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Direct Serum Assay for MicroRNA-21 Concentrations in Early and Advanced Breast Cancer

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BACKGROUND: MicroRNAs (miRs) are a class of small noncoding RNAs whose expression changes have been associated with cancer development and progression. Current techniques to isolate miRs for expression analysis from blood are inefficient. We developed a reverse-transcription quantitative real-time PCR (RT-qPCR) assay for direct detection of circulating miRs in serum. We hypothesized that serum concentrations of miR-21, a biomarker increased in breast tumors, would correlate with the presence and extent of breast cancer.

METHODS: The RT-qPCR applied directly in serum (RT-qPCR-DS) assay for circulating miR-21 was tested in sera from 102 patients with different stages of breast cancer and 20 healthy female donors.

RESULTS: The assay was sensitive for detection of miR-21 in 0.625 μL of serum from breast cancer patients. For differentiation of samples from patients with locoregional breast cancer from those from healthy donors, the odds ratio was 1.796 and the area under the curve was 0.721. In a multivariate analysis that included standard clinicopathologic prognostic factors, high circulating miR-21 concentrations correlated significantly ($P < 0.001$) with visceral metastasis.

CONCLUSIONS: A novel RT-qPCR-DS can improve the efficiency of miR assessment. Use of this assay to detect circulating miR-21 has diagnostic and prognostic potential in breast cancer.

Breast cancer was the second leading cause of cancer death among women in the United States in 2009 *(1)*. Although early detection through mammographic screening has reduced breast cancer mortality *(2)*, the sensitivity and specificity of mammography can be compromised in younger women who have dense breast tissue *(3)*. Minimally invasive and sensitive diagnostic approaches are needed to supplement breast imaging.

MicroRNAs (miRs)[4] are naturally occurring small noncoding RNA molecules (18–24 nucleotides) that interact with their target coding mRNAs to inhibit translation by promoting mRNA degradation or to block translation by binding to complementary sequences in the 3′ untranslated regions (3′ UTR) of mRNA *(4)*. miRs can be expressed in a tissue-specific manner and reportedly play pivotal roles in proliferation, apoptosis, and differentiation of mammalian cells *(5–7)*. miR-21 is one of the most significantly upregulated miRs in human breast cancer, and its expression has been associated with tumor progression and poor prognosis *(8–12)*. Evidence suggests that miR-21 targets and inhibits tropomyosin 1 (alpha) (*TPM1*)[5] *(9)*, programmed cell death 4 (neoplastic transformation inhibitor) (*PDCD4*) *(10)*, and phosphatase and tensin homolog (*PTEN*) *(13)* and other tumor-related genes.

miRs detected in serum or plasma reportedly are more stable than mRNA *(14)* in blood. Intrinsic miRs in serum are stable at room temperature and can withstand multiple freeze-thaw cycles and survive effects of RNase and DNase *(15, 16)*. Expression profiles of serum miRs can distinguish patients with specific cancers *(15, 17)*. However, the clinical utility of miR has not been investigated in a well-defined breast cancer–related study. We hypothesized that the serum concentration of circulating miR-21 is correlated with the presence and stage of breast cancer.

We previously established a direct quantitative PCR (qPCR) assay to study circulating DNA in blood from patients with breast and other cancers *(18, 19)*.

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[4] Nonstandard abbreviations: miR, microRNA; 3′ UTR, 3′ untranslated region; qPCR, quantitative PCR; RT-qPCR, reverse-transcription quantitative real-time PCR; RT-qPCR-DS, RT-qPCR directly applied in serum assay; PEAT, paraffin-embedded archival tissue; AJCC, American Joint Committee on Cancer; C_q, quantification cycle; dC_q, difference in real-time C_q between the target and the reference control; AUC, area under the curve; ddC_q, difference of $-dC_q$; T, Tween 20 surfactant; K, proteinase K.
[5] Human genes: *TPM1*, tropomyosin 1 (alpha); *PDCD4*, programmed cell death 4 (neoplastic transformation inhibitor); *PTEN*, phosphatase and tensin homolog.

