is central to reduce the morbidity and mortality, and is one of the major challenges in the struggle against this disease.[6,7]

Circulating microRNAs (c-miRNAs) are a class of endogenous, non-coding RNA, which regulate the expression of over 60% of target genes, and can circulate in plasma, serum, and whole blood samples. Their discovery in cancer patients making c-miRNAs have

TABLE 1 The clinicopathological features of breast cancer patients

Characteristics	Number of patients, n (%)
Age range: 25-64	
Mean age: 43	
Median age: 43	
ER status[a]	
Positive	39 (79.6)
Negative	8 (16.3)
Unknown	2 (4.1)
PR status[a]	
Positive	31 (63.2)
Negative	16 (32.7)
Unknown	2 (4.1)
HER-2 status[b]	
Positive	20 (40.8)
Negative	24 (49.0)
Unknown	5 (10.2)
Ki-67 protein[c]	
High (>14%)	45 (91.8)
Low	2 (4.1)
Unknown	2 (4.1)
Tumor size[d]	
Tis	3 (6.1)
T1	19 (38.8)
T2	20 (40.8)
T3	6 (12.2)
Unknown	1 (2.0)
Lymph nodes[e]	
N0	34 (69.4)
N1	8 (16.3)
N2	5 (10.2)
Unknown	2 (4.1)
Metastasis[f]	
M0	45 (91.8)
M1	2 (4.1)
Unknown	2 (4.1)
Histological tumor grade	
Tis (0)	3 (6.1)
I	16 (32.7)
II	20 (40.8)
III	6 (12.2)
IV	3 (6.1)
Unknown	1 (2.0)
Molecular subtypes	
HER-2 over-expression	7 (14.3)
Luminal A	4 (8.2)
Luminal B	31 (63.3)
Triple negative	2 (4.1)
Unknown	5 (10.2)

[a] ER (estrogen receptor)/PR (progesterone receptor) negative: Immunoreactive score (IRS) ≤2; ER/PR positive: IRS >2 but ≤12.
[b] HER-2 (human epidermal growth factor 2) negative: IHC-score ≤1; HER-2 positive: IHC-score >3.
[c] Ki-67 protein is a cellular marker for proliferation.
[d] Tis: tumor in situ; T1: Tumor ≤2 cm; T2: Tumor >2 cm but ≤5 cm; T3: Tumor >5 cm.
[e] N0: No regional lymph node metastasis; N1: Metastasis to movable ipsilateral lymph node(s); N2: Metastasis in ipsilateral axillary lymph node(s) fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis.
[f] M0: No distant metastasis; M1: Metastasis to distant organs (beyond regional lymph nodes).

enormous potential for using as specific and non-invasive cancer biomarkers.[8,9] In breast cancer patients, the levels of serum c-miRNA16 (c-miR-16) are significantly higher compared with healthy controls.[10,11] Serum c-miRNA21 (c-miR-21) is one of the most up-regulated c-miRNAs in breast cancer patients, indicating that it may serve as an important biomarker for breast cancer detection and progression.[12,13] The expression of serum c-miRNA155 (c-miR155) is also increased in breast cancer patients compared with healthy controls.[14-16] In addition, the expression of c-miRNA195 (c-miR-195) is increased in breast cancer patients, but not in other cancers, indicating that it might be used as a breast cancer specific biomarker.[17,18] However, to our knowledge, no previous studies have evaluated the diagnostic performance of the above four c-miRNAs in breast cancer early-stage detection.

Analytical methods of c-miRNAs have been hindered by measurement-associated inconveniency.[19] Some conventional techniques have been used for c-miRNA detection, such as northern blotting, microarrays, and quantitative real-time PCR (qRT-PCR).[20-22] However, these methods have low sensitivity, low selectivity, and labor-intensive steps, which limit their practical application. With the purpose of improving the sensitivity, specificity, and simplicity of c-miRNA analysis, many novel techniques have been developed. Among them, rolling circle amplification (RCA) is a simple and efficient isothermal enzymatic process that uses unique DNA or RNA polymerases to generate long single-stranded DNA (ssDNA) or RNA (ssRNA) with tens to hundreds of tandem repeats by continuously adding nucleotides (nt) to a primer of the circular template.[23-25] Unlike qRT-PCR, which not only needs two-step reactions (reverse transcription and polymerase chain reaction) but also requires a thermal cycler and thermostable DNA polymerases, RCA can be produced at an isothermal temperature from room temperature to 37°C by one-step reaction.[26] In addition, branched RCA (BRCA) is an exponential nucleic acid amplification method, where the products of RCA can be used as the template for further amplification by the addition of second and third primers.[27,28] Moreover, in principle, in one form or the other, BRCA has been used to detect miRNAs, including c-miRNAs in serum.[29-31]

