Identification of four candidates circulating microRNAs in plasma as a potential biomarker for early-stage breast cancer diagnosis in Vietnamese women

Luan Huu Huynh[1,2,3], Thuy Thi Chung Duong[2,3], Nga Thi Nguyen[2,3], Thanh Thi Ngoc Nguyen[1,2,3*] and Hue Thi Nguyen[1,2,3*]

1. Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, VIETNAM
2. Human genetic Laboratory, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, VIETNAM
3. Vietnam National University, Ho Chi Minh City, VIETNAM
*nthue@hcmus.edu.vn

Abstract

Breast cancer is the most commonly occurring malignant neoplasm among the female population. This study aims to ascertain novel biomarkers that may prove advantageous in the early detection of breast cancer. The present investigation involved a comparative analysis of the plasma expression of four distinct microRNA (namely, miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p) in a cohort of 80 Vietnamese patients who were newly diagnosed with breast cancer. The expression levels of these microRNA were compared with those of 80 healthy individuals who served as controls. The plasma total RNA was extracted and the miRNA expression levels were assessed using Stem-loop RT-qPCR followed by statistical analysis.

According to the findings of our study, the plasma levels of four microRNAs namely miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p, were significantly reduced in both breast cancer patients in stage 0-I and the general breast cancer patient population as compared to healthy controls. The diagnostic accuracy of both miR-9-3p levels and miR-15b-5p levels was satisfactory with 74% and 88% values respectively. The combined effect of miR-425-5p/miR-142-3p/miR-9-3p/miR-15b-5p revealed the highest level of diagnostic precision, attaining a rate of 98% and an area under the curve value of 0.98 (with a sensitivity of 87.5% and specificity of 100%). In summary, the microRNAs miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p exhibit potential as non-invasive diagnostic biomarkers for breast cancer, particularly in the initial stages of the disease.

Keywords: microRNA, breast cancer, diagnosis, circulating biomarkers, ROC curve.

Introduction

In women, breast cancer (BC) is by far the most prevalent form of cancer to be diagnosed and it is also the primary reason for mortality from cancer in the majority of countries[5]. It is critical to detect breast cancer at an early stage in order to enhance patient outcomes. It has been shown conclusively that individuals diagnosed at stage I of the disease have a relative survival rate of one hundred percent after five years. Despite the fact that screening programs have shown to be beneficial, only about 44% of people who have BC are detected at an early stage of the disease[8,16]. Consequently, it becomes essential to develop new screening procedures for BC that are both specific and effective.

MicroRNAs (miRNAs) have been proposed as key regulators of cellular activity over the past few decades. MiRNAs are non-coding RNAs that range in length from 21 to 25 nucleotides. They are involved in a wide variety of biological processes and influence gene expression on multiple levels. When considering cancer, it is important to note that miRNAs become dysregulated in tumor tissues. In these tissues, they can operate as oncogenes or tumor suppressors by targeting genes involved in cancer-related activities such as the development of a tumor, proliferation, cell death, angiogenesis, or invasion[2,20-22].

In addition, several authors have already shown that miRNAs may be effective as biomarkers for diagnosis, prognosis and response to therapy in various malignancies including breast cancer [3, 10, 17]. Importantly, microRNAs can be identified in biological fluids such as serum, plasma, or whole blood, making them intriguing candidates for non-invasive or minimally invasive biomarkers.[1]

Recent studies have revealed that abnormal expression of miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p in breast tumor tissue or breast cancer cell lines is related with the development of BC[15,19,23-25]. On the other hand, there have only been a few findings addressing the role of plasma miRNAs in BC. In this study, we studied and compared the expression of these four plasma miRNAs in patients diagnosed with breast cancer and healthy controls.

In addition, a statistical analysis was done to investigate the possibility of associations between the levels of these plasma miRNAs and the phases of BC. This study contributes to the establishment of miRNA profiles of Vietnamese breast cancer patients as well as the provision of novel insights. In the meantime, it will be helpful in the search for novel methods that can complement the existing standard diagnosis.

