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# Identification of MicroRNAs as New Blood Biomarkers to Predict Breast Cancer Recurrence

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## Abstract

MicroRNAs (microRNAs) are short sequences of RNA (about 22 nucleotides) that are involved in the regulation of gene expression. Previous studies have suggested that a number of microRNAs are recognized as new biomarkers for cancers. The aim of this study is to identify specific microRNAs in serum, which may serve as potential diagnostic and prognostic biomarkers and therapeutic targets for breast cancer. Quantitative real-time PCR (qRT-PCR) array analyses of microRNAs in sera from four pairs of recurrent and non-recurrent breast cancer patients were performed. Those differentially expressed microRNAs were verified in serum samples from 42 breast cancer patients. The prognostic values of the selected microRNAs were statistically analyzed, determined by the correlation between microRNA expression and tumor parameters. High serum levels of miR-134 and miR-483-5p were found to be associated with some aggressive tumor behaviors. Kaplan-Meier analysis of four up regulated microRNAs (miR-134, miR-483-5p, miR-493-3p and miR-139-3p) indicated that serum level of miR-134 can predict tumor recurrence in breast cancer patients after primary treatment. Identification of new blood biomarkers for prediction of recurrence may have a significant implication for breast cancer follow-up care and treatment.

**Keywords: microRNA; Biomarker; Recurrence; Breast cancer**

## Introduction

Breast cancer is the second most common cancer worldwide after lung cancer, the fifth most common cause of cancer death, and the leading cause of cancer death in women [1]. Recent statistics suggest that about 1.3 million females develop breast cancer each year and about 465,000 of them succumb to the disease [2,3]. Approximately 20% to 30% of early stage breast cancer cases will eventually experience recurrence. Previous studies have shown that the average rates of recurrence were 11% to 30% at 5 years and 20% to 36.8% at 10 years after completion of initial treatments. The 5-year Progression-Free Survival (PFS) and Overall Survival (OS) were found to be 45% and 71% in the local recurrence group and 34% and 58% in the regional recurrence group. Therefore, there is an urgent need to identify novel biomarkers that can predict which patients will develop recurrence, either at diagnosis or before clinical manifestation of recurrence. Many factors that have been shown to be associated with breast cancer recurrence include age, tumor size, locality, lymph node involvement, grade, Estrogen Receptor (ER) status, Progesterone Receptor (PR) status, and Human Epidermal growth factor Receptor 2 (HER2) status [4]. These factors are determined in the primary tumor and are obtained through traditional tumor biopsy. However, a tissue sample cannot be continuously monitored during therapy and follow-up of cancer patients.

MicroRNAs are small (about 21-25 nucleotides in length), non-coding RNA molecules. They regulate gene expression in a wide range of biological and pathological processes at the post-transcriptional level by binding to complementary sequences in the 3' Un Translated Region (UTR) of various target mRNAs, usually resulting in their silencing [5-8]. It has been well documented in the literature that microRNAs can control several key biological processes such as the cell cycle, apoptosis, differentiation, and stem cell regulation and have been found to be deregulated in cancer. MicroRNAs are released into blood circulation as a consequence of cell death or active secretion via exosomes. MicroRNA are easily accessible and reproducible, so they may have a good potential to serve as prognostic and predictive biomarkers for breast cancer.

A major advantage of blood-based biomarkers in the context of cancer recurrence is the fact that they can be monitored repeatedly, even after the primary tumor has been removed. Circulating microRNAs might be an ideal class of biomarkers for blood-based cancer detection because microRNA expression profiles are relatively cancer-specific. In addition, microRNAs are stable in

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**Table 1:** Clinical characteristics of breast cancer patients.