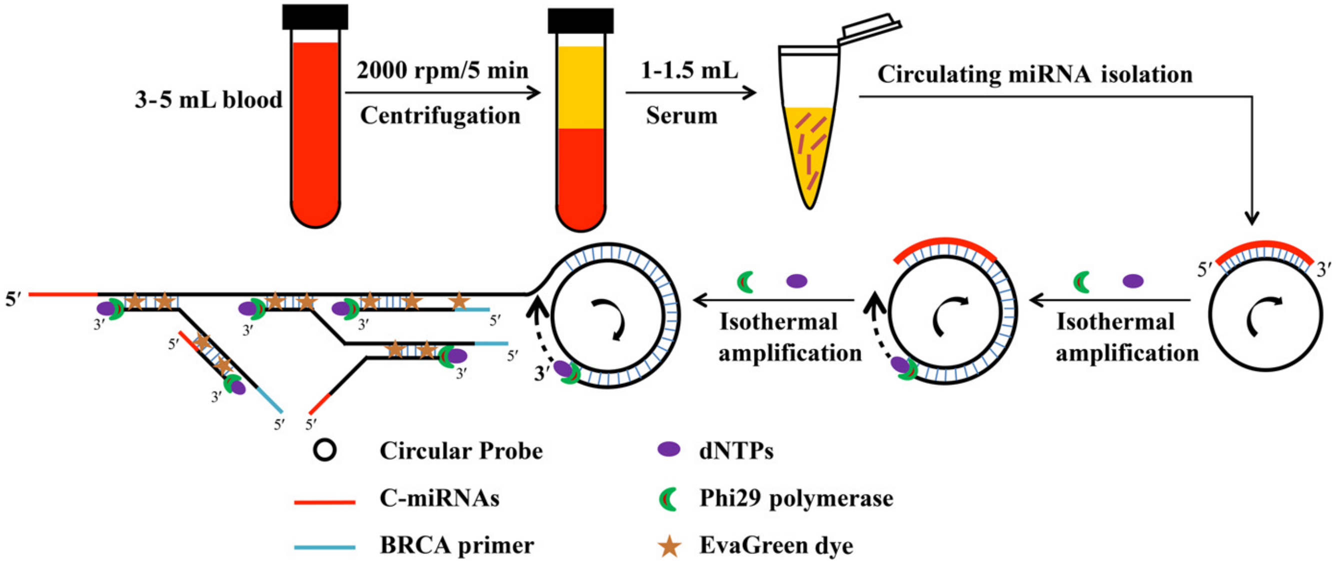

FIGURE 1 Schematic illustration of serum circulating microRNA detection based on branched rolling circle amplification for breast cancer early-stage diagnosis

Here, we have developed, for the first time, a simple and specific BRCA method for a rapid and convenient detection of c-miR16, c-miR21, c-miR155, and c-miR195 levels in human serum specimens. The BRCA products are long double-stranded DNAs (dsDNAs) that can be easily detected by EvaGreen dye, which is non-fluorescent by itself, but exhibits a great fluorescence enhancement upon binding to dsDNA. Furthermore, we used the BRCA method to determine the diagnostic performance of the four c-miRNAs to distinguish breast cancer patients with different histological tumor grades and molecular subtypes from healthy controls.[32,33] Meanwhile, we also used the conventional qRT-PCR method as a secondary independent assay to verify that the four c-miRNAs could serve as biomarkers.

2 | MATERIALS AND METHODS

2.1 | Reagents and consumables

The phi29 DNA polymerase, adenosine 5′-triphosphate solution (ATP), and deoxynucleotide solution mixture (dNTPs) were purchased from New England Biolabs (NEB, Beijing, China). RNase-free water, T4 Polynucleotide Kinase, T4 DNA Ligase were purchased from TaKaRa Biotechnology Co.,Ltd (Dalian, China). EvaGreen Dye (20× in water) was purchased from Biotium Inc. (Hayward, CA, USA). The oligonucleotides used in this study were synthesized by Invitrogen Biotechnology Co., Ltd (Shanghai, China). All of the pipette tips and centrifuge tubes were DNase and RNase-free, which were purchased from Axygen, Inc. (Union, CA, USA).

2.2 | Human serum specimens

The human blood specimens of breast cancer patients and healthy donors were obtained from the Peking University Shenzhen Hospital (Shenzhen, China), and the Shenzhen People's Hospital (Shenzhen, China) between March 2017 and May 2018. All cancer patients and healthy control donors were Chinese. Control blood specimens (n = 19) were collected from healthy volunteers with no history of breast cancer. Patient blood specimens (n = 49, women, aged 25-64) were collected at the time of diagnosis, before surgery. As shown in Table 1, patients' histopathological results and clinicopathological features were confirmed by surgical resection of the tumors and clinical immunohistochemical technical.

2.3 | Isolation of serum circulating microRNAs

Human serums were obtained from freshly drawn blood, which were collected by standard phlebotomy in vacuum blood tubes without clot activator, kept at 4°C for 2 h to clot, and then rotate at 300 *g* for 5 min at 4°C. The serum was transferred into RNase-free tubes, and stored at −80°C. Circulating microRNAs were extracted from serum using the miRCURY™ RNA Isolation Kit-Biofluids (EXIQON, Woburn, MA, USA) according to the manufacturer's protocol as shown in Doc. S1.

2.4 | Analysis of serum circulating miRNAs using BRCA approach

The main goal of this study was to use the BRCA method to detect serum levels of c-miRNAs that could be used for early-stage diagnosis in breast cancer patients. The study consisted of two parts: The first part was the isolation of serum circulating microRNAs, and the second part was the analysis of c-miR16 (5′-UAGCAGCACGUAAAUAUUGGCG-3′), c-miR21 (5′-UAGCUUAUCAGACUGAUGUUGA-3′), c-miR155 (5′-UUAAUGCUAAUCGUGAUAGGGGU-3′), and c-miR195 (5′-UAGCAGCACAGAAAUAUUGGC-3′) serum levels by BRCA (Figure 1).

