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A novel panel of serum miR-21/miR-155/miR-365 as a potential diagnostic biomarker for breast cancer

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Purpose: Insufficient sensitivity and specificity prevent the use of most existing biomarkers for early detection of breast cancer. Recently, it was reported that serum microRNAs (miRNAs) may be potential biomarkers in many cancer diseases. In this study, we investigated whether serum levels of 5 miRNAs including miR-21, miR-125b, miR-145, miR-155, and miR-365 could discriminate breast cancer patients and healthy controls.
Methods: Serum levels of miRNAs were measured by using quantitative real-time polymerase chain reaction in 99 breast cancer patients and 21 healthy controls. The abundance change of serum miRNAs were also evaluated following surgical resection in 20 breast cancer patients. Receiver operating characteristic (ROC) curve analysis was performed to assess the sensitivity and specificity of miRNAs as diagnostic biomarkers.
Results: Serum levels of miR-21 and miR-155 was significantly higher, while miR-365 was significantly lower in breast cancer as compared with healthy controls. The serum levels of miR-21 and miR-155 significantly decreased following surgical resection. Additionally, the serum level of miR-155 at stages I and II was significantly higher compared to stage III. The serum miR-145 level was remarkably higher in progesterone receptor (PR)-positive patients than PR-negative. The positivity of miR-21, miR-155, and miR-365 was high compared to CA 153 and CEA in breast cancer. ROC curve analyses of a combination of miR-21, miR-155, and miR-365 yielded much higher area under curve and enhanced sensitivity and specificity in comparison to each miRNA alone.
Conclusion: The combination of serum miR-21/miR-155/miR-365 may potentially serve as a sensitive and specific biomarker that enables differentiation of breast cancer from healthy controls.
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Key Words: Serum, MicroRNAs, miRNA-21, miRNA-155, miRNA-365, Breast neoplasms

INTRODUCTION

Breast cancer, one of the most common types of female cancers, is a highly heterogeneous disease that has multiple subtypes with distinct clinical outcomes and a high fatality rate globally [1]. In clinic, the status/expression level of hormone receptor including estrogen receptor (ER), progesterone receptor (PR), and human EGF-like receptor 2 (HER2) as well as tumor grade are often used for classification and target therapy indicators of breast cancers [1,2]. Although current early detection and target therapies based on the measurement of hormone receptors have remarkably reduced the rate of mortality from breast cancer, their application is still limited due to insufficient sensitivity and specificity as well as the invasive, unpleasant and inconvenient nature of diagnostic procedures. To identify unique therapeutic targets, it is necessary to develop predictive and prognostic biomarkers that can be conveniently and reliably used in clinic.

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MicroRNAs (miRNAs), a class of naturally occurring, small and noncoding RNA molecules with a length of 19–25 nucleotides, can specifically regulate gene mRNA expressions at post-transcriptional levels [3]. The dysregulation of miRNAs may affect some crucial biological processes of cells leading to tumor development by increasing proliferation, decreasing apoptosis, and enhancing the metastatic potential [3,4]. Recently, it has been reported that miRNAs expression level/profile is altered in various cancers exhibiting great potential in improving the diagnosis and prognosis of cancer [5]. Notably, it has been documented that certain secreted miRNAs originating from cancer tissues are protected from endogenous RNase by some unknown mechanisms [6], and thus can be detected in blood and other body fluids [7,8]. In breast cancer tissues, miRNAs have been shown to function as either oncogenes or tumor suppressors [9], and circulating miRNAs may correlate with disease progression, therapeutic responses and patient survival [10,11], suggesting that the evaluation of miRNAs levels in the blood, either serum or plasma, may be used as non-invasive blood-based biomarkers. Even though many studies have compared changes of miRNAs levels in blood between breast cancer patients and healthy controls in order to map the profiling of miRNAs and identify some specific miRNAs as potential biomarkers in breast cancer [12], the results are not always consistent due to the differences in study design, such as sample size, patient source and characteristics, and RNA preparation, as well as detection methods or profiling platforms that were used.

In our study, the serum levels of 5 miRNAs including miR-21, miR-155, miR-125b, miR-145, and miR-365 that have been indicated as a recurrent presence in breast cancer patients [12,13], were compared between breast cancer patients and healthy controls. We found that the serum level of miR-21 was significantly higher, while miR-155 and miR-365 was significantly lower in breast cancer than healthy control, and receiver operating curve (ROC) analyses showed that the combination of miR-21/miR-155/miR-365 led to higher sensitivity and specificity in distinguishing breast cancer from healthy controls, indicating a potential as biomarkers for the diagnosis and monitoring of breast cancer.

METHODS

Patients

Blood samples were collected from 99 patients with breast cancer (range, 31–77 years; mean, 48.95 years) and 21 age-matched healthy female volunteers (range, 35–59 years; mean, 45.38 years; without current or previous malignancy or inflammatory condition). In addition, the paired blood samples were collected from a 20-patient subset both before and 3 weeks after breast cancer surgery. All participants had signed an informed consent to participate in this study. The study was approved by the Ethics Review Board of Harbin Medical University.

All patients' breast cancer was histologically confirmed. The surgical patients' clinicopathological and relevant demographic characteristics were documented in our prospectively maintained breast cancer database. The clinical stage of breast cancer of all patients was classified according to the TNM classification system of the American Joint Committee on Cancer. The number of various groups' breast cancer patients is summarized in Table 1.

Serum preparation

A 5-mL sample of the whole blood was collected in a Vacutainer Serum Separator Tube (Becton Dickinson, Franklin Lakes, NJ, USA). The blood was left to clot at room temperature for 30 minutes, and then centrifuged at 2,000 rpm for 10 minutes at 4°C. The resulting serum was collected, aliquoted, and stored at –80°C.