These reports demonstrated that the integrity of circulating DNA as measured by Alu repeats was useful in detecting progression of breast and gastrointestinal cancers. In the present study, we applied our direct serum assay approach to detect miRs. Efficient extraction of circulating nucleic acids from plasma or serum has been difficult, particularly when the nucleic acids are small in length or when the available amount of nucleic acids or source material (i.e., blood) is limited. Our results demonstrated potential utility of the novel reverse-transcription quantitative real-time PCR (RT-qPCR) directly applied in a serum assay (RT-qPCR-DS) to detect and quantify the concentrations of circulating miR-21 in breast cancer patients without having to extract RNA from serum.

Patients and Methods

PARAFFIN-EMBEDDED ARCHIVAL TISSUE ANALYSIS

Paraffin-embedded archival tissue (PEAT) samples of the primary tumor and adjacent normal breast were obtained from 14 patients who underwent surgical treatment for invasive breast cancer at our breast center between 2000 and 2007. Patients had American Joint Committee on Cancer (AJCC) stage I (n = 4), stage II (n = 1), stage III (n = 5), or stage IV (n = 4) disease. All tissue samples for this study were obtained according to protocol guidelines set forth by the John Wayne Cancer Institute and approved by the Western Institutional Review Board.

SERUM SAMPLES FOR PILOT AND VALIDATION STUDIES

Blood samples collected in red tiger-top gel separator tubes (Fisher Scientific) from patients or healthy donors were processed within 2–5 h after collection as follows: the serum was separated by centrifugation and passed through a 13-mm serum filter (Fisher Scientific) to remove potential contaminating cells, as previously described *(18)*. Serum was divided into aliquots and immediately cryopreserved at −80 °C. For the pilot study, serum samples were obtained from 10 healthy female donors and 40 women with pathologic AJCC stage I (n = 10), II (n = 10), III (n = 10), or IV (n = 10) breast cancer. The 40 patients included all 14 patients in the PEAT study. For the validation study, serum samples were obtained from an additional 10 healthy women and 62 women with AJCC stage I (n = 21), stage II (n = 16), stage III (n = 12), or stage IV (n = 13) breast cancer. All patients with stage III disease had lymph node metastasis; all patients with stage IV disease had visceral metastasis. All patients underwent surgical treatment for invasive breast cancer at our center between 2000 and 2007. All serum samples for this study were obtained according to protocols approved by the institutional review board and after the sample donors provided informed consent.

RNA EXTRACTION FROM SERUM AND PEAT SAMPLES

Total RNA was extracted from 500 μL of serum by using TRI reagent BD (Molecular Research Center).

Ten sections, each 10-μm thick, were cut from each PEAT block. Deparaffinized tissue sections were digested by using proteinase K, and RNA was extracted by using a modified protocol of the RNAWiz Isolation kit (Applied Biosystems) *(20)*. The RNA was quantified and assessed for purity by using ultraviolet spectrophotometry and the Quant-iT RiboGreen RNA assay kit (Invitrogen) *(20)*.

RT-qPCR ASSAY WITH EXTRACTED RNA

We dissolved 10 ng of total RNA extracted from tumor tissue, normal tissue, or serum in 5 μL H_2O (2 ng/μL) for reverse transcription with the addition of 5 μL of a reaction mixture containing 5× first strand buffer, 10 mmol/L deoxynucleoside-5′-triphosphate, RNasin, Moloney murine leukemia virus reverse transcriptase, and miR-specific RT primers (Exiqon). After the mixture was incubated in 37 °C for 2 h, the transcribed specific cDNA was diluted 10-fold with molecular grade H_2O before use as a qPCR template. Each qPCR contained 2.5 μL of diluted cDNA, 5 μL of 2× PerfeCTa® SYBR® Green FastMix® for iQ™ (Quanta Bioscience), miR-specific, locked nucleic-acid–based forward primer targeting the specific miR (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue1), and universal reverse primer (Exiqon). Each sample in duplicates was assessed in 45 cycles of 95 °C for 1 min and 60 °C for 1 min. RNU6B was assessed as the reference control for tissue studies, and miR-16 was the reference control for serum studies.