| ID | Age | Grade | Size (cm) | Lymph Node | Last Contact/ Recurrence (Months) | Recurrence | ER Status | PR Status | HER2 Status |
|---|---|---|---|---|---|---|---|---|---|
| 41245 | 72 | 3 | 0.4 | 17 | 46 | 1 | POSITIVE | NEGATIVE | NEGATIVE |
| 41247 | 60 | NA | N/A | N/A | 141 | 0 | N/A | N/A | N/A |
| 41265 | 53 | 2 | 2.6 | 1 | 10 | 1 | NEGATIVE | NEGATIVE | NEGATIVE |
| 41266 | 53 | 3 | 2.1 | 1 | 135 | 1 | POSITIVE | NEGATIVE | N/A |
| 41320 | 53 | 3 | 2.5 | 0 | 45 | 1 | N/A | N/A | NEGATIVE |
| 41326 | 60 | 2 | 1.7 | 1 | 41 | 0 | POSITIVE | NEGATIVE | POSITIVE |
| 41333 | 51 | 3 | 3 | 13 | 41 | 1 | POSITIVE | NEGATIVE | POSITIVE |
| 41334 | 33 | 3 | 1.2 | 1 | 10 | 1 | NEGATIVE | NEGATIVE | POSITIVE |
| 41351 | 47 | 2 | 3 | 1 | 121 | 1 | N/A | N/A | N/A |
| 41354 | 33 | 3 | 1.7 | 6 | 20 | 1 | POSITIVE | NEGATIVE | NEGATIVE |
| 41356 | 49 | 3 | 2.4 | 0 | 30 | 1 | NEGATIVE | NEGATIVE | NEGATIVE |
| 41359 | 36 | 3 | 2.5 | 1 | 6 | 1 | POSITIVE | POSITIVE | POSITIVE |
| 41371 | 42 | 3 | 1.1 | 1 | 76 | 0 | NEGATIVE | NEGATIVE | POSITIVE |
| 41373 | 48 | 3 | 2.1 | 6 | 32 | 1 | POSITIVE | NEGATIVE | NEGATIVE |
| 41375 | 61 | 2 | 0.2 | 98 | 50 | 0 | POSITIVE | NEGATIVE | NEGATIVE |
| 41377 | 59 | 1 | 1.7 | 0 | 27 | 0 | POSITIVE | POSITIVE | N/A |
| 41383 | 63 | 3 | 1.5 | 0 | 53 | 0 | POSITIVE | NEGATIVE | N/A |
| 41389 | 65 | 2 | 1.1 | 2 | 54 | 0 | NEGATIVE | POSITIVE | POSITIVE |
| 41441 | 35 | 3 | 1.4 | 0 | 31 | 0 | POSITIVE | NEGATIVE | NEGATIVE |
| 41446 | 68 | 3 | 2.5 | 13 | 96 | 0 | N/A | N/A | POSITIVE |
| 41450 | 54 | 3 | 8 | 95 | 42 | 1 | POSITIVE | NEGATIVE | NEGATIVE |
| 41460 | 47 | 1 | 0.9 | 1 | 38 | 0 | POSITIVE | POSITIVE | N/A |
| 41460 | 47 | 1 | 0.9 | 1 | 38 | 0 | POSITIVE | POSITIVE | N/A |
| 41469 | 38 | 3 | 0.4 | 0 | 16 | 1 | POSITIVE | POSITIVE | NEGATIVE |
| 41473 | 61 | 3 | 2 | 0 | 55 | 0 | POSITIVE | POSITIVE | N/A |
| 41475 | 57 | 3 | 3 | 23 | 20 | 1 | NEGATIVE | NEGATIVE | POSITIVE |
| 41476 | 56 | 2 | 0.3 | 1 | 63 | 1 | POSITIVE | POSITIVE | NEGATIVE |
| 41477 | 30 | 2 | 3 | 8 | 34 | 1 | POSITIVE | POSITIVE | N/A |
| 41481 | 31 | 3 | 2.5 | 0 | 25 | 0 | POSITIVE | POSITIVE | POSITIVE |
| 41481 | 31 | 3 | 2.5 | 0 | 25 | 0 | POSITIVE | POSITIVE | POSITIVE |
| 41489 | 64 | 2 | 1.2 | 2 | 40 | 0 | POSITIVE | POSITIVE | NEGATIVE |
| 41503 | 61 | N/A | N/A | N/A | 242 | 1 | N/A | N/A | N/A |
| 41509 | 37 | 1 | 2.2 | 2 | 180 | 0 | POSITIVE | POSITIVE | N/A |
| 41510 | 63 | 1 | 1.4 | 1 | 36 | 0 | POSITIVE | POSITIVE | NEGATIVE |
| 41525 | 53 | 1 | 1.1 | 0 | 34 | 0 | POSITIVE | POSITIVE | NEGATIVE |
| 41545 | 61 | 3 | 2.3 | 4 | 48 | 1 | POSITIVE | POSITIVE | NEGATIVE |
| 41552 | 60 | 3 | 9.5 | 8 | 101 | 0 | POSITIVE | NEGATIVE | POSITIVE |
| 41560 | 34 | 3 | 4.3 | 0 | 11 | 1 | NEGATIVE | NEGATIVE | NEGATIVE |
| 41561 | 53 | 3 | 5 | 95 | 23 | 1 | POSITIVE | POSITIVE | NEGATIVE |
| 41612 | 71 | 2 | 1.1 | 0 | 36 | 0 | POSITIVE | POSITIVE | NEGATIVE |
| 41625 | 54 | N/A | N/A | N/A | 268 | 1 | N/A | N/A | N/A |
| 41626 | 67 | 3 | 1.4 | 0 | 35 | 0 | NEGATIVE | NEGATIVE | NEGATIVE |

