Research in Pharmaceutical Sciences, October 2025; 20(5): 689-700
Received: 19-08-2024
Peer Reviewed: 01-10-2024
Revised: 09-11-2024
Accepted: 10-11-2024
Published: 20-10-2025

School of Pharmacy & Pharmaceutical Sciences
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Original Article

Clinical relevance of circulating biomarkers in breast cancer metastasis detection: "insights from liquid biopsy technology"

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Abstract

Background and purpose: Breast cancer is a major global concern, especially because of high mortality linked to advanced stages. Metastasis may occur early, but current diagnostics struggle to detect small metastatic cells. Non-invasive liquid biopsies, such as circulating miRNA, ctDNA, and cfDNA, present a promising solution for screening and monitoring breast cancer. This study aimed to examine the clinical relevance of circulating levels of cfDNA, miR-182, CYFRA21-1, and CEA in metastatic and non-metastatic breast cancer patients and their association with clinical stage and distant metastasis.
Experimental approach: Ten mL blood samples were collected from 17 metastatic and 29 non-metastatic breast cancer patients pre-surgery. cfDNA was measured fluorometrically, miRNA-182 *via* quantitative real-time PCR, CYFRA21-1, and CEA using enzyme-linked immunosorbent assay ELISA.
Findings/Results: Results demonstrated significantly higher levels of cfDNA, miR-182, CYFRA21-1, and CEA in women with metastatic breast cancer compared to those with non-metastatic breast cancer. These markers were linked to advanced clinical stages and increased tumor size. Elevated levels of cfDNA, miR-182, and CYFRA21-1 were indicative of a higher risk for lymphatic metastasis, while cfDNA and CYFRA21-1 were associated with distant metastasis. ROC curve analyses revealed strong efficacy for cfDNA (AUC 0.94), miR-182 (AUC 0.91), CYFRA21-1 (AUC 0.88), and CEA (AUC 0.87) in detecting metastatic breast cancer.
Conclusion and implications: Combined analysis of these biomarkers will enhance the predictive accuracy of metastatic breast cancer and clarify the relationship between biomarker profiles and the characteristics of metastatic versus nonmetastatic patients using liquid biopsy technology.

Keywords: Biomarkers; cfDNA; CYFRA21-1; Liquid biopsy; Metastatic breast cancer; miR-182.

INTRODUCTION

Breast cancer (BC) is the predominant form of cancer in women globally. The origin of BC is most frequently observed within the inner lining of breast milk ducts or lobules (1). Despite considerable advancements in the identification and management of BC, it remains the foremost cause of cancer-related mortality among women worldwide. The median and 5-year survival rates for this patient population are approximately three years and 25%, respectively (2). Approximately 30% of women who receive an initial diagnosis of early-stage breast cancer will subsequently progress to metastatic breast cancer (3). Typical approaches for monitoring the treatment response of metastatic breast cancer (MBC) involve computed tomography scans and the application of Response Evaluation Criteria in Solid Tumor (RECIST) criteria (4). However, these methods are expensive, relatively less efficient, and difficult to obtain.

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Access this article online	
	Website: http://rps.mui.ac.ir
	DOI: 10.4103/RPS.RPS_138_24

It is essential to find out more convenient diagnostic techniques that can accurately prognose and diagnose metastasis, accurately monitor treatment response, and assist in clinical decision-making for MBC patients. Liquid biopsies, which analyze cancer-related signals in biological fluids, have gained significant attention in the past decade. This non-invasive technique measures tumor components such as circulating tumor DNA (ctDNA), circulating cell-free DNA (cfDNA), and circulating miRNA in the bloodstream for molecular screening and early cancer diagnosis (5). cfDNA in the bloodstream is due to a combination of apoptosis, necrosis, and active secretion from cancer cells. Its concentration is significantly higher in advanced cancer patients compared to both healthy individuals and those in the early stages of the disease (6). Circulating miRNAs can be consistently found in bodily fluids like blood, plasma, serum, and saliva (5). miRNA-182 has been identified with diverse functional roles, either as an oncogene or tumor suppressor, depending on the cancer type, location, and stage. In glioma, it promotes tumor aggressiveness, while in melanoma, it promotes migration and survival (7). Intermediate filaments primarily comprise epithelial keratins or cytokeratins (CKs), and there are 20 different CK types. These proteins are produced by all epithelial cells and are useful markers for epithelial differentiation. They can be detected in tumors and as partially degraded fragments in serum (8). Monitoring the blood levels of cytokeratin fragment antigen 21.1 (CYFRA 21-1) is valuable in cancer patients, as it indicates advanced-stage cancer characterized by tumor cell migration and metastasis formation (9). The quantification of soluble proteolytic fragments of CKs provides valuable insights into neoplastic burden and tumor progression (10-12). The widely used tumor marker in clinics is carcinoembryonic antigen (CEA), a cell surface glycoprotein involved in cellular adhesion, and is known to be elevated in some cancers. It is a marker for tumor presence in colorectal, gastrointestinal, lung, and breast cancers (13).