An iCycler iQ real-time PCR Detection System (Bio-Rad) was used for qPCR with melting-curve analysis. Target amplification was normalized with the reference control, and comparative quantification was recorded as the difference in real-time quantification cycle (C_q) between the target and the reference control (dC_q).

RT-qPCR DIRECTLY IN SERUM

This assay required only a small aliquot of serum. To deactivate or solubilize proteins that might inhibit the RT-qPCR, we mixed 2.5 μL of each serum sample with 2.5 μL of a preparation buffer that contained 2.5% Tween 20 (EMD Chemicals), 50 mmol/L Tris (Sigma-Aldrich), and 1 mmol/L EDTA (Sigma-Aldrich). We added 5 μL of RT reagent mixture, which contained the same RT reagents used for RT-qPCR with extracted

RNA, directly to 5 μL of serum in preparation buffer; a 2-h incubation at 37 °C was followed by a 5-min enzyme inactivation at 95 °C. The transcribed cDNA was diluted 10-fold by H_2O and then centrifuged at 9000*g* for 5 min to eliminate the protein precipitant. A 2.5-μL volume of the supernatant cDNA solution was used as the template for qPCR. qPCR conditions, primers and reagents, and data analysis were duplicated for those described in RT-qPCR with extracted RNA section.

BIOSTATISTICAL ANALYSIS

The Pearson correlation coefficient was used to compare the results generated by the 2 RT-qPCR methods. The Student-Newman-Keuls test was used to compare $-dC_q$ values according to AJCC stage of breast cancer; *P* values of <0.05 were considered significant. The Ryan-Einot-Gabriel-Welsch multiple range test, Tukey's honestly significant difference test, and Student-Newman-Keuls test were used for posthoc pairwise comparisons of RT-qPCR results across AJCC stages. Logistic regression analysis of circulating miR-21 concentrations was used to differentiate study participants by the presence and extent of breast cancer (no disease, AJCC stage I/II, or stage III/IV); ROC curves and the area under the curve (AUC) are reported. A general linear model was used for multivariate analysis to identify clinicopathologic factors significantly associated with the miR-21 concentration.

Results

RT-qPCR ANALYSIS OF BREAST TISSUE

RT-qPCR assay of 10 ng total RNA confirmed upregulation of miR-21 in PEAT from patients with breast cancer. The mean C_q value (95% CI) of miR-21 was 19.2 (18.7–19.8) in breast cancer tissue and 22.5 (21.6–23.4) in normal breast tissue. The mean C_q value (95% CI) of RNU6B (reference control) was 23.8 (22.9–24.6) in breast cancer tissue and 25.1 (24.2–26.0) in normal breast tissue. The comparative miR-21 expression in tumor tissue was measured by the difference of $-dC_q$ (ddC_q) from the tumor and the adjacent normal tissues [ddCq = (−dCq cancer) − (−dCq normal)]; and the ddC_qs were between 0.2 and 3.9 (95% CI 1.3–2.6).

RT-qPCR-DS OPTIMIZATION

Tween 20 surfactant (T) and 1 μg/μL proteinase K (K) are components of the preparation buffer of our direct serum assay for circulating DNA *(18)*. To develop our RT-qPCR-DS for circulating miR, we tested T and K in the following combinations: (A) no T or K, (B) K only, (C) 1.0% T and K, (D) 2.5% T and K, (E) 1.0% T only, and (F) 2.5% T only. Serum samples from a training set of 12 breast cancer patients, later included in the pilot study, were used; and results were compared to those for RT-qPCR with RNA. No miRs were detected for combinations A through D. Combination E showed improved sensitivity but no linear correlation ($r = -0.064$) to RT-qPCR; by contrast, combination F showed a linear correlation ($r = 0.796$). Thus 2.5% T was selected for the pilot and validation studies.