cell-free plasma or serum, can be uniformly amplified and quantified, and thus are readily detected by quantitative real-time PCR. MicroRNAs may also play a pathogenic role in the disease process by acting as oncogenes or tumor suppressor genes. In recent years blood-based biomarkers such as microRNAs have been investigated for the diagnosis and prognosis of breast cancer [9-14]. In this study, we aimed to investigate if microRNA can be a biomarker for predicting breast cancer recurrence during the follow-up of breast cancer patients after initial treatment.

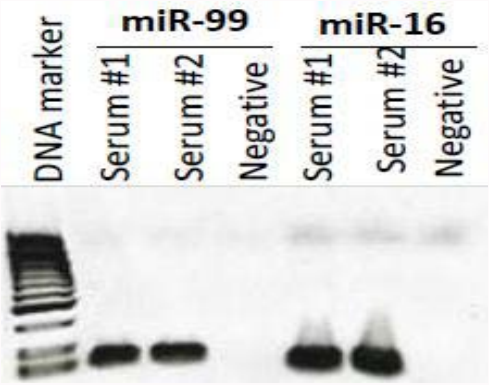


**Figure 1:** Representative image of PCR amplification of miR-99 and miR-16 from sera of two breast cancer patients. RNA was extracted from two individual patients' samples. These experiments were repeated once and similar results were obtained.

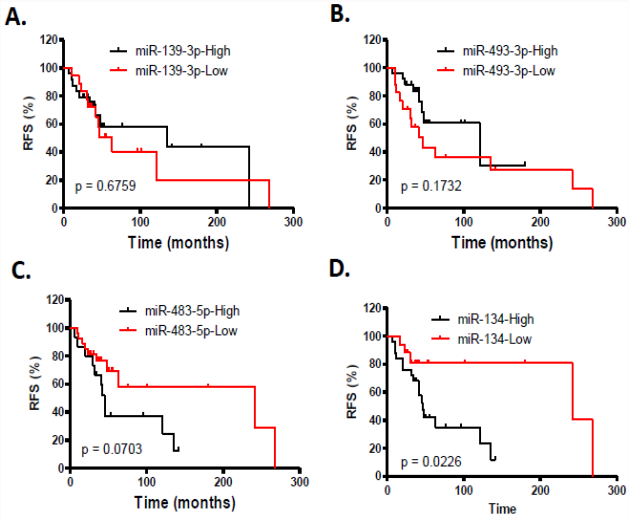


**Figure 2:** Kaplan–Meier analysis of recurrence-free survival (RFS) for breast cancer patients. The patient groups are stratified based on the expression levels of miR-139-3p (**A**) miR-493-3p, (**B**) miR-483-5p, (**C**) and miR-134 (**D**) in patients' sera.