Previous studies have separately evaluated cfDNA markers, miR-182, and CYFR 21-1 for early cancer detection in both healthy individuals and cancer patients (14-16). Our goal in this study was the early detection of metastatic breast cancer using the liquid biopsy technique. This research includes the simultaneous evaluation of these markers using molecular and biochemical techniques in metastatic and non-metastatic groups. This approach may improve the accuracy of early metastasis diagnosis and drive practical liquid biopsy methods, potentially decreasing the reliance on conventional tissue biopsies.

MATERIALS AND METHODS

Patient characteristics

The present study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Ethic No. IR.MUI.RESEARCH. REC.1400.414), and informed consent was provided by each participant, confirming that all experiments were performed in accordance with relevant guidelines and regulations. The samples were collected from metastatic (n = 29) and non-metastatic (n = 17, control group) cases of breast cancer between 2021 and 2023 at Isfahan-Ordibehesht-Surgery Center, Sina Hospital, Gharazi Hospital (Isfahan, Iran), and the Iran National Tumor Bank.

Before surgery, all patients underwent comprehensive assessments, which included a thorough clinical history review and physical examination, to determine the tumor location. Pathological examinations subsequently confirmed the diagnoses for all patients. The study included women with various stages of BC confirmed through pathological diagnosis. The target population consisted of two groups: the first group included women of all ages diagnosed with MBC (any kind of metastasis, including lymph vascular invasion, lymph node, and distant metastasis), and the second group included women of all ages diagnosed with non-MBC. Available diagnostic methods are required to confirm metastasis in any part of the body. Participants must have either not received chemotherapy or completed their last treatment at least three months before enrollment.

Blood specimen collection and processing

Peripheral blood samples (10 mL) were collected from BC patients. The samples were obtained either at the time of diagnosis or before surgery for BC. The blood was drawn from a peripheral vein and distributed into EDTA-coated tubes for plasma isolation and native tubes for serum. After collection, the blood samples underwent two consecutive centrifugation rounds at 3000 rpm for 10 min at room temperature to eliminate cellular components. The resultant plasma and serum were stored in 2 mL aliquots at -80 °C until further analysis.

Laboratory assessment

Circulating DNA quantification kit

The levels of circulating DNA present in blood plasma and cell supernatants were evaluated utilizing Abcam's circulating DNA quantification kit (CAT No. ab156898) according to the provided instructions. The DNA was incubated, digested, and isolated. Afterward, it was transferred to a column, centrifuged, and the resulting flow-through was discarded. After cleaning with ethanol, the DNA was eluted, and the samples and standards were plated. Finally, the fluorescence (BioTek, USA) was measured at excitation wavelengths between 480 and 500 nm and emission wavelengths of 520 and 550 nm.

2.4.2 miR-182 analysis by quantitative real-time polymerase chain reaction

To extract total RNA from 200 µL of plasma, the miRNeasy mini kit (Qiagen, Germany, CAT. No. 217184) was used as instructed by the manufacturer. A housekeeping gene (U6) was included in each sample and treated similarly to miRNA 182 to account for sample variation. The isolated RNA was stored at -80 °C until further analysis.

Quantitative real-time polymerase chain reaction was performed using SYBR green on the ABI StepOnePlus system (Applied Biosystems, Foster City, CA, USA). The experiment consisted of one cycle of 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 sec, and 56 °C for 1 min. The relative quantification of miR-182 expression differences between MBC and non-MBC samples was calculated using the (log $2^{-\Delta CT}$) method. The specific primer sequences employed can be found in Table 1.