The c-miRNAs intended for detection are isothermally amplified using BRCA,the DNA circular probes (miR-16 probe:

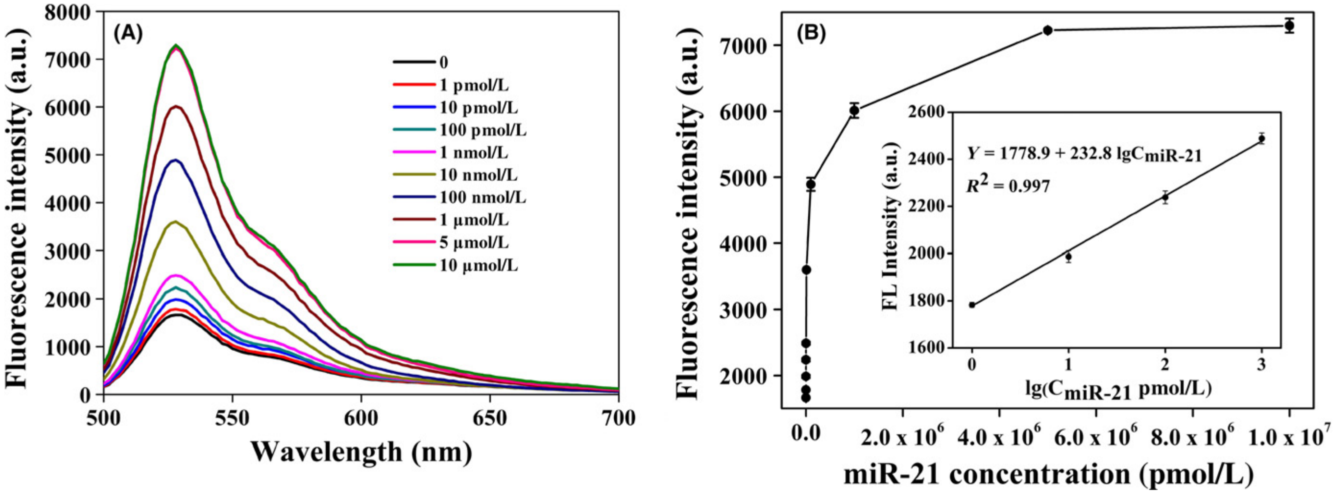

FIGURE 2 A, The fluorescence emission spectra for various concentrations of miR-21. From bottom to top: 0, 1, 10, 100 pM, 1, 10, 100 nM, 1, 5, 10 μM. B, The calibration curve between the fluorescence intensity and the logarithm of concentrations of miR-21. Experimental conditions: RCA temperature 30°C and RCA time 5 h. The error bars represent the standard deviations of three independent experiments

5′-ACGTGCTGCTAACACATCAAAGCCCATACTACAACAACTACAACACGCCATATTT-3′, miR-21 probe: 5′-TGATAAGCTAACACATCAAAGCCCATACTACAACAACTACAACATCAACATCAGTC-3′, miR-155 probe: 5′-GATTAGCATTAAACACATCAAAGCCCATACTACAACAACTACAACAACCCCTATCAC-3′, and miR-195 probe:5′-CTGTGCTGCTAACACATCAAAGCCCATACTACAACAACTACAACAGCCAATATTT-3′) are partially complementary to the target c-miRNA. In the presence of target c-miRNA, RCA is initiated by phi29 DNA polymerase that has exceptional strand displacement and processive synthesis properties, using the DNA circular probe as the template. As a result, a long ssDNA sequence is synthesized, generating multiple copies of the circular probe sequences. The resulting RCA products are used as the template for further amplification using the BRCA primer (5′-ATCAAAGCCCATACTACA-3′). The BRCA products are long dsDNAs that can be easily detected by the fluorescent EvaGreen Dye. Conversely, in the absence of target c-miRNA, the long dsDNA is not synthesized, resulting in a low background for the c-miRNA detection.

The biosynthesis of DNA circular probe is shown in Figure S1. The circular template serving as the padlock probe DNA, which was designed to be complementary to the both ends of the ligation template. Then, the circular template would hybridize by the ligation template and the circularization is accomplished with the T4 DNA ligase.

The assay was carried out in 50 μL solution containing 5 μL of miR-16 probe, miR-21 probe, miR-155 probe or miR-195 probe (10 μM), 5 μL of total serum c-miRNAs (various concentrations), 5 μL of dNTPs (10 mM), 0.5 μL of phi29 DNA polymerase (10 U/μL), 5 μL of RCA buffer (10×), 5 μL of BRCA primer (10 μM), 2.5 μL of EvaGreen Dye (20×), and 22 μL of RNase-free water. The mixture was incubated at 30°C for 5 h. Subsequently, 5 μL of RCA buffer (10 ×), 2.5 μL of EvaGreen Dye (20×), and 42.5 μL of ddH_2O were added to yield a total volume of 100 μL for fluorescence measurement. The apparatus of fluorescence measurement is shown in Doc. S1.

2.5 | Statistical analyses

Before statistical comparisons, the specimens were divided into multiple comparison groups including health controls, different histological tumor grades, and different molecular subtypes. C-miR-16, c-miR-21, c-miR-155, and c-miR-195 expression profiles were compared using *F* test, Student's *t* test, and multiple comparison groups. Bonferroni-corrected $P < 0.05$ was considered statistically significant and Bonferroni-corrected $P < 0.01$ was considered statistically extremely significant.[34-36]

Receiver operating characteristic (ROC) assay was carried out for determining the diagnostic performance of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 expression in patients with breast cancer from healthy controls. Sensitivity against (1-specificity) was plotted at each cutoff threshold, and the area under the curve (AUC) values that reflect the probability of correctly identifying breast cancer patients from healthy controls were calculated. This process was repeated 1000 times, and resulting mean values (95% confidence interval)[37] for sensitivity and specificity were calculated.[38,39] All statistical analyses were performed using the Origin version 8.6 (Hampton, MA, USA), IBM SPSS Statistics version 19.0 (Armonk, NY, USA), and MedCalc version 15.8 (Acacialaan, Ostend, Belgium) softwares.