RNA extraction

Total RNA was extracted from 300 μL of serum using the mirVana PARIS Kit (Ambion, Carlsbad, CA, USA), and finally eluted into 100 μL of preheated (95°C) Elution Solution according to the manufacturer's protocol. The eluate was then collected and stored at –20°C. RNA concentration and integrity were determined using NanoDrop spectrophotometry

Table 1. The number of patients with breast cancer in various groups (n = 99)

Variable	No. of patients
Family history	
Positive	14
Negative	85
TNM	
Stage I	49
Stage II	36
Stage III	14
miR-21, miR-145, miR-365	99
miR-155	49
miR-125b	50
Estrogen receptor	
Positive	48
Negative	42
Progesterone receptor	
Positive	65
Negative	26
Human epidermal growth factor receptor 2	
Positive	26
Negative	65
p53	
Positive	21
Negative	65

(NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). The RNA concentration (μg/mL) was calculated by the value of OD260 (OD260 × dilution factor × 40), and adjusted to 0.002 μg/μL. The total RNA was stored at –80°C.

RT and real-time qPCR

The level of miRNAs was quantified in duplicate using quantitative reverse transcription polymerase chain reaction (RT-PCR) and human TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). The RT reaction was carried out in a 15-μL TaqMan MicroRNA Reverse Transcription System containing 5 μL of RNA extract, 0.15 μL of 100mM dNTPs, 1 μL of Multiscribe Reverse Transcriptase (50 U/μL), 1.5 μL of 10 × RT buffer, 0.19 μL of RNase inhibitor (20 U/μL), 1 μL of gene-specific primer and 4.16 μL of nuclease-free water. For cDNA synthesis, the above mixtures were incubated at 16°C for 30

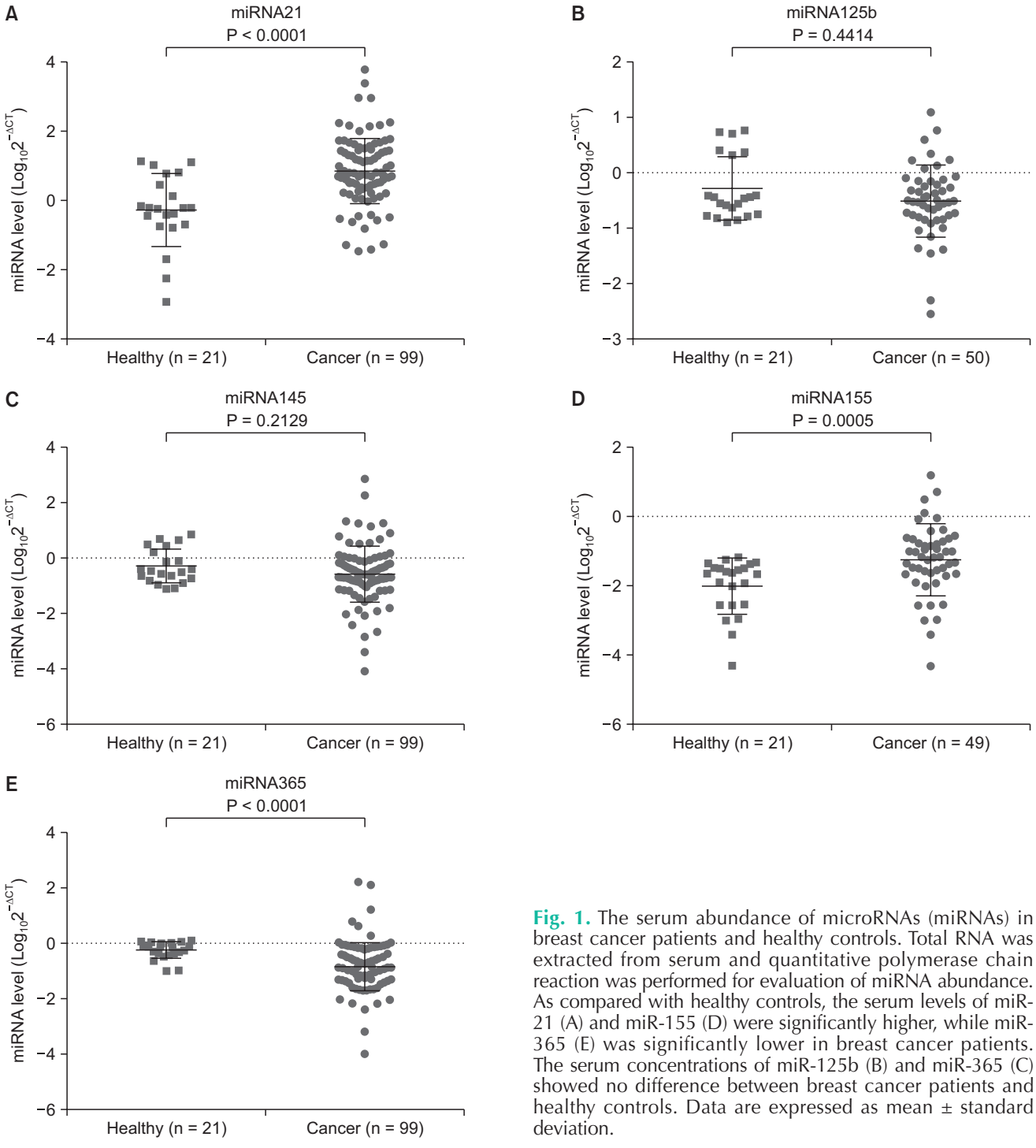

Fig. 1. The serum abundance of microRNAs (miRNAs) in breast cancer patients and healthy controls. Total RNA was extracted from serum and quantitative polymerase chain reaction was performed for evaluation of miRNA abundance. As compared with healthy controls, the serum levels of miR-21 (A) and miR-155 (D) were significantly higher, while miR-365 (E) was significantly lower in breast cancer patients. The serum concentrations of miR-125b (B) and miR-365 (C) showed no difference between breast cancer patients and healthy controls. Data are expressed as mean ± standard deviation.

minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

A 4-μL cDNA solution was amplified using 10 μL of TaqMan Gene Expression Master Mix, 1 μL of gene-specific primers/probe and 5 μL of nuclease-free water in a final volume of 20 μL. Quantitative PCR was performed on a 7000 Real-Time PCR system (Applied Biosystems) by incubation at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The values of cycle threshold (Ct) were calculated with SDS 1.4 software (Applied Biosystems).