## Materials and Methods

### Serum samples

Forty two breast cancer patient serum samples were provided by the South Carolina Cancer Center tissue bank with the approved IRB protocol. All subjects were females breast cancer patients (Table 1). For each patient, blood samples were collected during follow-up visits after initial therapy of the primary breast cancer. Recurrent patients' samples were taken before diagnosis of the recurrence and the recurrence did not occur within the one year after initial therapy. Non-recurrent patients were included for plasma samples which had been taken after at least 1 year of follow-up after therapy and at least 12 months of known, recurrence-free follow-up after sample collection. The Clinicopathologic characteristics of patients are summarized in Table 2.

### RNA isolation

RNA from serum samples was isolated by Trizol LS reagent using manufacturer protocol. Briefly, 250 uls of serum samples was used for RNA isolation. 750 uls of Trizol reagent was added to serum,

**Table 2:** Clinicopathologic characteristics of 42 breast cancer patients.

| Characteristics | No. of Patients | Percentage |
|---|---|---|
| **Recurrence** | | |
| Yes | 21 | 50.00% |
| No | 21 | 50.00% |
| **Age at Diagnosis** | | |
| < 55 | 24 | 57.10% |
| > 55 | 18 | 42.90% |
| **Tumor Grade** | | |
| I/II | 15 | 35.70% |
| III | 24 | 57.10% |
| NA | 3 | 7.10% |
| **Best AJCC Staging** | | |
| 0/1 | 4 | 9.50% |
| 2A | 3 | 7.10% |
| 2B | 32 | 76.20% |
| 3 | 1 | 2.40% |
| NA | 2 | 4.80% |
| **Tumor Size (cm)** | | |
| < 2.0 | 20 | 47.60% |
| ≥2.0 | 19 | 45.20% |
| Unknown | 3 | 7.10% |
| **Lymph Node Status** | | |
| Negative | 13 | 31.00% |
| Positive | 26 | 61.90% |
| Unknown | 3 | 7.10% |
| **ER Status** | | |
| Negative | 8 | 19.00% |
| Positive | 28 | 66.70% |
| Unknown | 6 | 14.30% |
| **PR Status** | | |
| Negative | 18 | 42.90% |
| Positive | 18 | 42.90% |
| Unknown | 6 | 14.30% |
| **HER2 Status** | | |
| Negative | 19 | 45.20% |
| Positive | 11 | 26.20% |
| Unknown | 12 | 28.50% |

vortexed and incubated for 3 min to 5 min at room temperature. 200 uls of chloroform was then added vortexed and incubated at room temperature for 3 min to 5 min. Samples were then centrifuged at 4°C for 15 min at 12000 g. Aqueous phase was collected in fresh tube. To precipitate RNA, the samples were treated with 500 uls isopropyl alcohol and 5 uls glycogen (5 ng/ul) overnight at -20°C. Following day, samples were centrifuged at 12000 g, 4°C for 10 min. Precipitated RNA was then washed twice with 75% Ethanol and centrifuged at 7500 g, 4C for 5 min. Finally, precipitated RNA was air-dried before re suspension with water.