CYFRA21-1 and CEA biomarkers measurement

CYFRA 21-1 and CEA were measured using enzyme-linked immunosorbent assay (ELISA). For CYFRA 21-1, polystyrene beads coated with the monoclonal antibody were mixed with serum. A standardized CYFRA 21-1 assay kit (ZellBio GmbH, Germany, CAT No. ZB-11619C-H9648) was used, and readings were obtained at 450 nm using a multi-mode microplate reader (BioTek, USA). Similarly, for CEA, polystyrene beads precoated with the antibody were incubated with serum. A standardized CEA assay kit (PISHTAZTEB, Iran) was used, and readings were obtained at 450 nm using a MultiMode Microplate Reader (BioTek, USA).

Statistical analyses

This study's statistical evaluation and figure generation involved using two software tools. These tools included SPSS version 25.0, developed by IBM in Armonk, NY, USA, and GraphPad Prism 9.0, developed by GraphPad Software in La Jolla, CA, USA. To determine differences between groups, the independent sample t-test was utilized. The diagnostic performance of the studied parameters was assessed through ROC curve analysis, and the sensitivity and specificity of each marker were calculated. The cfDNA, miR-182, CYFRA21-1, and CEA quantitative results were recorded as mean ± SD. *P*-values < 0.05 were considered statistically significant.

Table 1. Primers used in quantitative real-time polymerase chain reaction.

Gene	Primer sequence
MiR-182	Forward: 5'TTTGGCAATGGTAGAACTCACACT3'
	Reverse: Universal primer
U6	Forward: 5'CTCGCTTCGGCAGCACA3'
	Reverse: 5'AACGCTTCACGAATTTGCGT3'

RESULTS

Clinicopathological characteristics of patients

The study enrolled 46 patients with BC, divided into two groups: 29 metastatic patients (any kind of metastasis, including lymph vascular invasion, lymph node, and distant metastasis), and 17 non-metastatic. Table 2 gives an overview of the initial patient characteristics, such as demographics and clinicopathological features. The patients with BC were aged between 32 and 80 years with no statistically significant difference between the metastatic and non-metastatic groups. Tumor size > 2 cm was observed in 62.5% of cases (n = 30), and lymph vascular invasion was detected in 60.4% (n = 29). Among the metastatic patients, 60.4% had lymph node metastasis at diagnosis, while 26.1% had distant metastasis. Additional clinico-pathological characteristics, including tumor grade, stage, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) expression, and Ki67 proliferation index, are detailed in Table 2.

Plasma cfDNA as a potential prognostic marker in MBC

Plasma cfDNA concentration significantly increased in MBC compared to non-MBC

Although previous studies have suggested that cfDNA serve as a noninvasive biomarker for early breast cancer detection (17), its assessment in MBC compared to non-MBC has been relatively less investigated. The results of our study showed a statistically significant increase in the average concentration of cfDNA in MBC patients (1258.36 ± 433.5 ng/mL) compared to non-MBC (758.66 ± 142.09 ng/mL) (Fig. 1A).

Plasma cfDNA and clinicopathological features related to metastasis

Since there is a correlation between increasing tumor size and clinical stage, and the prevalence of metastases in breast cancer, we evaluated the relationship between cfDNA and these features in two patient groups (18). As shown in Table 3, there was a significant difference between the level of cfDNA in stage I-II (430.09 ± 69.85 ng/mL) and stage III-IV (1388.40 ± 88.69 ng/mL). Also, the cfDNA concentration was significantly elevated in patients with the tumor size more than 2 cm (1187.76 ± 91.10 ng/mL) compared the tumor size less than 2 cm (777.24 ± 204.48 ng/mL), and in lymph vascular invasion (1161.56 ± 111.31 vs. 791.23 ± 126.12 ng/mL), lymph node metastasis (1334.72 ± 104.54 vs. 810.09 ± 132.20 ng/mL), distant metastasis (1349.41 ± 125.56 vs. 656.10 ± 105.56 ng/mL), and Ki64 (1430.78 ± 97.43 vs. 604.52 ± 94.9 ng/mL). On the other hand, there is no significant relationship between cfDNA concentration and other clinicopathological parameters such as age, grade, and histology subtypes across the two study groups.

Table 2. Clinicopathological features of breast cancer patients. Data are presented as units (range) or n (%).