Measurement of hormone status, p53, CA 153, and CEA

The levels of CA 153 and CEA were measured through electrochemiluminescence assays, and the level of hormone status including ER, PR, HER2, and p53 was measured by immunohistochemistry assay in the Laboratory Department of the

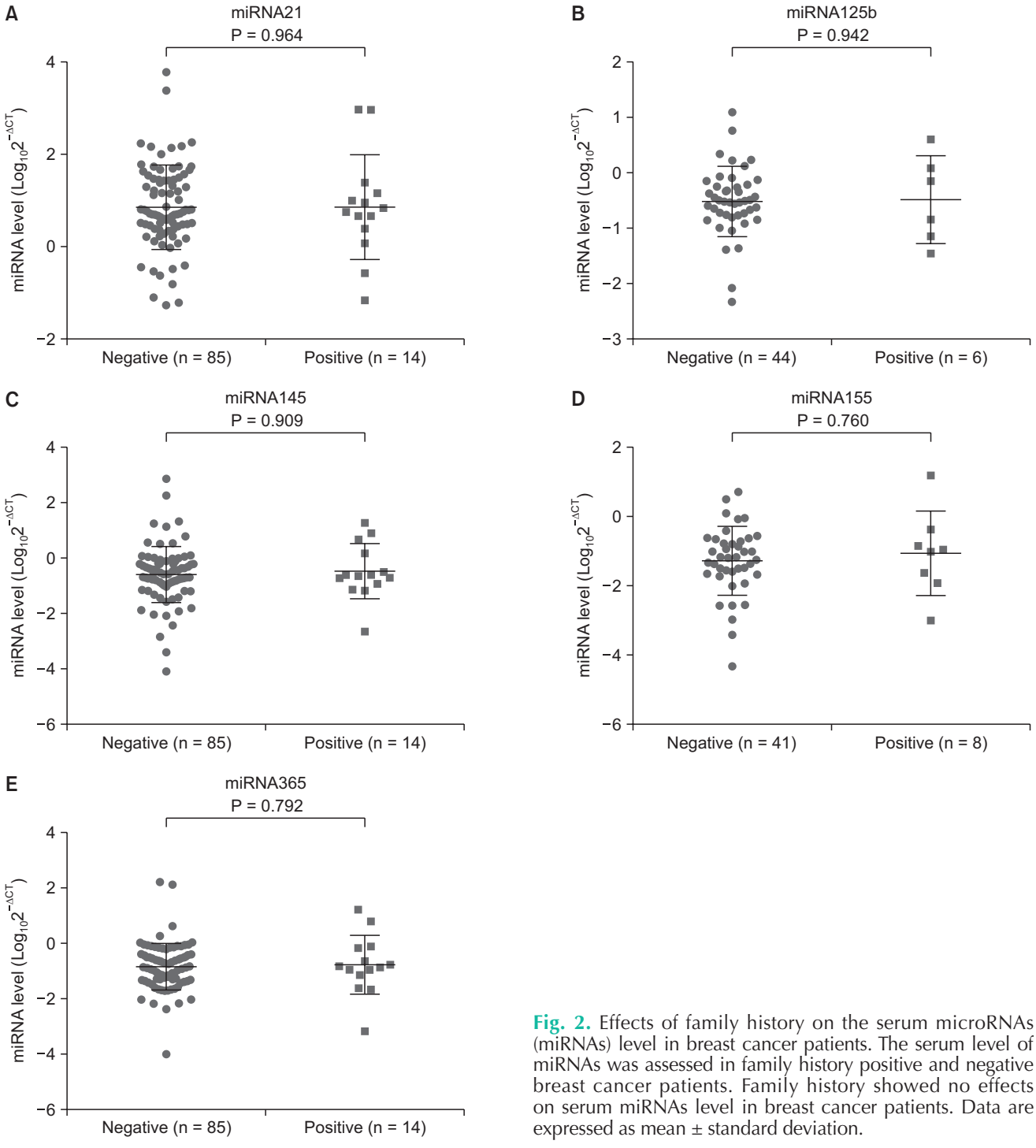

Fig. 2. Effects of family history on the serum microRNAs (miRNAs) level in breast cancer patients. The serum level of miRNAs was assessed in family history positive and negative breast cancer patients. Family history showed no effects on serum miRNAs level in breast cancer patients. Data are expressed as mean ± standard deviation.

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Statistical analysis

The unpaired t-test, a two-tailed Mann-Whitney test, was used to compare the differential expression of serum miRNAs between breast cancer and normal samples, between family history positive and negative patients, and between the patients with ER (or PR, HER2, p53) positive and negative. The paired t-test was used for comparison of serum miRNAs level between pre- and postoperative samples in breast cancer. The one-way