3 | RESULTS

3.1 | Optimization of detection system

We optimized a series of experiments to achieve the optimal detection conditions, such as the reaction temperature and the reaction time of BRCA. Firstly, as shown in Figure S2A, we optimized the temperature of BRCA (25-40°C range), the $(F\text{-}F_0)/F_0$ achieved a maximum value at 30°C, where F and F_0 are the fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 528 nm) with and without the target c-miRNA, respectively. Next, we optimized the time of BRCA reaction

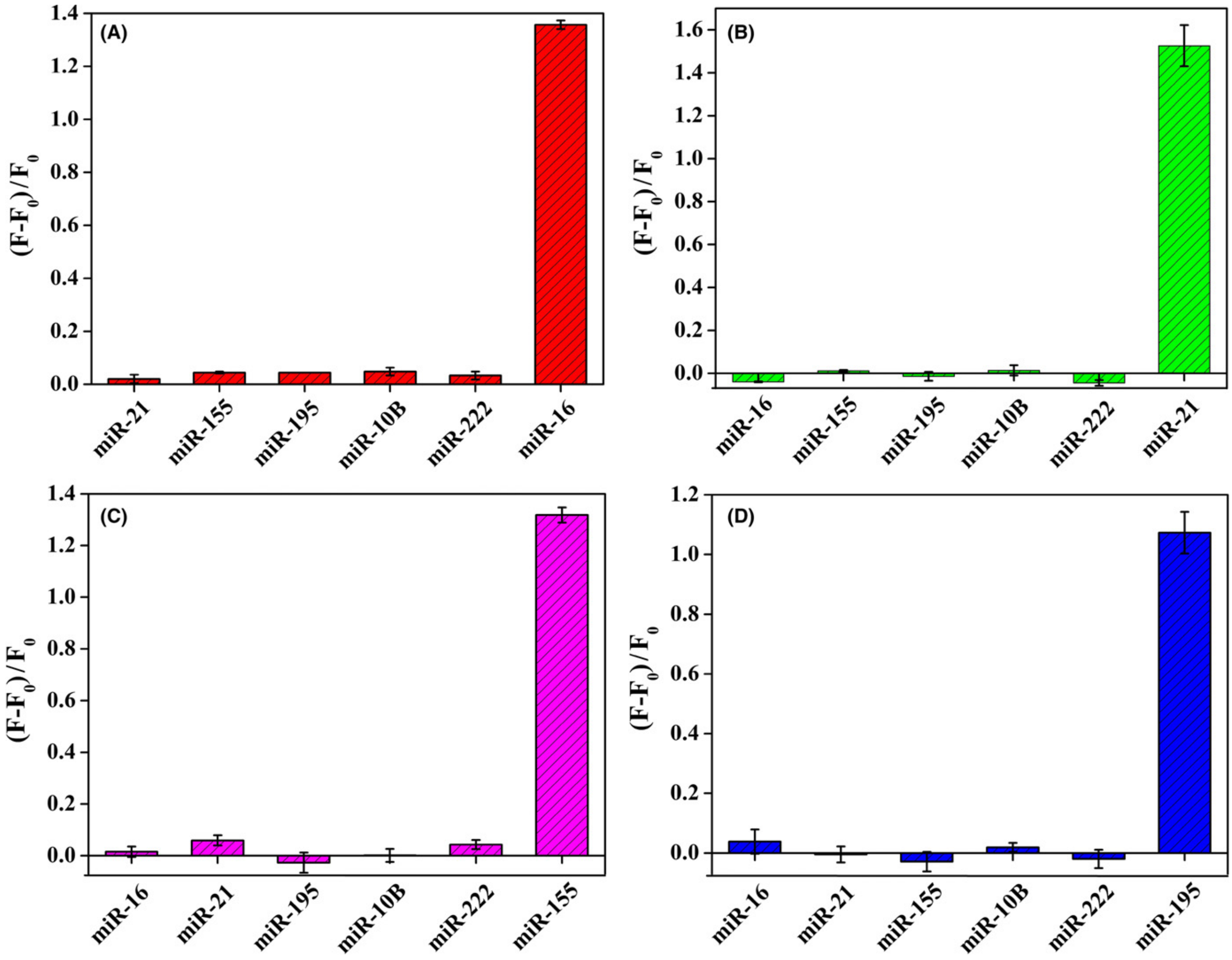

FIGURE 3 The specificity of the four serum circulating miRNA BRCA systems, miR-16 BRCA system (A), miR-21 BRCA system (B), miR-155 BRCA system (C), and miR-195 BRCA system (D). Experimental conditions: RCA temperature 30°C and RCA time 5 h. The error bars represent the standard deviations of three independent experiments

(Figure S2B), the value of $(F\text{-}F_0)/F_0$ increased distinctly from 1 to 5 h, and achieved a plateau after 5 h. Thus, the reaction temperature of 30°C and the BRCA reaction time of 5 h were applied for the following experiments.

3.2 | Sensitivity of the serum circulating miRNA assay

We further assessed the sensitivity of BRCA approach with the addition of various concentrations of miR-21. Allowing with the increase of miR-21 concentrations from 0 to 1 μM, a dramatic rise of the fluorescence intensity was obtained, which reached a plateau after 5 μM (Figure 2A). As shown in Figure 2B, the value of fluorescence intensity has a linear correlation with the logarithm of miR-21 concentrations over the range from 1 pM to 1 nM. The regression equation is $Y_{\text{fluorescence intensity}} = 1778.9 + 232.8\ \lg C_{\text{miR-21}}$ with a correlation coefficient of 0.997. The detection system has a wider linear range (4 orders of magnitude), which the limit of detection is 1 pM. Meanwhile, we have estimated the lowest measurement concentrations of total serum c-miRNAs and c-miR-21 isolated from four human serum specimens by this detection system, we found the lowest total c-miRNA measurement concentration is 0.001 ng/μL, and the lowest c-miR-21 measurement concentration is 4 pM as shown in Table S1.