ANALYTICAL SENSITIVITY AND SPECIFICITY OF RT-qPCR-DS

The serum dilution study demonstrated that variations in amount of serum assessed did not affect $-dC_q$ values measured by RT-qPCR-DS. When serum samples from 4 representative AJCC stage III breast cancer patients within the study were diluted by a factor of 2, 4, or 8, corresponding to 1.25 μL, 0.625 μL, or 0.3125 μL of serum, respectively, there was no significant difference in $-dC_q$ values for miR-21 normalized against miR-16 (Fig. 1A) in 1.25 μL and 0.625 μL of serum. However, miR-16 expression could not be detected in all samples using 0.3125 μL of serum; thus, the limit of detection for RT-qPCR-DS was demonstrated to be 0.625 μL of serum.

In 3 randomly selected sera from the study patients, the SD of C_q values for the triplicates of the same serum assessed in the same RT-qPCR-DS ranged from 0.115 to 1.27 with a mean of 0.59. The variability was within that of the inherent variability of qPCR for higher C_q *(21)*. When the same 3 sera were assessed again in a separate RT-qPCR-DS, the $-dC_q$ for each serum remained consistent at 3.68, 8.27, and 6.25 in run 1 and 3.83, 8.03, and 5.73 in run 2 with SDs of 0.21, 0.33, and 0.73 (mean SD = 0.42), demonstrating interassay reproducibility.

DNase treatment was assessed in 8 serum samples, and the RT-qPCR-DS results were similar to those of non–DNase-treated serum. The mean difference between C_q values of the DNase treated vs non–DNase-treated serum was 0.75 with an SD of 1, demonstrating that the universal reverse primer provided by Exiqon, a nonhuman sequence, amplified only the cDNA synthesized with the specific primer from Exiqon. The specificity of the Exiqon primers was further tested by using genomic DNA from breast cancer cell lines as template, and no amplification was found.

Stability of miR-21 was investigated in 4 serum samples randomly selected from the study patient group. There was no significant difference in $-dC_q$ values after 4 cycles of freezing at −80 °C and thawing at 23 °C (Fig. 1B).

RT-QPCR-DS VS RT-QPCR: PILOT STUDY

We performed a pilot study to compare the 2 RT-qPCR techniques in 50 serum samples from 10 healthy donors and 40 patients with AJCC stage I–IV breast cancer (10 patients for each stage).

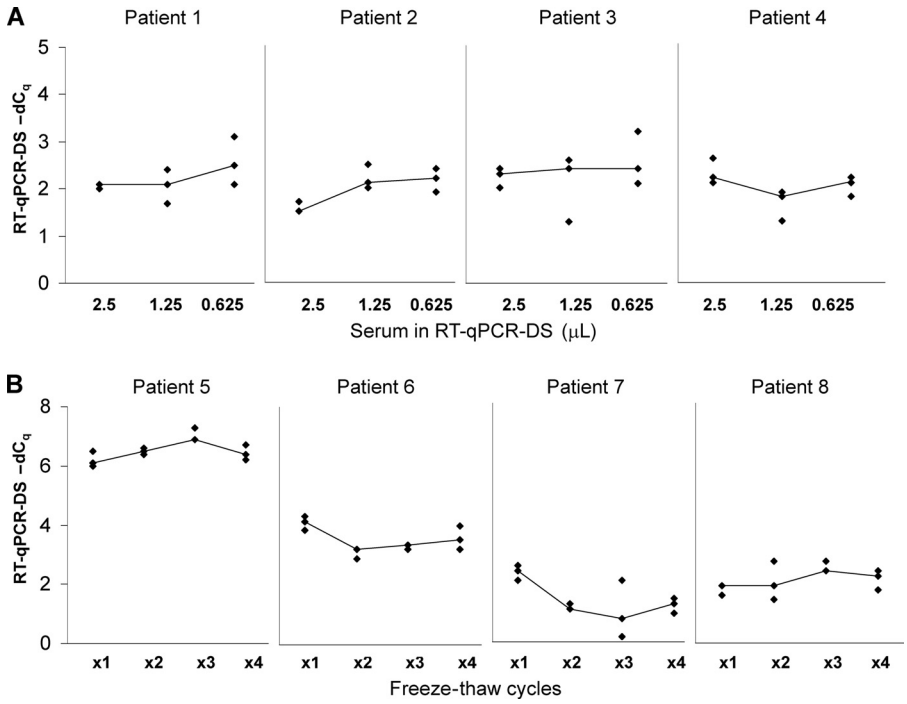


Fig. 1. Stability of circulating miR-21 by direct RT-qPCR-DS assay.