### Real-time PCR analysis of microRNA expression

For microRNA expression analysis, cDNA was synthesized

**Table 3:** Real-time PCR analysis of microRNA expression in serum samples from 4 pairs of recurrent and non-recurrent breast cancer patients.

| | Non-recurrent Patients (ΔCt) | | | | Recurrent patients (ΔCt)[a] | | | | | T-test |
|---|---|---|---|---|---|---|---|---|---|---|
| **miR Name** | No. 375 | No. 377 | No. 460 | No. 481 | No.265 | No. 333 | No. 245 | No. 266 | **Fold change** | (p value) |
| miR-146a-5p | 9.2 | 7.4 | 7.9 | 9.2 | 24.2 | 24.4 | 24.9 | 25 | 0 | 0 |
| miR-139-3p | 14.3 | 13.2 | 15.6 | 15.9 | 10.2 | 12 | 10.4 | 9.1 | **20.45** | **0.008** |
| miR-134 | 11.3 | 17.3 | 14 | 15.2 | 7.1 | 10.7 | 11 | 8.9 | **33.54** | **0.017** |
| miR-493-3p | 16.3 | 14.2 | 15.7 | 14.5 | 12.8 | 13.3 | 9.8 | 7.8 | **19.22** | **0.021** |
| miR-483-5p | 10 | 9.9 | 10.3 | 11.8 | 7.8 | 9.6 | 8.5 | 8.9 | **3.39** | **0.023** |
| miR-141 | 24.8 | 13.5 | 16 | 15.2 | 25 | 24.4 | 24.9 | 25.1 | 0.01 | 0.026 |
| miR-200a | 18.2 | 25 | 16.1 | 16.9 | 24.3 | 24.7 | 24.8 | 23.9 | 0.02 | 0.026 |
| miR-138-5p | 17.5 | 17 | 22.2 | 18.8 | 13 | 14.5 | 18.3 | 13.6 | 16.41 | 0.052 |
| miR-181b-5p | 12.4 | 11.5 | 12.5 | 14.7 | 13.9 | 15.1 | 24.7 | 24.9 | 0.01 | 0.067 |
| miR-424-5p | 6.3 | 4.8 | 6.3 | 7.1 | 9.2 | 6.1 | 7.5 | 9.8 | 0.25 | 0.087 |
| miR-199b-5p | 13.3 | 25 | 12.8 | 15.2 | 25 | 14.5 | 23.6 | 24.6 | 0.02 | 0.184 |
| miR-324-3p | 8.1 | 8.7 | 7.3 | 8.6 | 6.8 | 8.8 | 6.9 | 5.9 | 2.05 | 0.187 |
| miR-200b-3p | 14.7 | 13.4 | 14.4 | 14.7 | 6.9 | 25.3 | 24.6 | 24.7 | 0.01 | 0.222 |
| miR-182-5p | 13.7 | 25 | 13.9 | 14.7 | 24.3 | 11.7 | 25.3 | 24.6 | 0.03 | 0.303 |
| miR-142-3p | 9.2 | 9.3 | 10.5 | 12.6 | 14.4 | 10.7 | 10.3 | 10.9 | 0.44 | 0.376 |
| miR-188-5p | 12.9 | 24.8 | 14 | 13.5 | 15.3 | 13.3 | 12.3 | 15.2 | 5.04 | 0.463 |
| miR-105-5p | 24.6 | 24.7 | 24.8 | 20 | 11.2 | 24.5 | 24.3 | 24.7 | 4.53 | 0.574 |
| miR-127-3p | 12.6 | 16.6 | 17.2 | 15.1 | 12.7 | 10.2 | 25 | 6.4 | 3.49 | 0.679 |
| miR-486-5p | -1.2 | -1.9 | -0.4 | -0.3 | 0.5 | -0.6 | -0.5 | -2.6 | 0.89 | 0.819 |

[a]ΔCt value was calculated by subtracting miR-16 Ct value from the Ct values of the microRNAs of interest.

as per manufacturer's recommendation. Purified RNA was first polyadenylated using Poly A polymerase (NEB # M0276). cDNA was then prepared using M-MLV Reverase Transcription kit (Invitrogen # 28025013) and poly dT adapter primer. Qiagen Sybr green enzyme (Qiagen # 204141) was used to quantify microRNA expression. Real-Time PCR (ABI7900) instrument under the following conditions: hold at 95°C for 10 mins, then 45 cycles of 95°C for 15 seconds and 60°C for 1 min. The relative expression level of each microRNA expression was normalized using miR-16-5p.