Material and Methods

Sample collection: This study was approved by the Ethical Committee of The Oncology Hospital, Ho Chi Minh City, Vietnam (ethical no: 113/BVUB-HĐĐĐ). At The Oncology Hospital in Ho Chi Minh City, Vietnam, eighty blood samples from patients with pathologically confirmed breast cancer were collected. Healthy female volunteers provided eighty blood samples as a healthy control at the same facility. These volunteers have no prior cancer history. All participants were women of Vietnamese descent who gave their consent in writing to engage in the present study.

RNA extraction: Blood samples were centrifuged for 10 minutes at 3000 rpm at room temperature. Plasma was transferred to a new tube, aliquoted into microcentrifuge tubes and stored at -80°C prior to RNA extraction. Using a modified Trizol-based method, we extracted total RNA containing small RNA from 300 µl of thawed plasma containing small RNA. The RNA was eluted with 20 µl DEPC followed by the RNA to cDNA conversion. All RNA concentrations were measured using a NanoDrop 1000 (Thermo Scientific, USA).

Stem-loop Reverse Transcription Quantitative Polymerase Chain Reaction (Stem-loop RT-qPCR): cDNA was synthesized with stem-loop primer in 20 µl reaction by using the SensiFAST cDNA Synthesis kit (BIOLINE, England) according to the manufacturer's protocol. The reaction mixture was incubated at 16 °C for 10 min, at 42 °C for 15 min and at 85 °C for 5 min in a thermal cycler. cDNA was stored in 4 °C before RT-qPCR. To determine miRNA expression levels, SYBR green-based qPCR was performed by using SensiFAST HRM Kit (BIOLINE, England). A total of 2 µL of cDNA was amplified with 1X MasterMix, 0.2 – 0.4 µM of primer (Sigma, USA) and nuclease-free water. qPCR reaction was conducted on a Line-Gene 9660 System (Bioer, China) under the following conditions: 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 60 °C for 10 s and then HRM analysis was performed by default. The miR-16 was used as an endogenous control.

The relative expression of certain miRNA was first normalized to the endogenous control and then the results were compared to healthy control subjects. The relative levels of miRNA expression in individual case or control group were determined using the $2^{-\Delta CT}$ method with ΔCT equal CT value of target miRNA minus CT value of miR-16. The relative miRNA expression level between case and control group was determined as $2^{-\Delta\Delta Ct}$. ΔΔCt equals the average value of the case group (Ct target miRNA minus Ct miR-16) minus the average value of the control group (Ct target miRNA minus Ct miR-16).

Statistical analysis: GraphPad Prism 9 software was used to conduct statistical analysis. Using an unpaired t-test of $2^{-\Delta Ct}$, we could evaluate whether there was a significant difference between the fold change expression of miRNA in BC samples and healthy control samples. The one-way ANOVA analysis of variance was conducted to identify any significant dysregulation of the miRNA that occurred within two or more BC stages. p-values less than or equal to 0.05 were considered statistically significant. The $2^{-\Delta Ct}$ data were used to plot receiver operating characteristic (ROC) curves for each miRNA to establish its diagnostic accuracy and parameters.

Spearman's correlation coefficient was calculated for each pair of miRNAs based on fold change of expression values. The ROC curve of the combined miRNA was constructed using binary logistic regression. Youden's index was computed to determine each miRNA's optimal cut-off, sensitivity, specificity and accuracy.

Results and Discussion

Early breast cancer detection is a significant concern for clinicians. The majority of patients having later stages of cancer made the treatment process more challenging [18]. This study aims to find miRNA biomarkers to diagnose breast cancer early using less invasive methods. Several studies have suggested that the presence of miRNA in a cancer patient's blood circulation is indicative of breast cancer progression[4,6,7,9]. Stem-loop RT qPCR was used to examine miRNA expression in breast cancer patients and healthy controls' blood plasma. We examined the expression of four miRNAs, miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p which may be valuable breast cancer markers.