3.3 | Selectivity of the serum circulating miRNA assay

In order to estimate the specificity of the proposed four serum c-miRNA BRCA systems (c-miR-16, c-miR-21, c-miR-155, and c-miR-195 BRCA system), we carried out a series of contrast experiments by using miR-16, miR-21, miR-155, and miR-195 as perfectly matched miRNAs or control sequences, meanwhile, using miR-10B (5′-UACCCUGUAGAACCGAAUUUGUG-3′) and miR-222 (5′-AGCUACAUCUGGCUACUGGGU-3′) as the control sequences respectively. As shown in Figure 3A-D, these comparisons clearly show that the

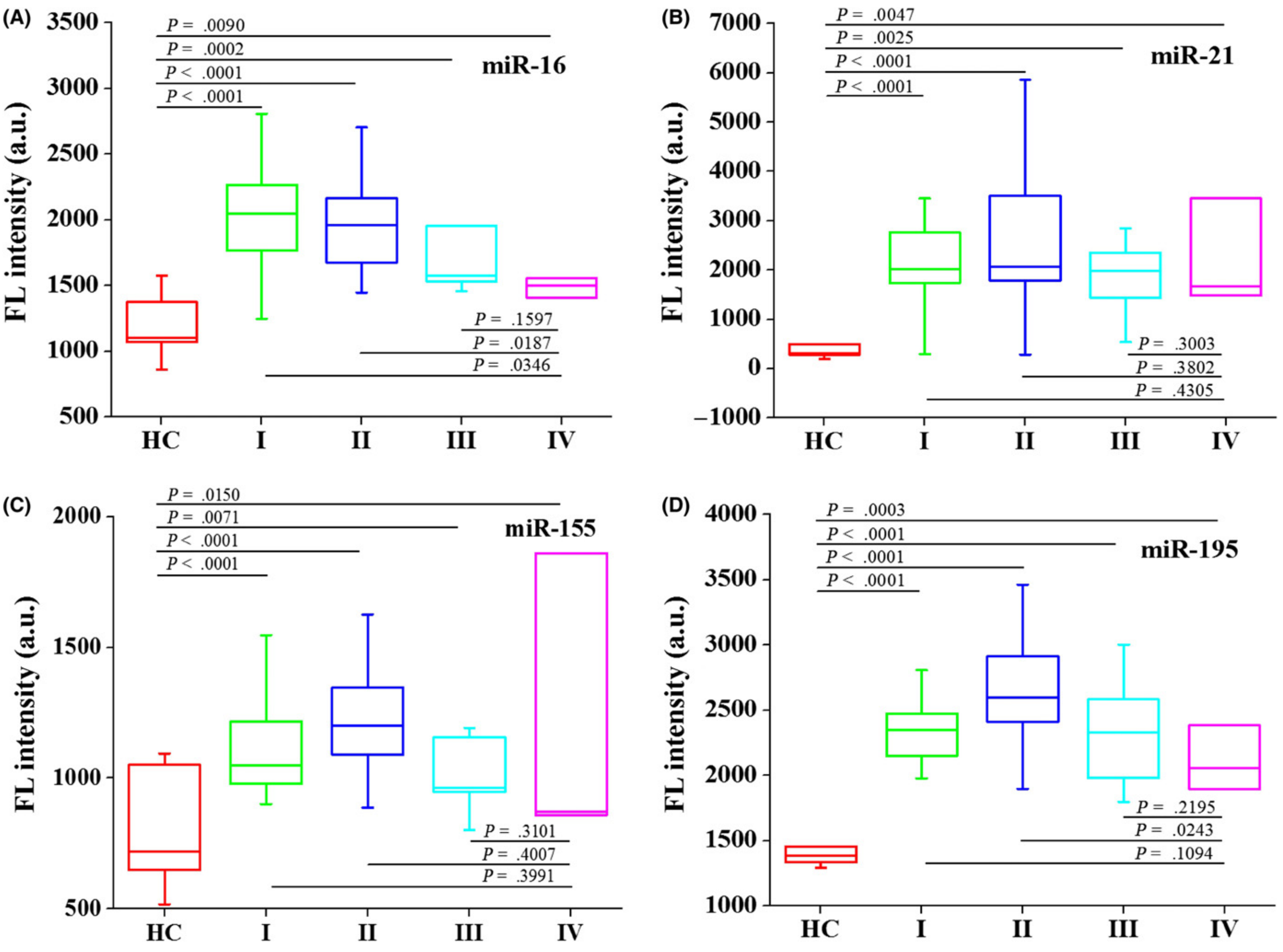

FIGURE 4 The levels of serum c-miR-16 (A), c-miR-21 (B), c-miR-155 (C), and c-miR-195 (D) compared between healthy controls (n = 15) and breast cancer patients with different histological tumor grades: stage I (n = 12), stage II (n = 20), stage III (n = 6), and stage IV (n = 3). HC, healthy controls; I, breast cancer patients at stage I; II, breast cancer patients at stage II; III, breast cancer patients at stage III; IV, breast cancer patients at stage IV. The *F* test and Student's *t* test were performed for comparisons between groups

four serum c-miRNA BRCA systems have higher selectivity in distinguishing discrepant miRNAs and have huge potential for discriminating the target c-miRNAs from their family members and other interference sequences.

3.4 | Identification of breast cancer patients using serum c-miRNA detection by BRCA

Breast cancer patients of different histological tumor grades (stages I-IV) and healthy controls were enrolled to validate the diagnostic ability of c-miR-16, c-miR-21, c-miR-155, and c-miR-195. In comparison with healthy controls, the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 (all $P < 0.0001$) were significantly increased in patients with early-stage breast cancer (stage I or II), as shown in Figure 4A-D. Furthermore, the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 (all $P < 0.01$) were significantly increased in patients with stage III or IV compared to healthy controls.