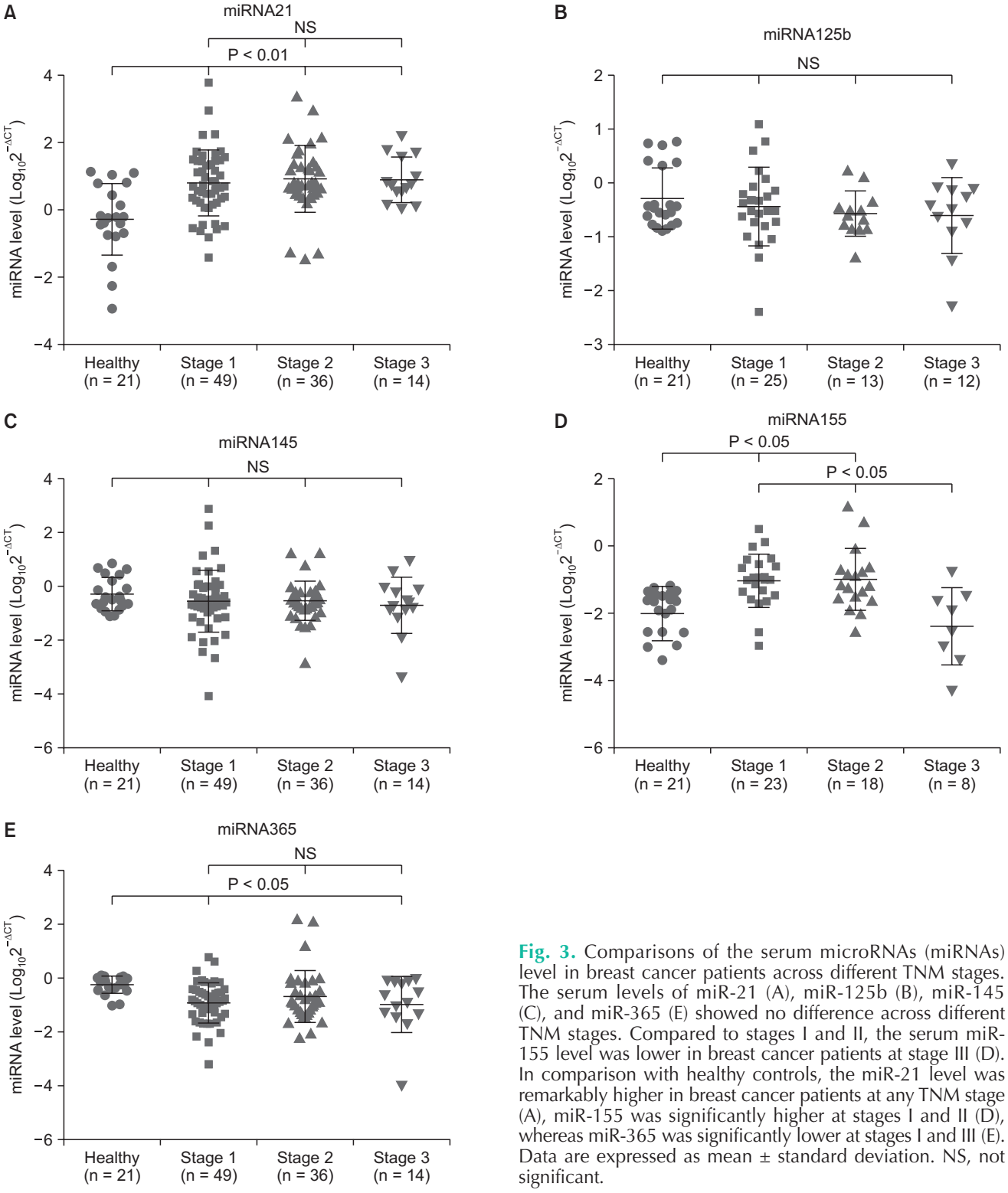

Fig. 3. Comparisons of the serum microRNAs (miRNAs) level in breast cancer patients across different TNM stages. The serum levels of miR-21 (A), miR-125b (B), miR-145 (C), and miR-365 (E) showed no difference across different TNM stages. Compared to stages I and II, the serum miR-155 level was lower in breast cancer patients at stage III (D). In comparison with healthy controls, the miR-21 level was remarkably higher in breast cancer patients at any TNM stage (A), miR-155 was significantly higher at stages I and II (D), whereas miR-365 was significantly lower at stages I and III (E). Data are expressed as mean ± standard deviation. NS, not significant.