Patient Characteristics: We analyzed the levels of microRNAs in 80 breast cancer patients and 80 healthy controls. Patients were compared to a healthy control group of the same age. The average age of the patients was 44.05 years while the average age of the healthy controls was 41.30 years (Table 1). At the time of diagnosis, 20% of the cases were in stages 0-I and 80% were in stages II-III.

MiRNA Expression in the Plasma of Breast Cancer Patients as compared with Healthy Subjects: After normalization, the four miRNAs, miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p showed lower expression with a relative expression (-ΔΔCt) of < -3 and a fold change ($2^{-\Delta\Delta Ct}$) of ≤ 0.1 (Table 2). Accordingly, these four miRNAs are downregulated and the independent T-Test was used to determine the significant difference between cancer and healthy control groups. Four microRNAs were found to have significantly different p-values including miR-425-5p ($p = 1 \times 10^{-12}$), miR-142-3p ($p = 1.35 \times 10^{-9}$), miR-9-3p ($p = 1.85 \times 10^{-7}$) and miR-15b-5p ($p = 2.54 \times 10^{-20}$) (Table 2).

Circulating miR-425-5p and miR-142-3p levels tended to increase in the control sample and decrease in the patient sample. Circulating miR-425-5p levels was significantly lower in samples from BC (mean: -1.335, 95% CI: -1.433 to -1.237) compared to healthy controls (mean: 0.3481, 95% CI: -0.05005 to 0.7462) (p = <0.0001) (Figure 1). miR-142-3p levels were significantly higher in BC (mean: -1.299,

95% CI: -1.406 to -1.193) than in volunteers' plasma samples (mean: 0.1392, 95% CI: -0.2804 to 0.5588) ($p < 0.0001$) (Figure 1). In the opposite trend, miR-9-3p and miR-15b-5p levels tended to decrease in both healthy and diseased samples, but in diseased samples, there was a greater reduction.

Circulating miR-9-3p levels was significantly lower in samples from BC (mean: -2.836, 95% CI: -2.944 to -2.728) compared to healthy controls (mean: -1.827, 95% CI: -2.178 to -1.477) ($p = <0.0001$) (Figure 1). miR-15b-5p levels were significantly higher in BC (mean: -2.141, 95% CI: -2.294 to -1.988) than in volunteers' plasma samples (mean: -0.7198, 95% CI: - -0.9350 to -0.5047) ($p < 0.0001$) (Figure 1).

Table 1
Characteristics of patients with breast cancer and controls

Variables		Case (n = 80)	Control (n = 80)	P
		Number (%)	Number (%)	
Age (year)	Mean ± SD	44.05 ± 10.5	41.3 ± 10.5	0.1
Stage	0-I	16 (20%)		
	II-III	64 (80%)		