Next, we used different molecular subtypes of breast cancer patients, HER-2 over-expression (HER-2 OE), Luminal A (LA), Luminal B (LB), and triple negative breast cancer (TN), and healthy controls to determine the diagnostic ability of c-miR-16, c-miR-21, c-miR-155, and c-miR-195. In comparison with healthy controls, the serum levels of c-miR-16, c-miR-21, and c-miR-155 were significantly increased in patients with LB, TN, HER-2 OE, and LA (Figure 5A-C). Moreover, the breast cancer specific biomarker c-miR-195 ($P < 0.0001$) was significantly increased in patients with HER-2 OE, LA, LB, and TN, compared with healthy controls (Figure 5D).

Finally, we used ROC curves to evaluate the performance of the four c-miRNAs as serum biomarkers for the diagnosis of early breast cancer (Figure 6A-E). AUC values for serum c-miR-16, c-miR-21, c-miR-155, and c-miR-195 in distinguishing patients with breast cancer from healthy controls were 0.936 (95% CI, 0.842-0.983; sensitivity at 97.78%, specificity at 80.00%), 0.884 (95% CI, 0.776-0.953; sensitivity at 93.33%, specificity at 80.00%), 0.793 (95% CI, 0.668-0.886; sensitivity at 100.00%, specificity at 60.00%), and 0.964 (95% CI, 0.881-0.995; sensitivity at 100.00%, specificity at 80.00%), respectively. Furthermore, combination of the four c-miRNAs maintained high diagnostic accuracy for patients with breast cancer AUC

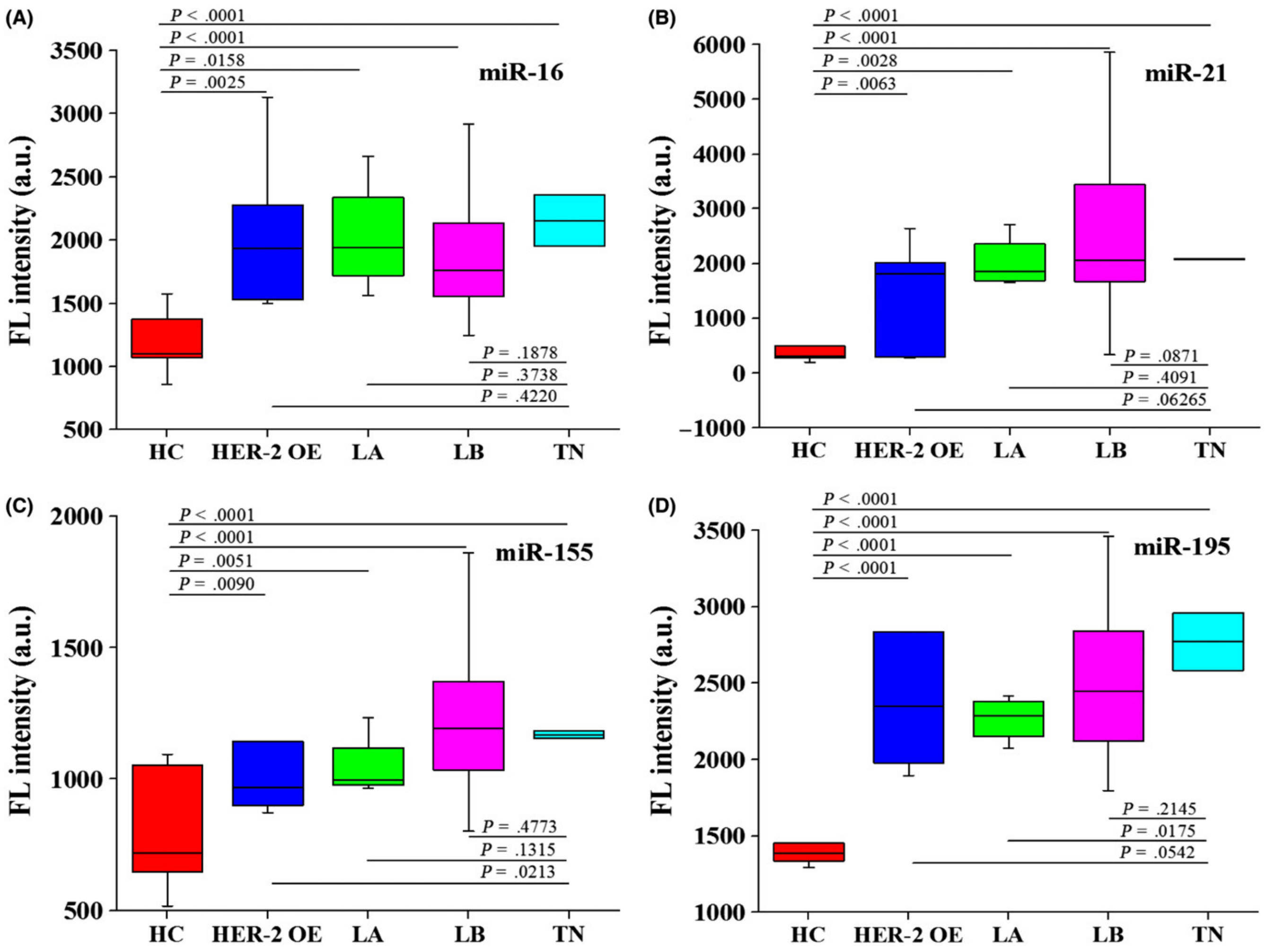

FIGURE 5 The levels of serum c-miR-16 (A), c-miR-21 (B), c-miR-155 (C), and c-miR-195 (D) compared between healthy controls (n = 15) and breast cancer patients with different molecular subtypes: HER-2 over-expression (n = 7), Luminal A (n = 4), Luminal B (n = 27), and triple negative breast cancer (n = 2). HER-2 OE, HER-2 over-expression breast cancer patients; LA, breast cancer patients with Luminal A subtype; LB, breast cancer patients with Luminal B subtype; TN, triple negative breast cancer patients. The *F* test and Student's *t* test were performed for comparisons between groups

0.936 (95% CI, 0.842-0.983; sensitivity at 88.89%, specificity at 86.67%).