Variable	All patients (%)
Total	46 (100)
Median age (years, range)	32-80
> 50	29 (58.0)
≤ 50	17 (34.0)
Grade	
I	12 (26.1)
II	24 (52.2)
III	10 (21.7)
Clinical stage	
I-II	17 (35.4)
III-IV	29 (60.4)
Tumor size	
T2-T4 (> 2 cm)	29 (60.4)
T1 (≤ 2 cm)	17 (35.4)
Histology subtype	
Ductal	44 (91.7)
Lobular	2 (4.2)
Lymph vascular invasion	
Positive	29 (60.4)
Negative	17 (35.4)
Lymph node metastasis	
N0	17 (35.4)
N1	29 (60.4)
Distant metastasis	
M0	17 (35.4)
M1	12 (26.1)
ER	
Positive (> 50)	36 (75.0)
Negative (≤ 50)	10 (20.8)
PR	
Positive (> 50)	26 (54.2)
Negative (≤ 50)	20 (41.7)
HER2	
Positive	20 (41.7)
Negative	26 (54.2)
Ki67	
Positive (> 20)	22 (44.0)
Negative (≤ 20)	24 (48.0)

M, Metastasis; M0, absence of distant metastasis; M1, indicates distant metastasis; N, node; N0, no lymph node metastasis; N1, indicates lymph node metastasis; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

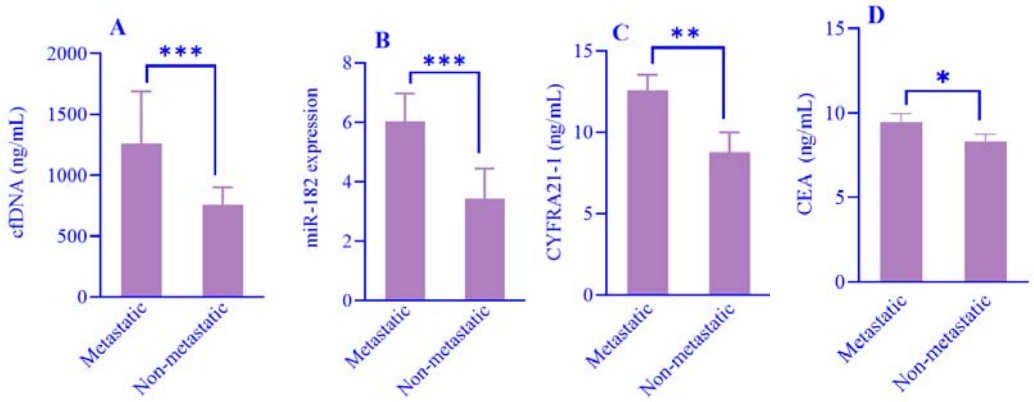

Fig. 1. Plasma concentrations of (A) cfDNA, (B) miR-182, serum concentration of (C) CYFRA21-1, and (D) CEA were analyzed in 29 patients with metastatic breast cancer, and 17 patients with non-metastatic breast cancer. Statistical analysis was performed by the independent sample t-test. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001 indicate significant differences between groups. cfDNA, Circulating cell-free DNA; miR-182, micro-RNA 182; CYFRA21-1, cytokeratin fragment antigen 21.1; CEA, carcinoembryonic antigen.

Table 3. The comparison between plasma cfDNA concentration, miR-182 expression, CYFRA21-1, and CEA concentration, and the clinicopathological features of breast cancer patients. The results represent the mean ± SD. Statistical analysis was performed with the independent-sample t-test (for two groups) and the one-way ANOVA test (for three groups). ***P*-values < 0.05 were considered statistically significant.