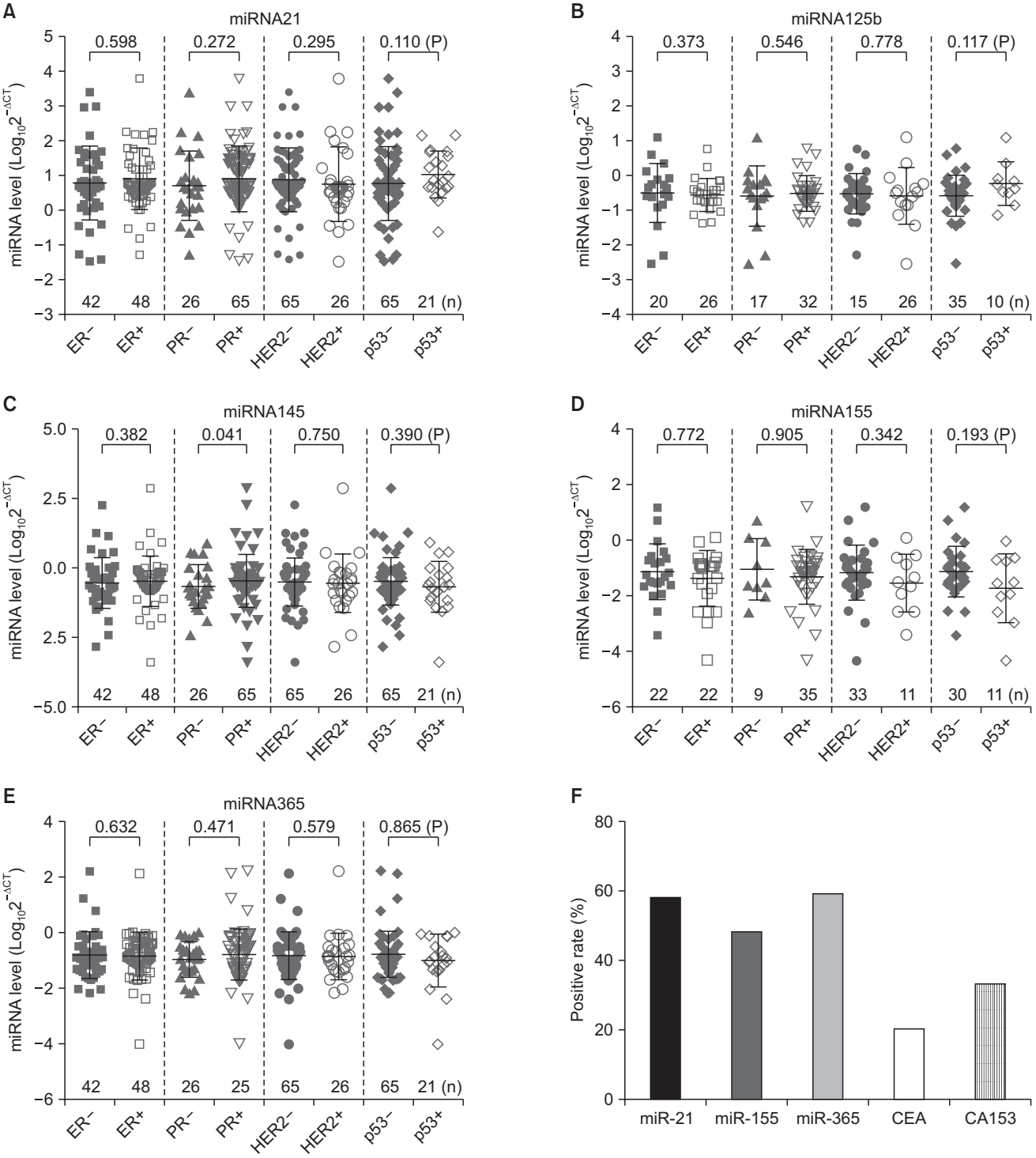

Fig. 4. Comparisons of the serum miRNAs levels in breast cancer patients with different hormone status. (A-E) The serum levels of miRNAs were compared in various groups of breast cancer patients classified by hormone status including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) as well as p53. The serum level of miR-145 was significantly higher in PR-positive patients as compared with PR negative. The number for each group was indicated in the images. Data are expressed as mean ± standard deviation. (F) The positive rates of miR-21, miR-155, and miR-365 as well as CEA and CA153 in breast cancer patients. Positivity of the three miRNAs was determined based on the confidence intervals (CI). The value of miR-21 and miR-155 level ≥ the CI, and the value of miR-365 level ≤ the CI, was considered positive.

analysis of variance was used to compare the differential serum miRNAs level between normal and breast cancer patients at different TNM stages. ROCs were generated using logistic regression models, to evaluate the diagnostic performance of various miRNAs and a combination of miRNAs. Area under curve (AUC) was used as the evaluation criteria; the higher AUC, the better diagnostic performance.

All analysis was performed using SAS 9.5 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism ver. 6 (GraphPad Software Inc., La Jolla, CA, USA). The $P < 0.05$ was considered as significant difference.

RESULTS

Serum miRNAs levels in breast cancer and healthy samples

It is difficult to extract RNA from serum due to its low abundance. In our experiments, the average concentration of the total RNA from 300 μL of serum was 0.118 μg/μL ranging from 0.042 to 0.208 μg/μL. As compared with normal samples, the level of miR-21 and miR-155 was significantly higher (miR-21: 0.86 ± 0.941 *vs.* –2.74 ± 1.055, P <0.0001; miR-155: –1.24 ± 1.022 *vs.* –2.01 ± 0.808, P = 0.0005), while miR-365 was significantly lower (–0.84 ± 0.873 *vs.* –0.24 ± 0.317, P < 0.0001) in breast cancer patients' serum. The level of serum miR-125b and miR-145 showed no significant difference between breast cancer and normal samples (Fig. 1).

Effects of family history and TNM stage on serum miRNAs level in breast cancer patients

We compared the serum level of the five miRNAs in breast cancer patients with or without family history. The five miRNAs levels were not significantly different between family history positive and negative patients with breast cancer (Fig. 2).

The serum levels of miR-21, miR-125b, miR-145, miR155, and miR-365 in breast cancer patients at different TNM stages were also evaluated to determine if the serum miRNAs could be detected in early-stage breast cancer (Fig. 3). Across 3 stages, the serum levels of miR-21, miR-125b, miR-145, and miR-365 showed no marked difference. In comparison to stages I and II,