3.5 | Quantitative real-time PCR assay

To validate BRCA approach for the detection of c-miRNAs in human serum, we used the conventional quantitative real-time PCR (qRT-PCR) as a secondary independent assay in which the levels of c-miRNA concentrations are verified. C-miRNAs were quantified by a commercial qRT-PCR kit (Doc. S1). The relative fluorescence units (RFU) and the threshold cycle (C_t) value were obtained on the 7500 Real-time PCR system (Applied Biosystems™, Bedford, OH, USA) and analyzed by 7500 Sequence Detection System software version 1.5.1 (Applied Biosystems™).

The fold change of c-miRNA expression in breast cancer serum specimens compared with healthy controls was calculated based on the C_t value using the fold change = $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t$ = (C_t miRNAs)$_{\text{breast cancer specimens}}$ - (C_t miRNAs)$_{\text{healthy controls}}$. As shown in Figure 7A-D, the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 (all $P < 0.05$) were significantly increased in patients with early-stage breast cancer (stage I) compared with healthy controls, which verify that the four c-miRNAs could serve as breast cancer biomarkers.

3.6 | Comparison of BRCA approach with qRT-PCR method

For comparison, we used the commercial kit of qRT-PCR to detect a series of miR-21 concentrations to establish a calibration curve (Figure 8), then, the calibration curve was applied to detect the concentration of miR-21 in human serum specimens by qRT-PCR method. Here, 10% human serums were injected with three different concentrations of miR-21 at 100 pM, 1 nM, and 10 nM were measured. The result (Table 2) can be clearly

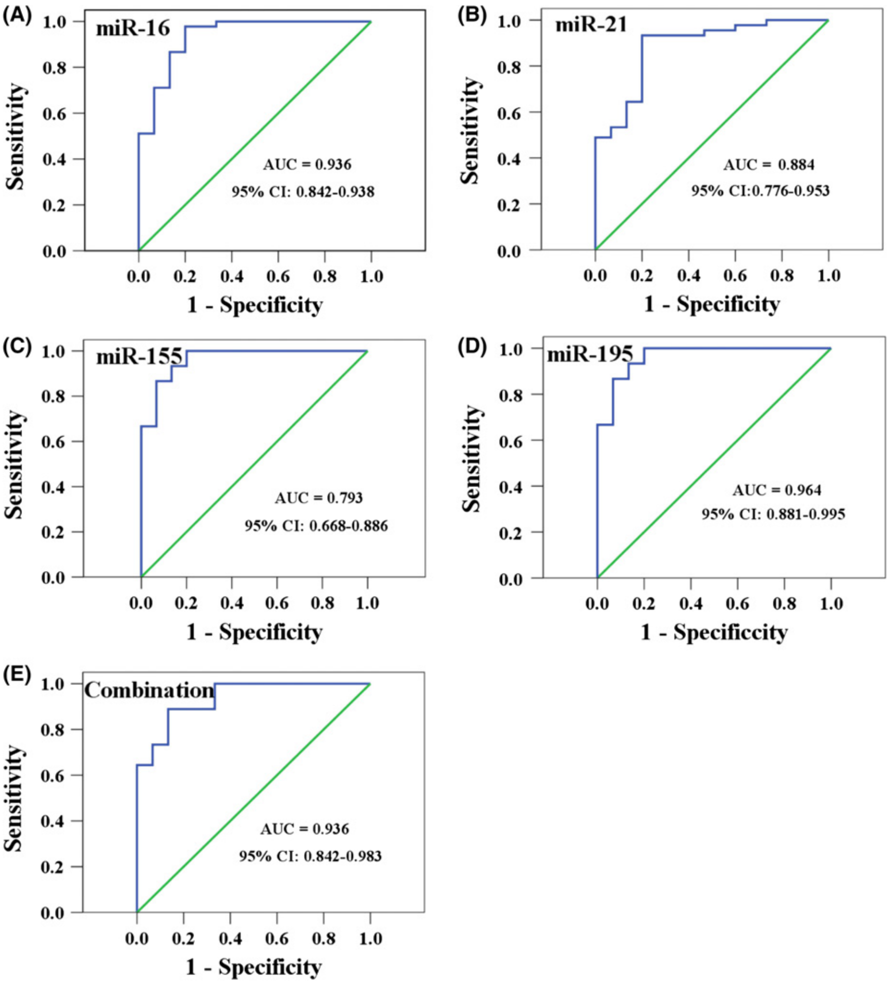

FIGURE 6 ROC curves for serum c-miR-16, c-miR-21, c-miR-155, c-miR-195, and the combination of the four c-miRNAs in distinguishing patients with breast cancer from healthy controls, AUC values are shown from A to E