Variable	N	cfDNA (ng/mL)	miR-182 expression	CYFRA21-1 (ng/mL)	CEA (ng/mL)
Age					
> 50	22	1226.44 ± 124.42	6.34 ± 0.90	11.82 ± 1.35	9.95 ± 0.59
≤ 50	24	904.25 ± 132.98	3.95 ± 1.02	9.19 ± 1.01	8.90 ± 0.40
P-value		0.3696	0.0661	0.2568	0.2014
Grade					
I	12	1064.46 ± 195.61	4.97 ± 1.30	8.90 ± 1.74	8.85 ± 1.03
II	24	979.92 ± 104.69	5.43 ± 1.01	11.73 ± 1.12	8.75 ± 0.43
III	10	1240.88 ± 22.041	4.57 ± 4.84	9.22 ± 1.85	8.94 ± 0.38
P-value		0.518	0.883	0.290	0.977
Clinical stage					
I-II	17	430.09 ± 69.85	0.54 ± 1.19	6.42 ± 1.14	7.52 ± 0.12
III-IV	29	1388.40 ± 88.69	7.01 ± 3.98	12.80 ± 0.92	9.58 ± 0.50
P-value		< 0.001	< 0.001	< 0.001	0.0037
Tumor size					
T2-T4 (> 2 cm)	29	1187.76 ± 91.10	6.00 ± 0.83	11.74 ± 0.04	9.43 ± 0.48
T1 (≤ 2 cm)	17	777.24 ± 204.48	2.70 ± 1.05	8.03 ± 1.28	7.68 ± 0.31
P-value		0.0200	0.0055	0.0359	0.0166
Histology subtype					
Ductal	43	1061.49 ± 100.11	5.24 ± 0.71	10.71 ± 0.89	8.73 ± 0.34
Lobular	3	696.55 ± 118.72	0.23 ± 0.00	5.97 ± 2.9	7.66 ± 0.16
P-value		0.2778	0.0911	0.0610	0.3284
Lymph vascular invasion					
Positive	29	1161.56 ± 111.31	5.95 ± 0.80	12.30 ± 0.66	9.69 ± 0.52
Negative	17	791.23 ± 126.12	2.58 ± 1.13	9.27 ± 1.84	8.75 ± 0.62
P-value		0.0397	0.0333	0.0651	0.2706
Lymph node metastasis					
N0	17	810.09 ± 132.20	3.42 ± 1.04	8.69 ± 1.03	8.10 ± 0.38
N1	29	1334.72 ± 104.54	6.90 ± 0.78	12.74 ± 1.26	9.75 ± 0.58
P-value		0.0284	0.0119	0.0163	0.1175
Distant metastasis					
M0 (stage III)	17	656.10 ± 105.56	5.03 ± 0.85	9.11 ± 0.82	8.75 ± 0.45
M1 (stage IV)	12	1349.41 ± 125.56	5.34 ± 1.27	14.23 ± 1.93	9.02 ± 0.42
P-value		< 0.001	0.8411	< 0.001	0.5628

cfDNA, Circulating cell-free DNA; miR-182, micro-RNA 182; CYFRA21-1, cytokeratin fragment antigen 21.1; CEA, carcinoembryonic antigen; M, metastasis; M0, absence of distant metastasis; M1, indicates distant metastasis; N, node; N0, no lymph node metastasis; N1, indicates lymph node metastasis.

Plasma cfDNA and its association with ER, PR, and HER2 receptor in BC

Previous studies have demonstrated that these hormone receptors play critical role in BC progression and metastasis (19, 20). We assessed the relationship between plasma cfDNA and ER, PR, and HER2 receptors. No significant differences were observed in the cfDNA concentration between patients with positive versus negative receptors of these rceptors (Table 4).

Prognostic value of miR¦182 in MBC patients

Higher Plasma levels of miR-182 in MBC compared to non-MBC patients

miR-182 is an important oncogenic microRNA involved in BC development (21). To evaluate the effect of miR-182 overexpression on the metastasis of breast cancer, its expression was detected in the plasma of two groups. The results revealed that miR-182 expression in the MBC group was markedly increased, approximately more than two-fold higher compared to the non-MBC group (Fig. 1B).

Plasma miR-182 and clinicopathological features related to metastasis

The relationship between miR-182 and clinicopathological features was compared between the two patient groups. As summarized in Table 3, there was a significant difference between the level of miR-182 in the stage I-II (0.54 ± 1.19) and stage III-IV (7.01 ± 3.98). Also, the miR-182 expression was significantly different in patients with the tumor size more than 2 cm (6.00 ± 0.83) compared to the tumor size less than 2 cm (2.70 ± 1.05); In the case of lymph vascular invasion (5.95 ± 0.80 vs. 2.58 ± 1.13), lymph node metastasis (3.42 ± 1.04 vs. 6.90 ± 0.78), distant metastasis (1349.41 ± 125.56 vs. 656.10 ± 105.56) and Ki64 (1.69 ± 0.71 vs. 7.40 ± 0.78), a significant difference was also observed. However, no significant relationship was found between miR-182 expression and other clinical pathological parameters such as age, grade, distant metastasis, and histology subtypes in the two groups.

