Original Article

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

Tingting Fan1,2 | Yu Mao3 | Qinsheng Sun2 | Feng Liu2 | Jin-Shun Lin2 | Yajie Liu4 | Junwei Cui4 | Yuyang Jiang1,2

1Department of Chemistry, Tsinghua University, Beijing, China
2State Key Laboratory of Chemical Oncogenomics, Key Laboratory of Chemical Biology, The Graduate School at Shenzhen, Tsinghua University, Shenzhen, Guangdong, China
3School of Food Science and Engineering, Hefei University of Technology, Hefei, China
4Peking University Shenzhen Hospital, Shenzhen, Guangdong, China

Correspondence: Yu Mao, School of Food Science and Engineering, Hefei University of Technology, 193 Tunxi Road, Hefei 230009, China (maoyu@hfut.edu.cn)
and Yuyang Jiang, State Key Laboratory of Chemical Oncogenomics, Key Laboratory of Chemical Biology, the Graduate School at Shenzhen, Tsinghua University, 2279 Lishui Road, Shenzhen 518055, China (jiangyy@sz.tsinghua.edu.cn).

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 over-expression, 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

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
enormous potential for using as specific and non-invasive cancer biomarkers. In breast cancer patients, the levels of serum c-miRNA16 (c-miR-16) are significantly higher compared with healthy controls. 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. The expression of serum c-miRNA155 (c-miR155) is also increased in breast cancer patients compared with healthy controls. 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. 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 inconvenience. Some conventional techniques have been used for c-miRNA detection, such as northern blotting, microarrays, and quantitative real-time PCR (qRT-PCR). 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. 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. Moreover, in principle, in one form or the other, BRCA has been used to detect miRNAs, including c-miRNAs in serum.

| Characteristics | Number of patients, n (%) |
|-----------------|---------------------------|
| **Age range**   | 25-64                     |
| **Mean age**    | 43                        |
| **Median age**  | 43                        |
| **ER status**   |                           |
| Positive        | 39 (79.6)                 |
| Negative        | 8 (16.3)                  |
| Unknown         | 2 (4.1)                   |
| **PR status**   |                           |
| Positive        | 31 (63.2)                 |
| Negative        | 16 (32.7)                 |
| Unknown         | 2 (4.1)                   |
| **HER-2 status**|                           |
| Positive        | 20 (40.8)                 |
| Negative        | 24 (49.0)                 |
| Unknown         | 5 (10.2)                  |
| **Ki-67 protein**|                         |
| High (>14%)     | 45 (91.8)                 |
| Low             | 2 (4.1)                   |
| Unknown         | 2 (4.1)                   |
| **Tumor size**  |                           |
| Tis             | 3 (6.1)                   |
| T1              | 19 (38.8)                 |
| T2              | 20 (40.8)                 |
| T3              | 6 (12.2)                  |
| Unknown         | 1 (2.0)                   |
| **Lymph nodes** |                           |
| N0              | 34 (69.4)                 |
| N1              | 8 (16.3)                  |
| N2              | 5 (10.2)                  |
| Unknown         | 2 (4.1)                   |
| **Metastasis**  |                           |
| 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)                 |

(Continues)
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. 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 sera 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′-UAGCAGACGUAACAUUUUGGCG-3′), c-miR21 (5′-UAGCUUAUCAGACUGAUGUA-3′), c-miR155 (5′-UUAAUUGCCUAUUGGUGG-3′), and c-miR195 (5′-UAGCAGACGAAAUUUGGC-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:
5′-ACGTGCTGCTAACATCAAAGCCCATACTACAACACATTACCCATATTT-3′, miR-21 probe: 5′-TGATAAGCTAACATCAAAGCCCATACTACAACACTACACACCAATATTT-3′, mir-155 probe: 5′-GATTACCATAAACATCAAAGCCCATACTACAACACTACACACCAATATTT-3′, and miR-195 probe: 5′-CTGTGCTGCTAACAATAAGCCCATACTACAACACAACAGCCATATTT-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 for fluorescence 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 both ends of the ligation template. As a result, the circular template would hybridize by the ligation 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 for fluorescence detection.