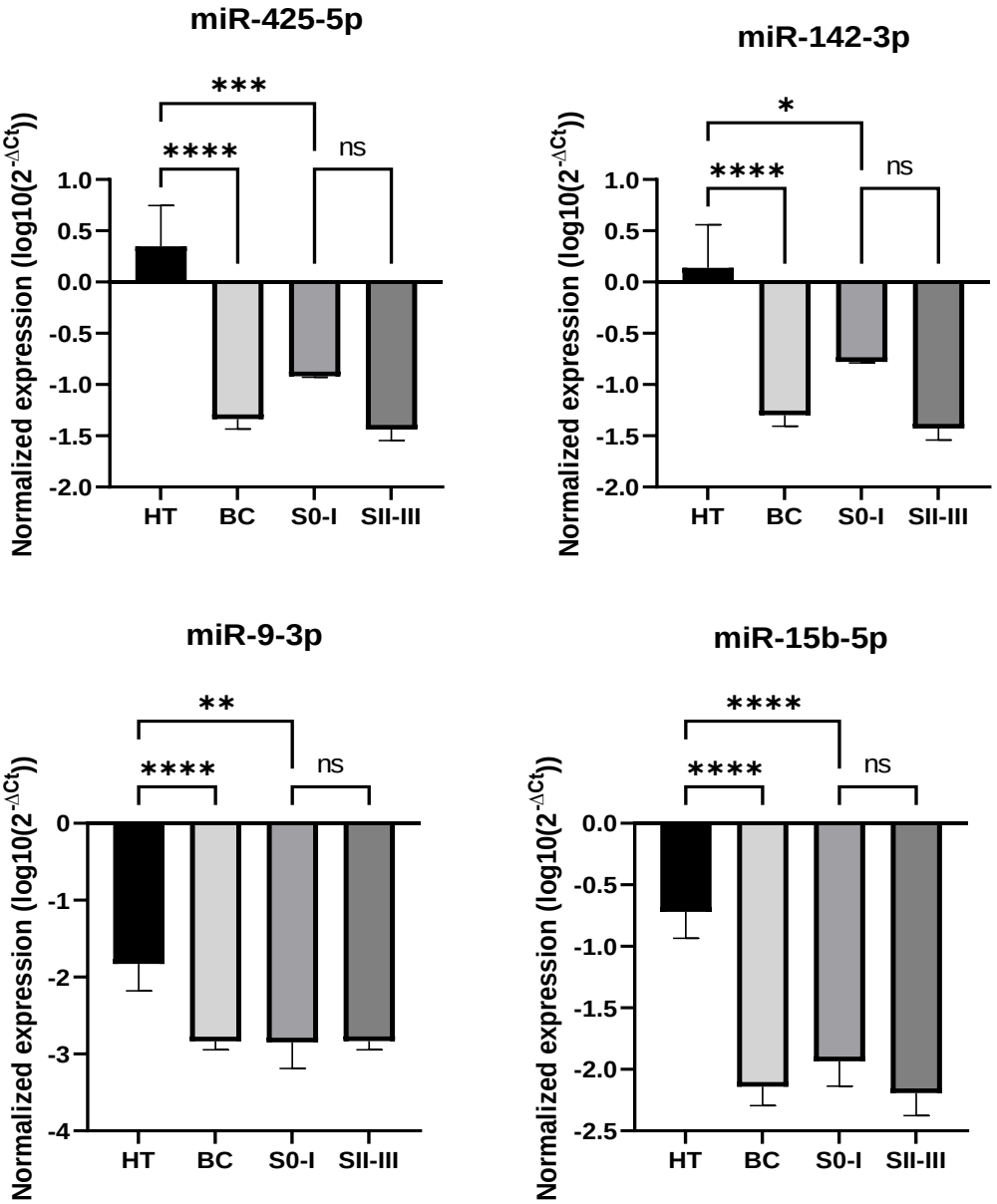

Figure 1: Analysis of normalized miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p expression in plasma between BC patients and healthy controls. HT: Healthy controls. BC: Breast cancer patients. S0-I: Breast cancer patients stage 0 and I. SII-III: Breast cancer patients stage II and III. p-value style GP: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

Table 2
MiRNAs expression in plasma of breast cancer patient as compared to healthy control

miRNA	Minus delta delta Ct (-ΔΔCt)	Fold change ($2^{-\Delta\Delta Ct}$)	Regulation (vs. control)	Cohen's Effect size	p-value (-ΔCt case vs. -ΔCt control)	p-value (-ΔCt case stage 0-I vs. -ΔCt control)
miR-425-5p	-5.59	0.02	down	1.29	<0.0001	0.0002
miR-142-3p	-4.78	0.04	down	1.05	<0.0001	0.0202
miR-9-3p	-3.35	0.10	down	0.86	<0.0001	0.0013
miR-15b-5p	-4.72	0.04	down	1.69	<0.0001	<0.0001

Our study findings indicate a statistically significant decrease in miR-425-5p and miR-142-3p levels among breast cancer patients compared to the healthy control group. These investigations were similar to Mangolini et al's[14] findings in Americans, where they also found miR-425-5p down-regulated in breast cancer patients.