(A), The $-dC_q$ values of serum samples from 4 breast cancer patients were assessed in triplicate before dilution (2.5 μL serum) and after dilution (1.25 μL, 0.625 μL serum). (B), Assay consistency across 4 freeze-thaw cycles was examined in serum samples obtained from 4 other breast cancer patients. The line connects the median $-dC_q$ of the triplicates.

By RT-qPCR, the median C_q values (range) of miR-16 were 36.4 (34.5–37.6) in healthy donors and 36.0 (34.8–37.4), 36.6 (35.1–37.7), 36.8 (33.3–37.8), and 36.9 (34.0–37.9) in patients with stage I, II, III, and IV breast cancer, respectively (Fig. 2A). By RT-qPCR-DS, the median C_q values (range) of miR-16 were 36.3 (30.3–36.7) in healthy donors and 34.8 (31.7–38.5), 34.4 (31.1–38.1), 33.1 (30.8–37.3), and 34.4 (30.0–38.6) in patients with stage I, II, III, and IV breast cancer, respectively (Fig. 2B). Both assays demonstrated no significant differences in miR-16 C_q values between healthy donors and patients with any stage of breast cancer. These results indicate that miR-16 is present in serum at a consistent concentration and can be used as an internal control to normalize sampling and PCR variations in both RT-qPCR methods.

By RT-qPCR, the median $-dC_q$ values (range) of miR-21 were 4.2 (2.1–5.2) in healthy donors and 6.3 (4.5–7.6), 5.9 (4.3–7.4), 5.5 (4.2–7.8), and 6.7 (5.1–10.3) in patients with stage I, II, III, and IV breast cancer, respectively. By RT-qPCR-DS, the median $-dC_q$ values (range) were 2.2 (−1.5 to 3.1) in healthy donors and 4.1 (2.1–5.7), 3.8 (2.2–4.7), 3.3 (2.7–4.4), and 5.0 (3.2–6.6) in patients with stage I, II, III, and IV breast

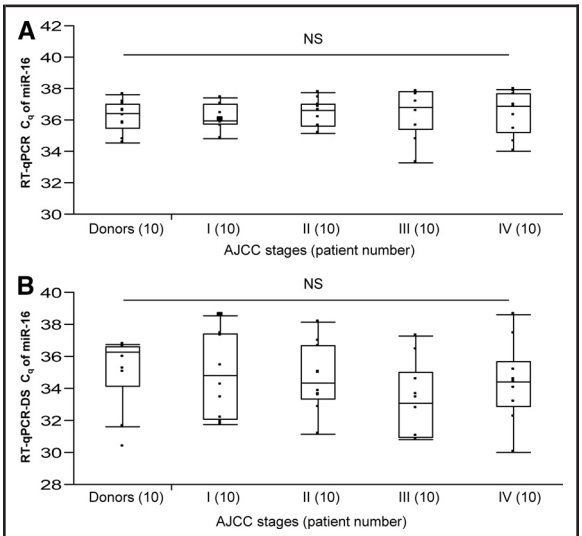


Fig. 2. C_q values of circulating miR-16 in the pilot study.

(A), Results by RT-qPCR. (B), Results by RT-qPCR-DS. NS, not significant.