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 (10x), 5 μL of BRCA primer (10 μM), 2.5 μL of EvaGreen Dye (20X), and 22 μL of RNase-free water. The mixture was incubated at 30°C for 5 h. Subsequently, 5 μL of RCA buffer (10 X), 2.5 μL of EvaGreen Dye (20X), and 42.5 μL of ddH2O 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.

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) for sensitivity and specificity were calculated. 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/F0)F0 achieved a maximum value at 30°C, where F and F0 are the fluorescence intensity (λex = 485 nm, λem = 528 nm) with and without the target c-miRNA, respectively. Next, we optimized the time of BRCA reaction...
Figure S2B), the value of \((F-F_0)/F_0\) 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 \(Y_{fluorescence\ intensity} = 1778.9 + 232.8 \ lgC_{miR-21}\) 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′-UACCCUGUAGAACCGAAUUUGU-3′) and miR-222 (5′-AGCUACUACUGGG-3′) as the control sequences respectively. As shown in Figure 3A-D, these comparisons clearly show that the
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 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 < 0.0001) 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.
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 (Ct) 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 Ct value using the fold change = 2−ΔΔCt method, where ΔΔCt = (Ct c-miRNAs) breast cancer specimens - (Ct c-miRNAs) 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 sera 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
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 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.
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. 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.

**ACKNOWLEDGMENTS**

The authors thank the State Key Laboratory of Chemical Oncogenomics, Key Laboratory of Chemical Biology, the Graduate School at Shenzhen, Tsinghua University for providing article research platform. They also thank the Peking University Shenzhen Hospital and the Shenzhen People’s Hospital for providing serum specimens.

**CONFLICT OF INTEREST**

The authors have no potential conflict of interest to disclose.

**ORCID**

Tingting Fan [http://orcid.org/0000-0003-3338-4154](http://orcid.org/0000-0003-3338-4154)

**REFERENCES**

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.
2. Maxmen A. The hard facts. Nature. 2012;485:550-551.
3. Molina-Montes E, Pollan M, Payer T, Molina E, Davila-Arias C, Sanchez MJ. Risk of second primary cancer among women with breast cancer: a population-based study in Granada (Spain). Gynecol Oncol. 2013;130:340-345.

4. Moon PG, Lee JE, Cho YE, et al. Identification of developmental endothelial locus-1 on circulating extracellular vesicles as a novel biomarker for early breast cancer detection. Clin Cancer Res. 2016;22:1757-1766.

5. Henry NL, Hayes DF. Cancer biomarkers. Mol Oncol. 2012;6:140-146.

6. Cuk K, Zucknick M, Heil J, et al. Circulating microRNAs in plasma as early detection markers for breast cancer. Int J Cancer. 2013;132:1602-1612.

7. Inns J, James V. Circulating microRNAs for the prediction of metastasis in breast cancer patients diagnosed with early stage disease. Breast. 2015;24:364-369.

8. Montani F, Marzi MJ, Dezi F, et al. miR-Test: a blood test for lung cancer early detection. J Natl Cancer Inst. 2015;107:djv063.

9. Wang X, Ivan M, Hawkins SM. The role of MicroRNA molecules and MicroRNA-regulating machinery in the pathogenesis and progression of epithelial ovarian cancer. Gynecol Oncol. 2017;147:481-487.

10. Hu Z, Dong J, Wang LE, et al. Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. Carcinogenesis. 2012;33:828-834.

11. Stuckrath I, Rack B, Janni W, Jager B, Pantel K, Schwarzenbach H. Aberrant plasma levels of circulating miR-16, miR-107, miR-130a and miR-146a are associated with lymph node metastasis and receptor status of breast cancer patients. Oncotarget. 2015;6:13387-13401.

12. Asaga S, Kuo C, Nguyen T, Terpenning M, Giuliano AE, Hoon DS. Direct serum assay for microRNA-21 concentrations in early and advanced breast cancer. Clin Chem. 2011;57:84-91.

13. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Annu Rev Med. 2009;60:167-179.

14. Roth C, Rack B, Muller V, Janni W, Pantel K, Schwarzenbach H. Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. Breast Cancer Res. 2010;12:R90.

15. Pimentel F, Bonilla P, Ravishankar YG, et al. Technology in microRNA profiling: circulating microRNAs as noninvasive cancer biomarkers in breast cancer. J Lab Autom. 2015;20:574-588.