Nevertheless, our findings are in opposition to the pattern identified in prior investigations. Another studies on African American[26] and Malaysian[12] subjects revealed that the plasma concentrations of miR-425-5p and miR-142-3p, respectively were generally higher in cases than in controls. The preceding trends indicate that various populations may have distinct miRNA profile patterns.

The expression of miR-9-3p was comparatively lower in breast cancer patients' plasma compared to that of healthy controls. The aforementioned assertion is corroborated by research conducted by Li et al[13] in which the aberrant modulation of miR-9 is identified as a substantial catalyst for the development of breast cancer. The present study's results align with a prior observation that miR-9-3p exhibited decreased expression in diverse breast cancer cell lines[25]. The findings of our study indicate that miR-15b-5p exhibits decreased expression in breast cancer in comparison to individuals without the disease.

The present findings are in opposition to the research conducted by Wu et al[23] who reported a rise in the expression of miR-15b-5p in breast cancer tissues. The researchers additionally discovered that miR-15b-5p facilitates the proliferation and metastasis of breast cancer cells through the targeting of HPSE2. The dissimilarities could potentially be attributed to variations in the sample and patient origins utilized in each respective study.

Expression of miRNAs in Early-Stage BC Patients: The potential of miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p to detect breast cancer in its early stages was analyzed. The 16 stage 0-I BC patients included in the initial screening population were chosen for this study. The miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p levels were statistically lower in stage 0-I BC patients (miR-425-5p mean: -0.9176, 95% CI: -0.9286 to -0.9066; miR-142-3p mean: -0.7785, 95% CI: -0.7876 to -0.7695; miR-9-3p mean: -2.849, 95% CI: -3.189 to -2.510; and miR-15b-5p mean: -1.934, 95% CI: -2.136 to -1.732) than in healthy donors (Table 2 and figure 1). Then, comparisons of miRNA level between stage 0-I and II-III BC patients were conducted to analyze the potential of miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p to discriminate BC stages. There was no significant difference between stage 0-I and II-III BC patients in terms of miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p levels (Figure 1). The findings indicate that the four miRNAs show considerable promise as a plasma biomarker for diagnosis during the early phases of breast cancer.

Diagnostic Accuracy of miRNA in BC: This analysis was conducted on miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p to identify the most accurate miRNA biomarkers to distinguish between breast cancer and healthy control. We found that miR-425-5p and miR-142-3p with AUC value > 0.6 ($p < 0.001$) were considered as acceptable biomarkers for early detection of breast cancer (Table 3 and figure 2 A-B). In addition, miR-9-3p and miR-15b-5p were able to discriminate between BC patients and controls with an AUC of > 0.70 ($p < 0.0001$) (Table 3 and figure 2 C-D). At the cutoff value -0.77 for miR-9-3p, the sensitivity and specificity were 87.50% and 82.50%. At the cutoff -1.349 for miR-15b-5p, the sensitivity and specificity were 87.50% and 86.25%. Thus, these miRNAs, miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p, can be considered as the suitable biomarkers to undergo a validation process. Similar observations were reported by Orangi et al[19] for miR-425-5p[26], miR-142-3p[12] and miR-9.

Spearman's correlation coefficients were computed to examine the correlations among the expressions of the four miRNAs that were significantly dysregulated (Table 4). The results indicate a noteworthy and robust positive association between miR-425-5p and miR-142-3p with a correlation coefficient of 0.61 and a p-value of less than 0.0001. The study also found a statistically significant and positive correlation between miR-9-3p and miR-15b-5p with a correlation coefficient of 0.25 and a p-value of less than 0.01. The ROC analysis was performed to evaluate the collective diagnostic potential of the miRNAs showing statistically significant and robust correlations, as determined through correlation testing. Table 3 presents the calculated optimal cut-off values, specificity, sensitivity, diagnostic accuracy and 95% confidence intervals for each combination of miRNAs. The combination of miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p exhibited the most excellent diagnostic efficacy with an area under the curve (AUC) of 0.98 (Figure 2).