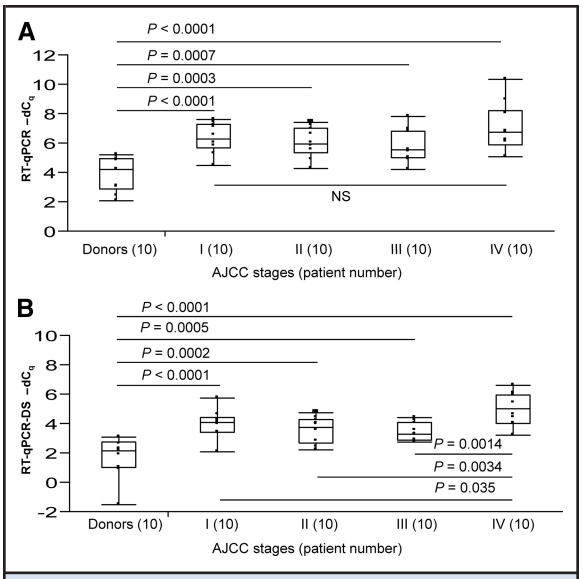


Fig. 3. Pilot study comparing $-dC_q$ values of circulating miR-21 in healthy female donors and breast cancer patients.

(A), Results by RT-qPCR. (B), Results by RT-qPCR-DS. NS, not significant.

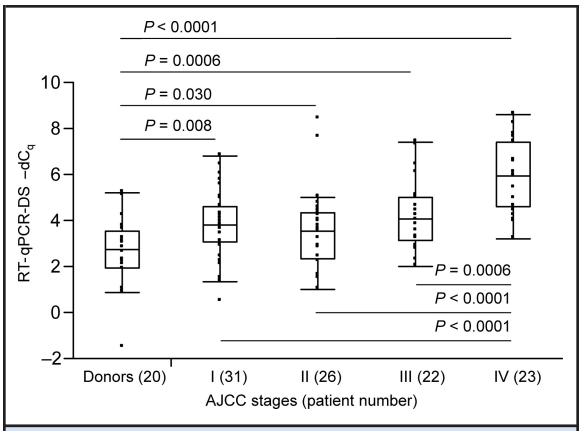


Fig. 4. Validation study comparing $-dC_q$ values of circulating miR-21 in 20 healthy female donors and 102 breast cancer patients by RT-qPCR-DS.

cancer, respectively. There was a significant linear correlation in $-dC_q$ values between both assays ($r = 0.796$).

The RT-qPCR assay demonstrated that $-dC_q$ differences were significant between healthy female donors and breast cancer patients but not between patients with different stages of breast cancer (Fig. 3A). By contrast, the RT-qPCR-DS showed that $-dC_q$ differences were significant not only between healthy female donors and breast cancer patients but also between patients with AJCC stage I, II, or III breast cancer and those with AJCC stage IV breast cancer (Fig. 3B). The same results were obtained with 3 different statistical procedures: Student-Newman-Keuls test, Ryan-Einot-Gabriel-Welsch multiple range test, and Tukey's honestly significant difference test.

CLINICAL UTILITY OF CIRCULATING miR-21: VALIDATION STUDY

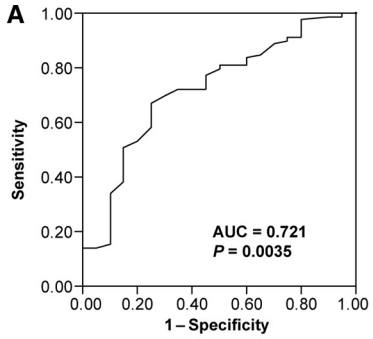
Based on results of the pilot study with 50 participants, we further performed RT-qPCR-DS to validate the clinical utility of circulating miR-21 concentrations for breast cancer. In serum analysis of all patients in the pilot and validation groups, the median (range) $-dC_q$ values were 2.7 (−1.5 to 5.2) in 20 healthy donors and 3.8 (0.5–6.8), 3.6 (1.0–6.8), 4.1 (2.0–7.4), and 5.9 (3.2–8.6) in 102 patients with stages I (n = 31), II (n = 26), III (n = 22), and IV(n = 23) breast cancer, respectively. The detected miR-21 concentration was significantly lower in healthy donors compared to breast cancer patients with any stage of disease (Fig. 4). Furthermore, circulating miR-21 concentrations were significantly higher in patients with AJCC stage IV breast cancer than in patients with other stage breast cancer (Fig. 4).