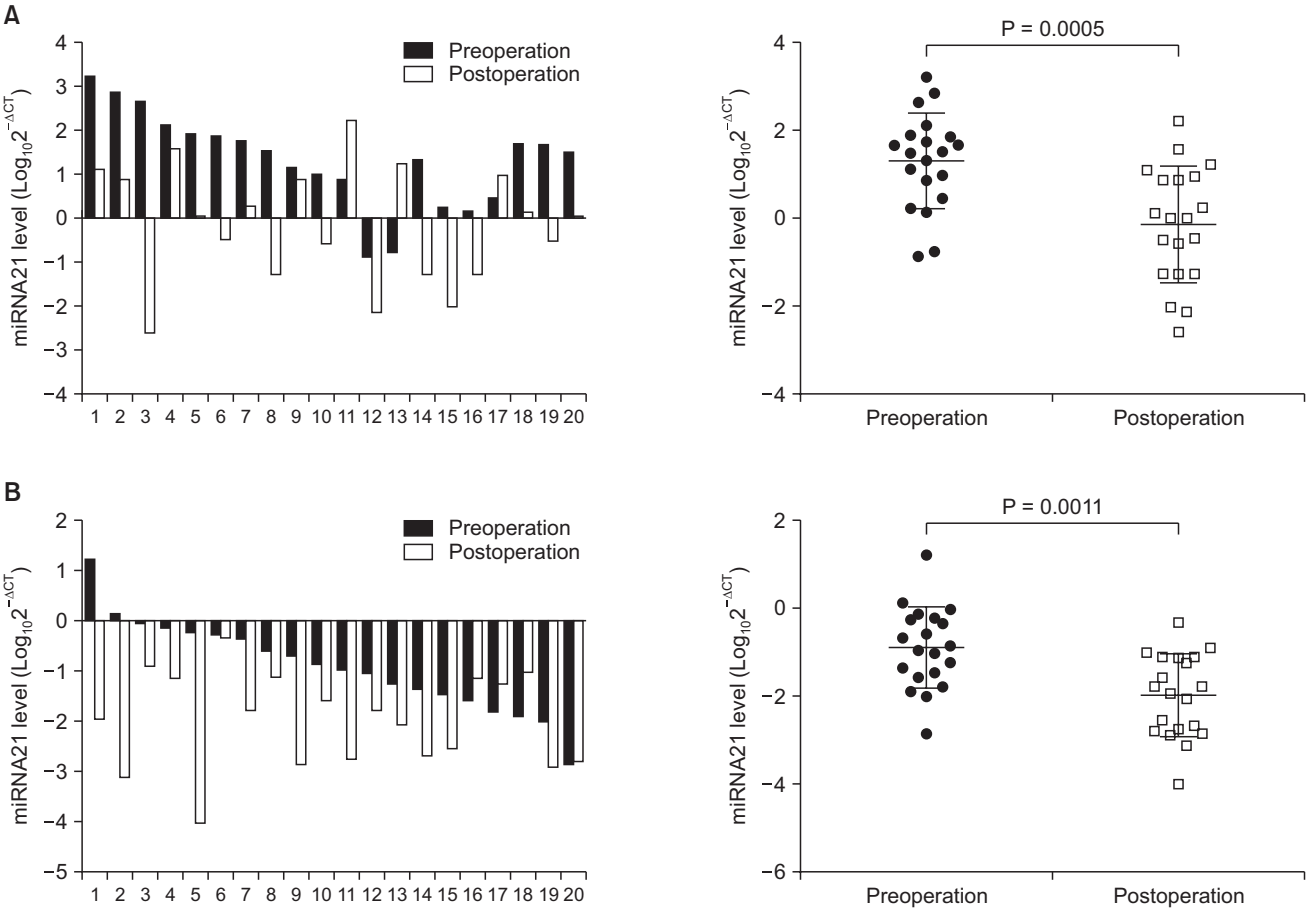

Fig. 5. Alteration of the serum miR-21 and miR-155 levels in breast cancer patients receiving surgery. Total serum RNA was extracted preoperation and postoperation in 20 breast cancer patients, and quantitative polymerase chain reaction was performed for evaluation of changes of miRNAs levels. In comparison with preoperation, the serum levels of miR-21 (A) and miR-155 (B) were significantly reduced 3 weeks postsurgery in breast cancers. Data are expressed as mean ± standard deviation.

the serum miR-155 level was remarkably lower (stage III: –2.40 ± 1.151 *vs.* stage I: –1.03 ± 0.790, stage II: –1.00 ± 0.919, $P < 0.05$) in breast cancer patients at stage III. Notably, compared to normal samples, the serum level of miR-21 was significantly higher (stage I: 0.80 ± 0.980, stage II: 0.92 ± 0.994, stage III: 0.87 ± 0.674 *vs.* healthy controls: –0.27 ± 1.055; $P < 0.01$) in breast cancer at any TNM stage. Nevertheless, in comparison with normal controls, the serum level of miR-365 was significantly lower (stage I: –0.92 ± 0.753, stage III: –0.97 ± 1.043 *vs.* healthy control: –0.24 ± 0.317; $P < 0.05$) at both stages I and III.

Comparison of serum miRNAs levels in various groups of breast cancer patients

We compared the serum levels of miR-21, miR-125b, miR-145, miR-155, and miR-365 in various groups of breast cancer patients classified by hormone status including ER, PR, and HER2 as well as p53 (Fig. 4). We only detected that the serum level of miR-145 was significantly higher (–0.46 ± 0.953 *vs.* –0.66 ± 0.777, $P = 0.041$) in PR-positive patients as compared with PR negative (Fig. 4C). Additionally, in breast cancer patients, the positivity of miR-21, miR-155 and miR-365 was 58%,

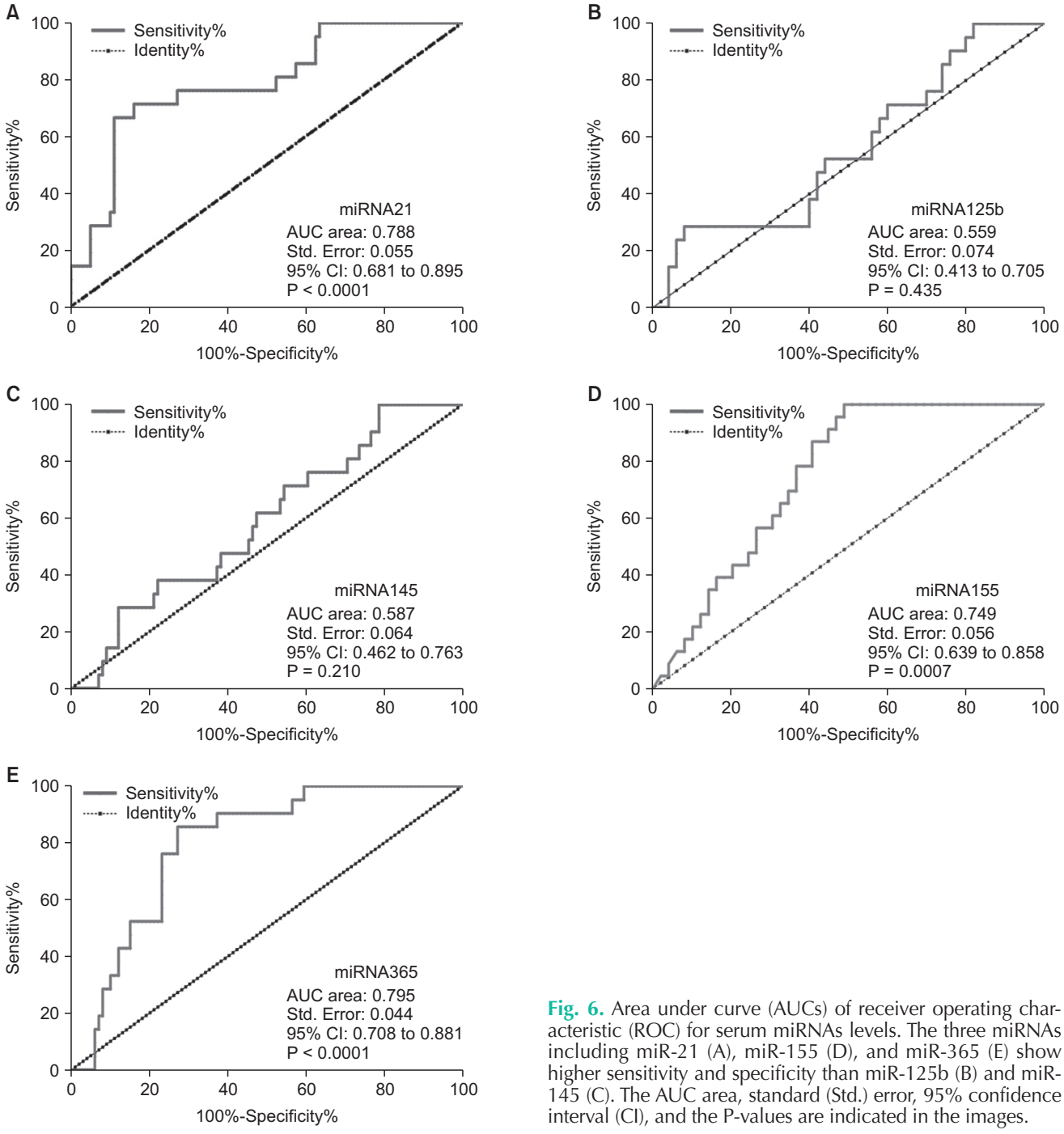

Fig. 6. Area under curve (AUCs) of receiver operating characteristic (ROC) for serum miRNAs levels. The three miRNAs including miR-21 (A), miR-155 (D), and miR-365 (E) show higher sensitivity and specificity than miR-125b (B) and miR-145 (C). The AUC area, standard (Std.) error, 95% confidence interval (CI), and the P-values are indicated in the images.