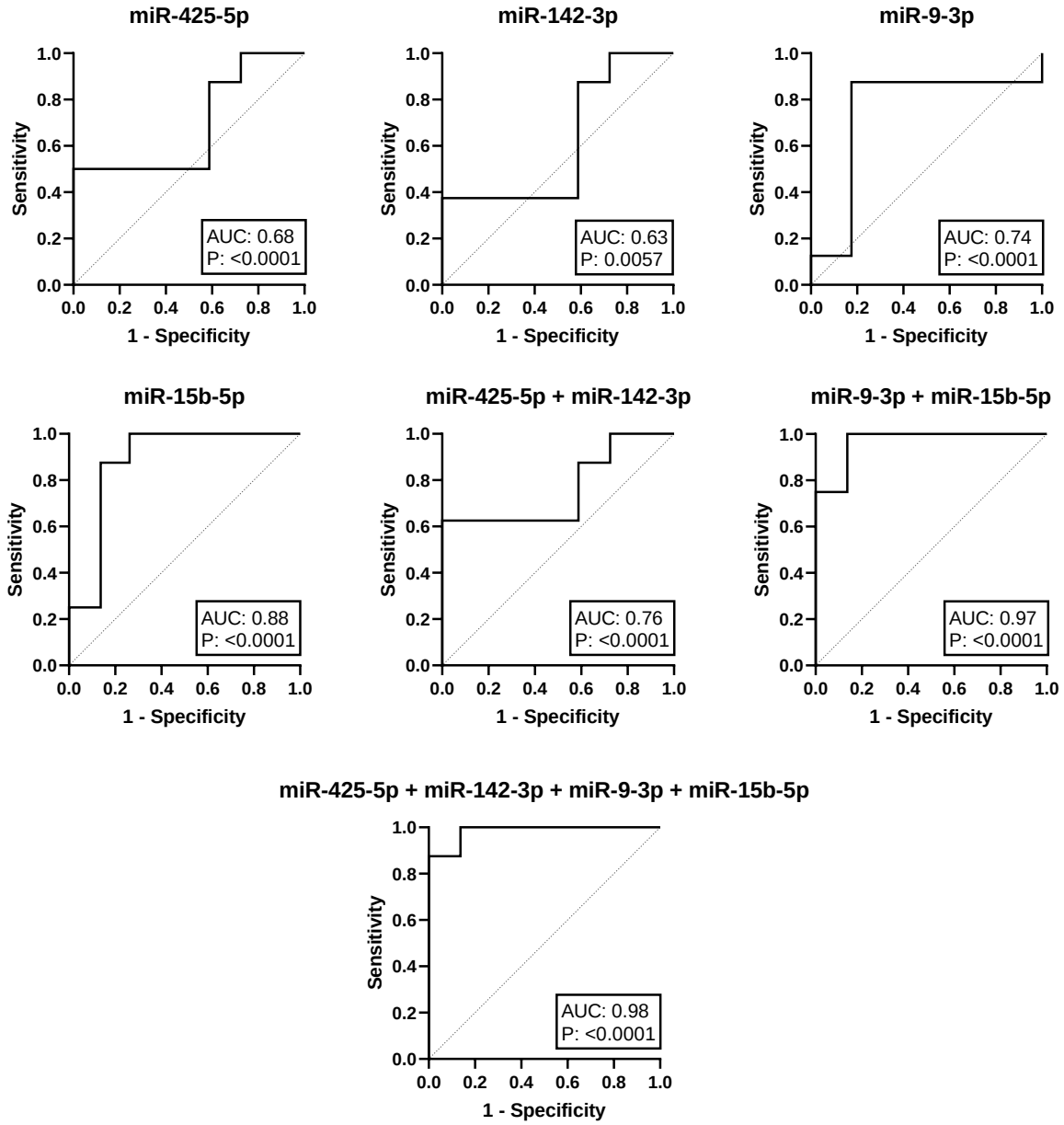

Figure 2: Diagnostic accuracy of miR-425-5p, miR-142-3p, miR-9-3p, miR-15b-5p and combine miRNA for BC detection in plasma