ROC analysis was performed to assess sensitivity and specificity of the RT-qPCR-DS assay. For distinguishing locoregional breast cancer patients from healthy donors, the odds ratio was 1.796 (95% CI 1.213–2.661) and the AUC was 0.721 (Fig. 5A). When the cutoff value was set to the optimal point, 3.3, specificity was 75%, sensitivity was 67%, and positive predictive value was 91% (Fig. 5B). We also explored whether circulating miR-21 could distinguish patients with AJCC stage IV breast cancer from patients with earlier stages of breast cancer. The ROC results demonstrated that the odds ratio was 2.153 (95% CI 1.514–3.062) and the AUC was 0.833 (Fig. 5C). When the cutoff value was set to the optimal point, 5.4, specificity was 86%, sensitivity was 70%, and positive predictive value was 59% (Fig. 5D).

We assessed the correlation between circulating miR-21 concentrations and 11 clinicopathologic factors. Univariate analysis showed that visceral metastasis and lymph node metastasis were significant factors associated with higher circulating miR-21 concentrations. However, multivariate analyses showed that AJCC stage IV disease was the only clinicopathologic factor significantly correlated to higher concentrations of circulating miR-21 (Table 1).

Discussion

This is the first reported investigation that demonstrated the utility of an RT-qPCR-DS assay for circulating miR. It is also one of the first studies to demon-

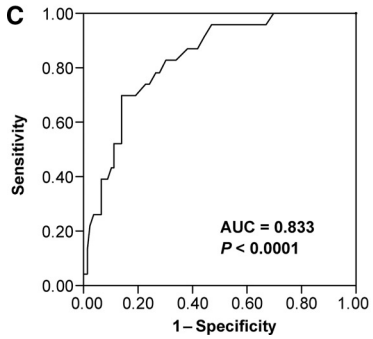


B

Patients' Status and $-dC_q$
AJCC Stage I–III Breast Cancer vs Healthy Female Donors

	$-dC_q$ value >3.3	<3.3	Total
Stage I–III patients	53	26	79
Healthy donors	5	15	20
Total	58	41	99

Cutoff: $-dC_q = 3.3$



D

Patients' Status and $-dC_q$
AJCC Stage IV vs Stage I–III Breast Cancer

	$-dC_q$ value >5.4	<5.4	Total
Stage IV patients	16	7	23
Stage I–III patients	11	68	79
Total	27	75	102

Cutoff: $-dC_q = 5.4$

Fig. 5. Identification of the presence and extent of breast cancer by RT-qPCR-DS of circulating miR-21.
(A), ROC analysis for patients with AJCC stage I, II, and III breast cancer vs healthy females. (B), The correlation between disease status and test results using a $-dC_q$ of 3.3 as cutoff. (C), ROC analysis for AJCC stage IV breast cancer vs AJCC stage I, II, or III breast cancer. (D), The correlation between disease status and test results with the use of a $-dC_q$ of 5.4 as cutoff.

strate the diagnostic and prognostic potential of circulating miR-21 for detecting and staging breast cancer. Finally, our results confirm miR-16 as a reference control for measurement of circulating miRs *(17, 22, 23)*; we found that serum miR-16 was consistently expressed and was not influenced by breast cancer status.

Our RT-qPCR-DS assay was effective and robust for detection of circulating miR. Unlike standard RT-qPCR, the direct assay could distinguish patients with AJCC stages I, II, or III breast cancer from those with AJCC stage IV breast cancer. By eliminating the extraction step, the RT-qPCR-DS assay avoided miR loss, streamlined the procedure, minimized human and mechanical errors, and reduced time and overall cost. This success may in part reflect the incorporation of Tween 20, which can dissociate lipid-bound nucleic acids from protein in serum. Circulating miRs in blood have been found in free form *(15)* and encapsulated in exosomes *(24–26)*. Although there is still little information about the structure of exosome-involving miRs, cancer-derived exosomes are soluble in detergents *(27)*.