16. Mattiske S, Suehani RJ, Neilsen PM, Callen DF. The oncogenic role of miR-155 in breast cancer. Cancer Epidemiol Biomark Prev. 2012;21:1236-1243.

17. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg. 2010;251:499-505.

18. Heneghan HM, Miller N, Kelly R, Newell J, Kerin MJ. Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. Oncologist. 2010;15:673-682.

19. Fan T, Mao Y, Liu F, Zhang W, Yin J, Jiang Y. Dual signal amplification strategy for specific detection of circulating microRNAs based on thiolflavin T. Sens Actuators B Chem. 2017;249:1-7.

20. Valoci A, Hornyk C, Varga C, Burgyan J, Kauppinen S, Havelda Z. Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res. 2004;32:e175.

21. Li W, Ruan K. MicroRNA detection by microarray. Anal Bioanal Chem. 2009;394:1117-1124.

22. Yan J, Zhang N, Qi C, Liu X, Shangguan D. One-step real time RT-PCR for detection of microRNAs. Talanta. 2013;110:190-195.

23. Hamblin GD, Carneiro KM, Fakhoury JF, Bujold KE, Sleiman HF. Rolling circle amplification-templated DNA nanotubes show increased stability and cell penetration ability. J Am Chem Soc. 2012;134:2888-2891.

24. Mao Y, Liu M, Tram K, et al. Optimal DNA templates for rolling circle amplification revealed by in vitro selection. Chemistry. 2015;21:8069-8074.

25. Konry T, Smolina I, Yarmush JM, Irinia D, Yarmush ML. Ultrasensitive detection of low-abundance surface-marker protein using isothermal rolling circle amplification in a microfluidic nanoliter platform. Small. 2011;7:395-400.

26. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33:e179.

27. Cheng Y, Zhang X, Li Z, Jiao X, Wang Y, Zhang Y. Highly sensitive determination of microRNA using target-primed and branched rolling-circle amplification. Angew Chem Int Ed Engl. 2009;48:3268-3272.

28. Ali MM, Li F, Zhang Z, et al. Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. Chem Soc Rev. 2014;43:3324-3341.

29. Li Y, Liang L, Zhang CY. Isothermally sensitive detection of serum circulating miRNAs for lung cancer diagnosis. Anal Chem. 2013;85:11174-11179.

30. Zhang LR, Zhu G, Zhang CY. Homogeneous and label-free detection of microRNAs using bifunctional strand displacement amplification-mediated hyperbranched rolling circle amplification. Anal Chem. 2014;86:6703-6709.

31. Hong C, Baek A, Hah SS, Jung W, Kim DE. Fluorometric detection of microRNA using isothermal gene amplification and graphene oxide. Anal Chem. 2016;88:2999-3003.

32. Liedtke C, Rody A, Gluz O, et al. The prognostic impact of age in different molecular subtypes of breast cancer. Breast Cancer Res Treat. 2015;152:667-673.

33. Cheng H-T, Huang T, Wang W, et al. Clinicopathological features of breast cancer with different molecular subtypes in Chinese women. J Huazhong Univ Sci Technol Med Sci. 2013;33:117-121.

34. Toyiama Y, Takahashi M, Hur K, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst. 2013;105:849-859.

35. Shimomura A, Shino S, Kawauji J, et al. Novel combination of serum microRNA for detecting breast cancer in the early stage. Cancer Sci. 2016;107:326-334.

36. Freres P, Wениc S, Boukerroucha M, et al. Circulating microRNA-based screening tool for breast cancer. Oncotarget. 2016;7:5416-5428.

37. Koperski L, Kotlarek M, Swierkami M, et al. Next-generation sequencing reveals microRNA markers of adenocortical tumors malignancy. Oncotarget. 2017;8:49191-49200.

38. Yamada A, Horimatsu T, Okugawa Y, et al. Serum miR-21, miR-29a, and miR-125b are promising biomarkers for the early detection of colorectal neoplasia. Clin Cancer Res. 2015;21:4234-4242.

39. Werner S, Krause F, Rolny V, et al. Evaluation of a 5-marker blood test for colorectal cancer early detection in a colorectal cancer screening setting. Clin Cancer Res. 2016;22:1725-1733.

SUPPORTING INFORMATION

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