### Statistical analysis

All results were confirmed in at least three independent experiments, and all quantitative data are presented as mean ± SD or SEM as indicated. Student's t test or one-way ANOVA test was employed for analyzing quantitative variables. The patients were stratified based on the mean values of microRNA expression levels. The association between microRNA expression and the clinicopathologic parameters of the breast cancer patients was evaluated by the Fisher extract or Chi-square test. Survival curves were evaluated using Kaplan-Meier method and the differences between those survival curves were tested by log-rank test.

## Results

### MicroRNAs are differentially expressed in the serum of recurrent and non-recurrent breast cancer patients

To determine whether the microRNA profiles of recurrent and non-recurrent breast cancer serum samples may differ, total RNA was isolated from four pairs of recurrent and non-recurrent patient serum and converted into cDNA for microRNA screening. The cDNA quality was first examined by PCR analysis of expression of two high abundant microRNAs: miR99 and miR16. As shown in Figure1, two microRNA could be specifically amplified from two serum cDNAs. We used miR-16 as internal control for our analysis [15]. In our previous studies, we did RT-PCR array analysis of microRNA expression in breast cancer cells and studied the role of miR-489 in breast cancer cells [16-18]. In these studies, we screened about 350 microRNAs by RT-PCR analysis in breast cancer cells and found that about 60 of them are commonly differentially expressed in breast cancer cells [16]. Most these differentially expressed miRNAs are not necessarily secreted. We then compared our list with the exosomal microRNAs derived from breast cancer cells and circulating microRNA found in cancer sera samples [19-21]. And selected 22 cancer related microRNAs in these four recurrent and four non-recurrent patient samples. The expression of three microRNAs (miR-96-3p, miR-340-5p and miR-373-3p) was not detectable in the any of the serum samples. Seven out of 22 selected microRNAs were differently expressed in recurrent and non-recurrent samples based on the p value cutoff of 0.05 (Table 3). Three microRNAs (miR-146, miR-200a and miR141) were down regulated and four microRNAs (miR-134, miR-139-3p, miR-483-5p and miR-493-3p) were up regulated in recurrent samples compare to non-recurrent samples. We reasoned that the up regulated microRNA may be secreted by the recurrent tumor cells and potentially serve as prognostic biomarkers.

### Correlation of serum microRNA levels with clinical characteristics

We decided to examine the expression levels of four up regulated microRNAs including miR-134, miR-483-5p, miR-493 and miR-139-3p in the sera of 42 breast cancer patients (Table 2,4). Based on the expression levels of each microRNA, we stratify the patients into two groups and performed Chi-square test to analyze the associations of this microRNA with various primary tumor parameters, including tumor grade, tumor size, nodal status, ER status, PR status and HER2 status. We found that tumors with high levels of miR-134 were more