There is urgent need for a rapid and reliable serum assay for diagnostic as well as prognostic assessment of breast cancer. Two serum-based tumor biomarkers, CA15–3 and carcinoembryonic antigen, are used to assess advanced breast cancer, but neither is recommended for diagnostic use *(28)*. Circulating tumor cells in blood have been used for prognostic assessment in patients with metastatic breast cancer *(29)*; but circulating tumor cells are of limited use for detection of early disease *(30)*, because tumor burden is low and fewer cells are shed into the circulation *(31)*. Mammography remains the primary tool for breast cancer screening but is not recommended in younger women *(32)*, because costs and false-positive results are high while clinical impact is low *(33)*.

Our multivariate analysis showed that the concentration of circulating miR-21 was correlated with AJCC stage and was independent of estrogen-receptor status or age. Therefore, circulating miR-21 may be a potential biomarker for breast cancer progression and detection. This finding is in accordance with several reports that miR-21 expression in breast tumors is correlated with advanced clinical stage, lymph node metastasis, and poor prognosis *(11, 12)*. In addition, miR-21 reportedly stimulates, in vitro and in vivo, cell invasion and metastasis in breast and other tumors *(5)*, which

Table 1. Clinicopathologic characteristics of breast cancer patients.[a]

Characteristic	Patients, n	$-dC_q$ value, mean (SD)	95% CI	Univariate analysis, *P*	Multivariate analysis, *P*
Visceral metastasis					
Negative (stages I–III)	79	3.9 (1.6)	3.5–4.2	<0.0001	<0.0001
Positive (stage IV)	23	5.9 (1.5)	5.2–6.5		
Patient age, y					
≥50	73	4.4 (1.8)	4.0–4.8	NS[b]	NS
≤49	25	3.7 (1.4)	3.2–4.3		
Estrogen receptor					
Negative	32	4.0 (1.5)	3.4–4.5	NS	NS
Positive	63	4.3 (1.7)	3.8–4.7		
Progesterone receptor					
Negative	55	4.0 (1.5)	3.6–4.4	NS	NS
Positive	40	4.4 (1.9)	3.8–5.0		
Her2					
Negative	69	4.2 (1.7)	3.8–4.6	NS	NS
Positive	26	4.1 (1.6)	3.5–4.7		
Ki67 index					
Low	33	4.5 (1.5)	3.9–5.0	NS	NS
Intermediate–high	59	4.0 (1.7)	3.5–4.4		
p53					
Negative	63	4.1 (1.7)	3.7–4.5	NS	NS
Positive	29	4.2 (1.6)	3.6–4.8		
Histological grade					
1	25	4.3 (1.4)	3.7–4.9	NS	NS
2–3	67	4.0 (1.8)	3.6–4.5		
Lymphovascular invasion					
Negative	53	4.0 (1.5)	3.6–4.4	NS	NS
Positive	39	4.3 (1.9)	3.7–4.9		
Lymph node metastasis					
Negative	41	3.9 (1.5)	3.4–4.4	0.05	NS
Positive	61	4.6 (1.8)	4.1–5.1		
Histological type					
Ductal	78	4.0 (1.6)	3.6–4.3	NS	NS
Lobular	14	4.8 (1.7)	3.9–5.8		

[a] Age could not be obtained for 4 patients, and estrogen receptor/progesterone receptor/Her2 could not be obtained for 7 patients. Data on Ki67 index, p53 status, histological grade, and lymphovascular invasion were not provided for 10 patients.
[b] NS, not significant.

might be due to its direct suppression of maspin, PDCD4, and urokinase plasminogen activator surface receptor *(34)*.

In conclusion, we observed that circulating miR-21 concentrations can distinguish patients with breast cancer from healthy females and further distinguish patients with distant metastases from those with locoregional disease. The concentration of circulating miR-21 may be an important blood biomarker for breast cancer screening and could be used as a biomarker for disease progression. Our findings warrant further studies with a large cohort of patients to validate and develop the serum biomarker as a critical tool for breast cancer care.

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