Table 3
Diagnostic parameters to evaluate the BC diagnostic ability of individual and combined studied miRNA

miRNA	AUC[a]	95% CI[b]	p-value	Youden's Index	Cut-off	Sen.[c] (%)	Spe.[d] (%)
miR-425-5p	0.68	0.60 - 0.77	<0.0001	0.50	> -0.18	50.0	100
miR-142-3p	0.63	0.54 - 0.71	0.0057	0.38	> 0.56	37.5	100
miR-9-3p	0.74	0.65 - 0.83	<0.0001	0.70	> -0.77	87.5	82.5
miR-15b-5p	0.88	0.82 - 0.94	<0.0001	0.74	> -1.60	100	73.8
miR-425-5p + miR-142-3p	0.76	0.69 - 0.84	<0.0001	0.63	>0.64	62.5	100
miR-9-3p + miR-15b-5p	0.97	0.94 - 0.99	<0.0001	0.86	>0.25	100	86.3
miR-425-5p + miR-142-3p + miR-9-3p + miR-15b-5p	0.98	0.97 - 0.99	<0.0001	0.88	>0.62	87.5	100

a. AUC, area under the curve; b. CI, confidence interval; c. Sen., Sensitivity; d. Spe., Specificity

Table 4
Spearman's correlation coefficients between each pair of miRNAs

	miR-142-3p	**miR-425-5p**	**miR-9-3p**	**miR-15b-5p**
miR-142-3p	1	0.61****	0.047	-0.11
miR-425-5p		1	0.12	0.15
miR-9-3p			1	0.25**
miR-15b-5p				1

**denotes significant correlation coefficient (p-value < 0.01); ****denotes significant correlation coefficient (p-value < 0.0001)

These findings are consistent with a prior investigation conducted on Lebanese females which demonstrated that the combination of four miRNAs including miR-425-5p showed the most significant sensitivity, specificity and diagnostic accuracy (97%, 91% and 95% respectively) among all individual and combined miRNAs for the identification of early-stage breast cancer patients[11].

Despite limitations, such as a relatively small sample size and a narrow focus on breast cancer stages, this investigation has revealed unique miRNA expression patterns indicating their potential as non-invasive diagnostic molecular biomarkers for patients with early-stage breast cancer. It is imperative to assess dysregulated microRNAs in larger experimental cohorts. This phenomenon can be attributed to the latter's association with greater tumor size, superior individual diagnostic precision and its inclusion in the combination of mRNAs that achieved the highest diagnostic accuracy.

Although dysregulated circulating miRNAs have the potential to serve as a diagnostic signature for early-stage breast cancer cases, there are some inconsistencies with prior research findings. The observed variations may be attributed to dissimilarities in the selected sample size, ethnic background, age cohorts and disparities in the molecular and histopathological subtypes of breast cancer.

Conclusion

The present investigation has successfully identified four miRNAs namely miR-425-5p, miR-142-3p, miR-9-3p and miR-15b-5p, which have potential as biomarkers and warrant further validation. The four-miRNA combination that was proposed, has been shown to possess significant potential as a biomarker for the diagnosis of breast cancer which is crucial for early detection without invasive procedures.

This study successfully identified miRNAs and combinations with high diagnostic accuracy, further validation studies in independent cohorts of breast cancer patients and more extensive multi-institutional settings are necessary to confirm its potential for clinical implementation.

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