**Table 4:** The association of miR-134, miR-483-5p, miR-139-3p and miR-493-3p with clinical parameters.

| | miR-134 | | Chi- | miR-483-5p | | Chi- | miR-139-3p | | Chi- | miR-493-3p | | Chi- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Characteristics** | **Low n(%)** | **High n(%)** | **square (p value)** | **Low n(%)** | **High n(%)** | **square (p value)** | **Low n(%)** | **High n(%)** | **square (p value)** | **Low n(%)** | **High n(%)** | **square (p value)** |
| **HER2 Status** | | | 0.4473 | | | 0.4786 | | | 0.2557 | | | 0.2789 |
| Negative | 6 (54.5) | 13 (66.7) | | 12 (60.0) | 8 (72.7) | | 11 (73.3) | 8 (53.3) | | 9 (75.0) | 10 (55.6) | |
| Positive | 5 (45.5) | 6 (31.6) | | 8 (40.0) | 3 (27.3) | | 4 (26.7) | 7 (46.7) | | 3 (25.0) | 8 (44.4) | |
| **ER Status** | | | 0.3609 | | | 0.8423 | | | 0.4648 | | | 0.1203 |
| Negative | 2 (14.3) | 6 (27.3) | | 6 (23.1) | 2 (20.0) | | 4 (28.6) | 4 (18.2) | | 5 (35.7) | 3 (13.6) | |
| Positive | 12(85.7) | 16 (63.7) | | 20 (76.9) | 8 (80.0) | | 10 (71.4) | 18 (81.8) | | 9 (64.3) | 19 (86.4) | |
| **PR Status** | | | 0.1715 | | | **0.0256** | | | 0.4941 | | | |
| Negative | 5 (35.7) | 13 (59.1) | | 10 (38.5) | 8 (80.0) | | 8 (57.1) | 10 (45.5) | | 10 (71.4) | 8 (36.4) | **0.0402** |
| Positive | 9 (64.2) | 9 (40.9) | | 16 (61.5) | 2 (20.0) | | 6 (42.9) | 12 (54.5) | | 4(28.6) | 14 (63.6) | |
| **Tumor Size** | | | 0.305 | | | **0.0436** | | | 0.749 | | | 0.2718 |
| < 2 cm | 8 (61.5) | 11 (44.0) | | 15 (62.5) | 4 (28.6) | | 9 (52.9) | 11 (47.8) | | 9 (64.3) | 11(45.8) | |
| ≥ 2 cm | 5 (39.8) | 14 (56.0) | | 9 (37.5) | 10 (71.4) | | 8 (47.1) | 12 (52.2) | | 5 (36.7) | 13 (54.2) | |
| **Lymph Status** | | | **0.0362** | | | 0.6369 | | | 0.7401 | | | 0.6369 |
| Negative | 8 (53.3) | 5 (20.8) | | 9 (36.0) | 4 (28.6) | | 5 (29.4) | 8 (36.4) | | 4 (28.5) | 9 (36.0) | |
| Positive | 7 (46.7) | 19 (79.2) | | 16 (64.0) | 10 (71.4) | | 12 (70.6) | 14 (63.6) | | 10 (71.5) | 16 (64.0) | |
| **Tumor Grade** | | | 0.6279 | | | 0.1018 | | | 0.3072 | | | 0.3421 |
| I/II | 6 (42.9) | 9 (36.0) | | 12 (48.0) | 3 (21.4) | | 5 (29.4) | 10 (45.5) | | 4 (28.5) | 11 (44.0) | |
| III | 8 (57.1) | 16 (64.0) | | 13 (52.0) | 11 (78.6) | | 12 (70.6) | 12 (54.5) | | 10 (61.5) | 14 (66.0) | |

likely to spread into lymph node (p=0.0362) (Table 4). The miR-483-5p expression was positively associated with tumor size (p=0.0436) but negatively associated with PR expression status (p=0.0256) (Table 4). High levels of miR-493 expression were weakly associated with positive PR status. Other microRNA did not showed significant association with the primary parameters, likely due to the limited sample size.

### miR-134 expression in patient serum predicts breast cancer recurrence

To evaluate if any of these microRNAs can predict breast cancer recurrence, we performed a Kaplan-Meier analysis (Figure 2A-2D). High expression of miR-134 and miR-483-5p in patient serum tend to be associated with high risk of impending breast cancer recurrence (Figure 2C,2D). The association of recurrence and miR-134 remained significant (P=0.0226) (Figure 2D). Taken together, these observations suggested that selected microRNA can be very promising biomarkers to predict the breast cancer recurrence.