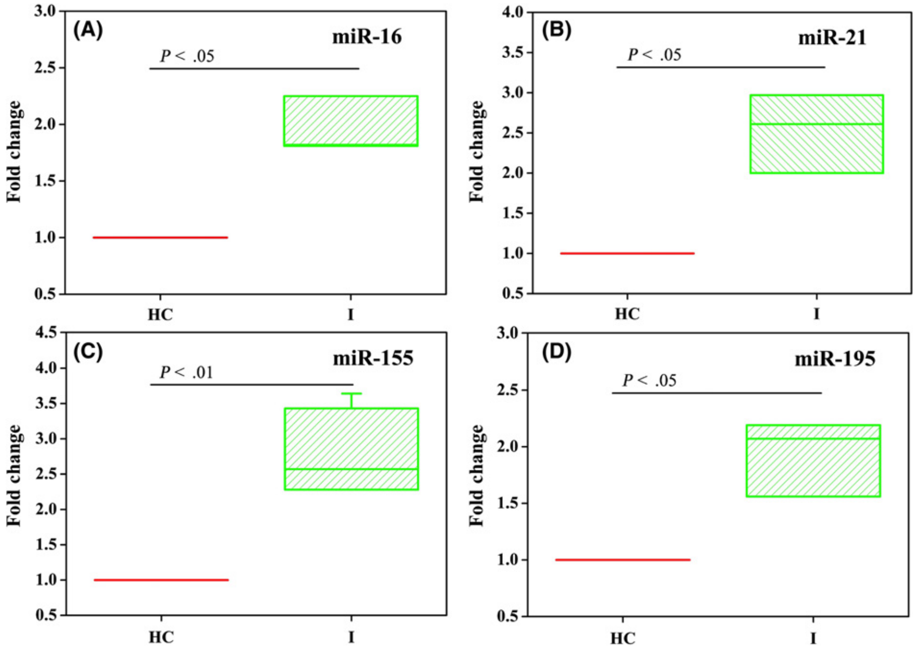

FIGURE 7 The fold change of c-miR-16 (A), c-miR-21 (B), c-miR-155 (C), and c-miR-195 (D) serum levels compared between healthy controls (n = 4) and breast cancer patients at stage I (n = 4). The *F* test was performed for comparisons between groups

shown that BRCA approach exhibits a better recovery rates of standard addition from 101.5% to 104.7% compared with qRT-PCR method from 91.4% to 110.4%, which indicated that BRCA technology shows stronger anti-interference ability in clinical diagnosis of cancers compared with conventional qRT-PCR method.

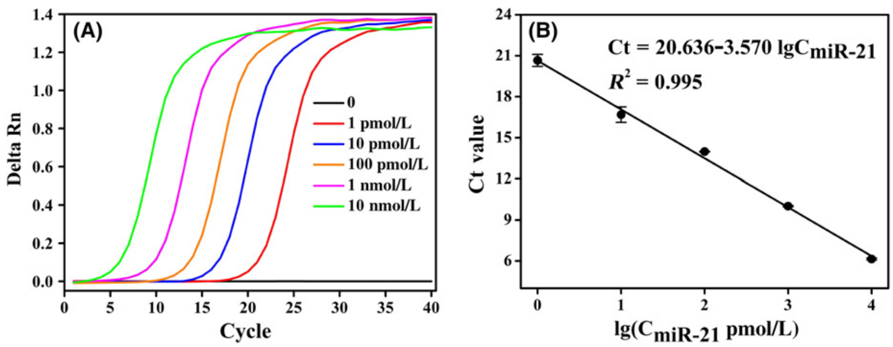

FIGURE 8 A, The fluorescence monitoring of quantitative real-time PCR amplification reaction triggered by various concentrations of miR-21 (0, 1, 10, 100 pM, 1, 10 nM). B, The calibration curve between threshold cycle value (C_t) and the logarithm of the different concentrations of miR-21. The error bars show the standard deviation of three replicate determinations. C_t defined as the fractional cycle number where the fluorescence passes the fixed threshold value

TABLE 2 Detection of miR-21 in human serum specimens compared with qRT-PCR

	BRCA assay				qRT-PCR assay			
Specimen	Added/(nM)	Mean Found[a]/(nM)	Mean Recovery[b](%)	RSD[c]	Added/(nM)	Mean Found/(nM)[a]	Mean Recovery (%)[b]	RSD[c]
1	0.1	0.103	102.659	0.045	0.1	0.110	110.440	1.249
2	1	1.043	104.328	0.355	1	0.914	91.401	0.162
3	10	10.153	101.527	1.155	100	0.938	93.787	0.172

[a] Mean concentration of three replicates.

[b] Mean recovery(%) = ($C_{mean\ found}/C_{added}$) × 100%.

[c] Relative standard deviation of three determinations.

4 | DISCUSSION

In this study, we analyzed a cohort of 68 serum specimens including 49 breast cancer patients (stages I-IV) and 19 healthy controls, which found that the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 could identify patients with early breast cancer, and distinguish them from healthy controls using one-step BRCA and qRT-PCR. To our knowledge, it is the first study that demonstrates the detection of c-miRNA biomarkers by the one-step BRCA method in clinical serum specimens.

Our study has demonstrated that the levels of c-miR-21, c-miR-155, and c-miR-195 were higher in breast cancer patients than healthy subjects by BRCA approach and conventional qRT-PCR method. Moreover, we also have verified that the serum c-miR-16 levels are not consistent during breast cancer progression, and are influenced by breast cancer status, which is consistent with previous studies.[10] In addition, the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 can not only distinguish early-stage breast cancer patients (stage I or II) from healthy controls, but also can distinguish different molecular breast cancer subtypes from healthy controls.

In conclusion, it is the first study that demonstrates that the BRCA assay can be used to measure the serum levels of c-miRNAs to screen and detect early breast cancer. The results demonstrate that the serum levels of c-miR-16, c-miR-21, c-miR-155, and c-miR-195 can identify patients with early breast cancer, and distinguish them from healthy controls. Most importantly, our research highlights the value of BRCA in the analysis of c-miRNAs in clinical serum specimens, and validates serum c-miRNAs as biomarkers for early breast cancer detection.

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CONFLICT OF INTEREST

The authors have no potential conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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