48%, and 59%, respectively. Nevertheless, the positivity of CA 153 and CEA was 33% and 20%, respectively (Fig. 4F).

Comparison of serum miRNAs levels before and after surgery in breast cancer patients

The serum level of miRNAs was analyzed in the paired pre- and postoperative samples from 20 patients with breast cancer. As compared with the preoperative samples, the level of miR-21 and miR-155 decreased significantly (miR-21: –0.01 ± 1.318 *vs.* 1.31 ± 1.082, P = 0.0005; miR-155: –1.98 ± 0.944 *vs.* –0.89 ± 0.921, P = 0.0011) in the post-operative samples (Fig. 5).

ROC curve analysis

ROC curve analysis was performed to evaluate the diagnostic value for the five miRNAs. The AUCs closer to 1 reflect more substantial differences between breast cancer and normal samples. ROC curve analysis revealed that the three miRNAs, miR-21, miR-155, and miR-365, had significantly higher AUCs with values of 0.788, 0.749, and 0.795, respectively (Fig. 6, Table 2). At the optimal cutoff value of –0.089, with the values of sensitivity plus specificity considered to be maximal for miR-21, the sensitivity and specificity were 66.67% and 88.89%, respectively. At the optimal cutoff value of –1.171 for miR-155, the sensitivity and specificity were 100% and 51.02%, respectively. At the optimal cutoff value of –0.4722 for miR-365, the sensitivity and specificity were 85.71% and 72.73%, respectively.

We further performed ROC curve analyses for combinations of miR-21, miR-155, and miR-365. Compared to miR-21 alone, the combination of miR-21/miR-365 yielded higher AUC (0.8677 *vs.* 0.7879, P = 0.0027), and better sensitivity (96.99%) and specificity (66.67%) (Fig. 7A). A combination of the three miRNs also created higher AUC (0.9184 *vs.* 0.8134; P = 0.0261), and better sensitivity (85.71%) and specificity (85.72%) (Fig. 7B).

Table 2. The area under curve, sensitivity and specificity of miRNAs

miRNAs	AUCs	Sensitivity (%)	Specificity (%)	P-value
miR-21	0.788	66.67	88.89	<0.001*
miR-125b	0.559	-	-	0.435
miR145	0.587	-	-	0.210
miR155	0.749	100	51.02	<0.001*
miR-365	0.795	85.71	72.73	<0.001*
miR-21/miR155	0.868	96.99	66.67	0.003*
miR-21/miR-155/ miR-365	0.918	85.71	85.72	0.026*

AUC, area under curve; miRNA, microRNA.
*P < 0.05, statistically significant.

DISCUSSION

Endocrine treatment is conventionally used for ER^+ patients, and trastuzumab for $HER2^+$ patients [14,15]. Although these biomarkers are commonly used in clinic for target therapy and management of breast cancer, there is still room for improvement, such as early diagnosis of tumor lesions, identification of high-risk patients, development of predictive biomarkers for monitoring progress and therapy effects, etc. By mapping the circulating miRNAs signature and profiling, some miRNAs have been found differentially expressed in breast cancer and normal tissues [16]. Although the clinical application of serum miRNAs as a noninvasive diagnostic strategy is promising, the miRNA signatures should be further investigated and validated for different subtypes of breast cancers. In the current study, we analyzed the serum level of five miRNAs including miR-21, miR-125b, miR-145, miR-155, and miR-365 in breast cancers and

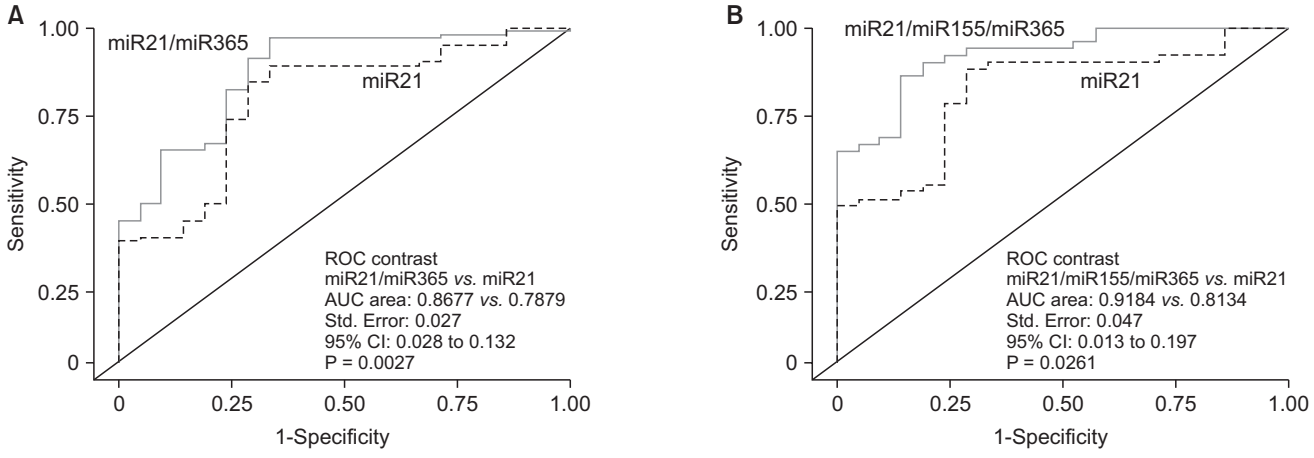

Fig. 7. Area under curve (AUCs) of receiver operating characteristic (ROC) for combination of microRNAs (miRNAs). The combination of miR-21 and miR-365, as well as miR-21, miR-155 and miR-365, shows higher sensitivity and specificity than each miRNA alone, e.g. miR-21. The AUC area, standard (Std.) error, 95% confidence interval (CI), and the P-values are indicated in the images.

healthy controls, followed by construction of ROC curves to determine the sensitivity and specificity of circulating miRNAs as potential biomarkers for breast cancer.