## Discussion

Circulating miRNAs are an emerging field of cancer biomarker research [10,11,15,22,23]. Global and focused screening approaches have been used to identify circulating miRNAs, which can serve as the biomarker for predict cancer progression. In this study, we demonstrated that circulating miRNA can be used to predict breast cancer recurrence. By screening circulating miRNA expression profiles in sera samples from patients after initial treatment, we identified four miRNAs (miR-134, miR-483-5p, miR-493-3p and miR-139-3p) that were consistently up regulated in recurrent patients compared to non-recurrent patients. Further statistical analysis revealed that miR-134 was directly correlated with recurrence. High miR-134 expression was associated with lymph node metastasis and predicted higher recurrence rate. miR-134 has been previously reported to be present in serum and reported to be secreted through exosomes [24]. Previous study has found increased miR-134 in serum of pancreatic cancer. Another study showed that miR-134 might be an emerging diagnostic biomarker in early stage of acute myocardial infarction because its expression was markedly increased in patients' plasma. In another study, miR-134 expression was down regulated in patients' tissue and associated with the size of tumor and terminal pathologic grade and advancement of tumor development in gastrointestinal stromal cancer. Decreased miR-134 expression contributed to lower total survival as well [25]. miR-134 expression in serum of breast cancer patients has not been reported yet. We found that it was highly expressed in serum of recurrent patients when compared to non-recurrent patients. In contrast, miR-134 has been previously shown as tumor suppressor microRNA in breast cancer and shown to suppress proliferation, migration and invasion [26]. It is possible that cancer cells may expel miR-134 to prevent its growth suppressive effect. Further study comparing miR-134 expression in cancerous tissue and serum of same patient side by side might reveal more insight on this complex observation. Nonetheless, our study shows that miR-134 can be used as a potential biomarker to predict recurrence in breast cancer patient. MiR-483-5p has been shown to be dys regulated in adrenocortical carcinoma is a diagnostic and prognostic marker of ACC [27]. It was highly expressed in adrenocortical tumor tissue as well as in serum of malignant tumors compared to benign. Consistent with this result, our study also found strong positive correlation of miR-483-5p with tumor size. Another study also reported increased serum miR-483-5p levels in oral squamous cell carcinoma. miR-483-5p has been shown largely to function as oncogenic microRNA [28]. Interestingly, consistent with our result recent study comparing serum level of miR-483-5p showed increased expression level in serum of breast cancer patients when compared to serum of healthy individuals [29]. Several other microRNAs including miR-139-3p and miR-483-5p were observed differentially present in serum of breast cancer patients. Consistently,

miR-139-3p has been shown to present in serum. Interestingly, study exploring miR-139-3p in breast cancer found that its down regulation in cancer tissue is indicative of aggressive cancer. However, study could not find significant difference in serum level of miR-139-3p [30]. Our study did detect miR-139 in the breast cancer patient serum but seemed not to be associated any breast cancer pathological parameters including recurrence. The major pitfall of this study is the limited sample size; we suggest expanding this study in a large cohort of patients to further confirm the potential application of selected microRNAs a diagnostic marker. Increasing ample size can also help minimizing the problems associated with a relatively large variation among samples which was generated at least partially due to data normalization using miR-16 as the internal control. The common issue for quantification of circulating miRNA is the lack of a solid and common normalization strategy to account for inter-individual or intergroup variability [31]. Most of the results were obtained by qPCR, using different reference genes for normalization such as miR-16 [15,19,32], miR-1207 [33], miR-484 [20,34], and miR-24 [32]. MiR-16 is most frequently used as endogenous control for data normalization of circulating miRNAs [15], because this miRNA is highly expressed in plasma or serum, and has been described as being relatively invariant across diverse blood samples of breast cancer patients [15,19,21,35]. Our data also showed that miR-16 is most stably expressed in our samples. We therefore selected miR-16 as the internal control for our qRT-PCR analysis. Further studies with a large samples size and different normalization methods may help to clarify this issue. In summary, this study identified microRNA with differential serum levels in recurrent and non-recurrent breast cancer patients. Assessing their levels in serum of breast cancer patient might help predicting patients who are predisposed to recurrence and who require more aggressive therapy and closely monitoring after primary treatment.

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