Our results showed that the serum concentrations of miR-21 and miR-155 were significantly elevated, while miR-365 was significantly downregulated in patients with breast cancer compared with healthy controls (Fig. 1). MiR-21 is involved in regulating the expression of multiple tumor suppressor genes, and plays a crucial role in the occurrence and development of a variety of cancer diseases [17]. In breast cancer, several studies have shown that up-regulation of miR-21 was detected in both serum and tissues [18], and that serum miR-21 could be used as an non-invasive biomarker for diagnosis and prognosis of breast cancer as well as an indicator for the invasiveness of the tumor [19]. MiR-155 plays a role in various physiological and pathological processes, and oversilencing by miR-155 may result in apoptotic resistance and thus triggering oncogenic cascades. It has been reported that the amount of both circulating miR-155 in serum and non-circulating miR-155 in tissue were elevated in patients with breast cancer [20,21]. Therefore, miR-21 and miR-155 may act as oncogenic factors, and thus may be potential targets for breast cancer therapy. Additionally, we found that miR-155 level was remarkably higher at stages I and II compared to stage III in breast cancer patients (Fig. 3). Similarly, it has been shown that miR-7 was associated with breast cancer grades [22]. Reduction of tissue miR-365 has been reported in different types of cancers such as hepatocellular carcinoma (HCC) [23] and lung cancer [24]. An *in vitro* experiment showed that overexpression of miR-365 remarkably suppressed proliferation and migration capacities of HCC cell [23]. Kodahl et al. [25] also showed that the serum miR-365 was downregulated in early stage breast cancer patients. Family history is an important predisposing factor in breast cancer. However, we did not find differential expression of the five miRNAs between family history positive and negative breast cancer patients (Fig. 2).

In this study, the serum abundance of miR-125b and miR-145 displayed no remarked difference between breast cancer and healthy control (Fig. 1). Nevertheless, by comparing the miRNAs profile between 61 breast cancers and 10 healthy controls in the Mexican population, it was found that the serum levels of miR-145 and miR-125b were significantly higher in patients with breast cancer than healthy controls [26]. The current analysis just found that serum miR-145 abundance was significantly higher in PR-positive breast cancer patients compared to the PR-negative (Fig. 4C), indicating that serum miR-145 level may separate the PR-positive subtypes of breast cancer. A previous study also examined the association of miRNAs expression in serum with different tumor hormone status, and they found 7 miRNAs with differential expression for women whose breast cancer differed by HER2 expression [27]. Notably, it was reported that the level of miR-145 was downregulated both in the *in vitro* cultured breast cancer cell lines [28] and in the serum from early stage breast cancer patients [25]. CA153 and CEA are the most widely used circulating biomarkers in monitoring patients with breast cancer. Nevertheless, we found that the positivity of miR-21, miR-155 and miR-365 was higher in comparison with CEA and CA 153 in breast cancer patients (Fig. 4F). Similarly, serum miR-21 and miR-30a has a higher sensitivity in diagnosis of breast cancer compared with CEA and CA153 [29,30].

The serum level of miR-21 and miR-155 was further analyzed in 20 patients with breast cancer before surgery and 3 weeks after tumor removal. The abundance of serum miR-21 and miR-155 was significantly reduced in the postoperative samples as compared with the paired pre-operative samples (Fig. 5). Consistently, Sochor et al. [20] found that early breast cancer patients significantly overexpressed several oncogenic miRNAs such as miR-155, miR-181b, miR-19a, and miR-24, which dramatically decreased following surgical resection. Kodahl et al. [25] reported that circulating miR-338-3p, miR-223, and miR-148a exhibited lower, and miR-107 exhibited higher levels postoperatively than the preoperative samples from 24 postmenopausal women with ER^+ early-stage breast cancer. These findings suggest that serum oncogenic miRNAs may be potentially used for diagnostic purpose and relapse judgement.

To determine the diagnostic performance of miRNAs for breast cancer, we performed ROC curve analysis, and the AUC was used as the evaluation criteria; the higher AUC, the better diagnostic performance. Our data revealed that the 3 miRNAs miR-21, miR-155, and miR-365 had significantly higher AUC with the values of 0.788, 0.749, and 0.795, respectively (Fig. 6), suggesting that we were able to discriminate breast cancer from healthy controls. The sensitivity for the three miRNAs (miR-21, miR-155, and miR-365) was 66.7%, 100%, and 85.7%, respectively, and the specificity was 88.9%, 51.02%, and 72.7%, respectively (Fig. 6, Table 2). It has been reported that a miRNA panel could accurately distinguish cancers from normal subjects [4,7,8,11,12]. A combination of ROC curve analyses of miR-145, miR-155, and miR-382 exhibited much better sensitivity and specificity than each miRNA alone [30]. In this study, ROC curve was analyzed in a panel of 3 miRNAs (miR-21, miR-155, and miR-365). Our data demonstrate that a combination of miR-21 and miR-365 yielded a significantly high AUC (0.868), sensitivity (96.97%), and specificity (66.67%). The combination of miR-21, miR-155, and miR-365 further generated much higher AUC (0.918), accompanied by relatively higher sensitivity (85.71) and specificity (85.72) (Fig. 7, Table 2). Accordingly, our results implied that a panel of miRNAs (e.g., a combination of miR-21/miR-155/miR-365) remarkably enhanced the diagnostic performance with high sensitivity and specificity, and thus may provide an improved indicator for breast cancer diagnosis and

screening.

Taken together, our study provides evidence that evaluation of serum miRNAs level can be used as biomarkers for diagnosis of breast cancer, and that a combination of miR-21/miR-155/miR-365 may potentially serve as a sensitive and specific biomarker that enables the differentiation of breast cancer from healthy controls.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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