Plasma miR-182 and its association with ER, PR, and HER2 receptors in BC patients

After assessing the relationship between plasma miR-182 and ER, PR, and HER2 receptors. A significantly higher expression of miR-182 was observed in patients with HER2-negative status compared to HER2-positive tumors (6.47 ± 0.94 vs. 3.39 ± 0.94). No significant differences were found in miR-182 in the patients with positive versus negative ER or PR receptors (Table 4).

Table 4. The comparison between plasma cfDNA concentration, miR-182 expression, and serum CYFRA21-1, CEA concentration, and ER, PR, Ki67, and HER2 receptor of breast cancer patients. The results represent the mean ± SD. Statistical analysis was performed with the independent-sample t-test (for two groups) and the one-way ANOVA test (for three groups). *P*-values < 0.05 were considered statistically significant.

Variable	N	cfDNA (ng/mL)	miR-182 expression	CYFRA21-1 (ng/mL)	CEA (ng/mL)
ER					
Positive (> 50)	36	1000.96 ± 108.14	4.41 ± 0.76	10.27 ± 0.85	8.47 ± 0.38
Negative (≤ 50)	10	1229.48 ± 188.29	7.30 ± 1.51	11.07 ± 2.53	10.08 ± 0.77
P-value		0.3099	0.0788	0.0700	0.0594
PR					
Positive (> 50)	26	980.55 ± 145.10	4.13 ± 1.01	8.95 ± 0.91	8.48 ± 0.48
Negative (≤ 50)	20	1147.01 ± 108.48	6.15 ± 0.94	12.40 ± 1.46	9.25 ± 0.50
P-value		0.3854	0.1540	0.0105	0.2910
HER2					
Positive	20	1036.11 ± 153.90	3.39 ± 0.94	9.73 ± 1.19	7.82 ± 0.33
Negative	26	1065.87 ± 120.82	6.47 ± 0.94	11.00 ± 1.19	9.58 ± 0.52
P-value		0.8022	0.0278	0.1031	0.0116
Ki67					
Positive (> 20)	20	1430.78 ± 97.43	1.69 ± 0.71	7.45 ± 1.18	7.81 ± 0.18
Negative (≤ 20)	26	604.52 ± 94.9	7.40 ± 0.78	13.26 ± 0.88	9.66 ± 0.58
P-value		< 0. 001	0.0004	0.5559	0.0089

cfDNA, Circulating cell-free DNA; miR-182, micro-RNA 182; CYFRA21-1, cytokeratin fragment antigen 21.1; CEA, carcinoembryonic antigen; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

The role of circulating CYFRA21-1 in the early detection of MBC

Significant elevation of serum CYFRA 21-1 levels in MBC versus non-MBC patients

CYFRA 21-1 is monitored in various cancers due to its association with advanced-stage cancer disease, tumor cell migration, and metastatic progression (22). As shown in Fig. 1C, serum CYFRA21-1 levels were notably higher in MBC patients versus non-MBC patients (12.75 ± 0.97 vs. 8.77 ± 1.2 ng/mL, respectively).

Serum CYFRA 21-1 and clinicopathological features related to metastasis

As shown in Table 3, there was a significant difference between the level of CYFRA 21-1 in stage I-II (6.42 ± 1.14 ng/mL) and stage III-IV (12.80 ± 0.92 ng/mL) advanced disease. Serum CYFRA 21-1 was significantly different in patients with the tumor size more than 2 cm (11.74 ± 0.04 ng/mL) compared to the tumor size less than 2 cm (8.03 ± 1.28 ng/mL) as well as in lymph node metastasis (12.74 ± 1.26 vs. 8.69 ± 1.03 ng/mL), distant metastasis (14.23 ± 1.93 vs. 9.11 ± 0.82 ng/mL). In contrast, there is no significant relationship between miR-182 expression and other clinicopathological parameters such as age, grade, lymph vascular invasion, Ki64, and histology subtypes.

Relationship between Serum CYFRA 21-1 and ER, PR, and HER2 receptor in MBC patients

It was found that CYFRA 21-1 levels were significantly higher in the patients with negative PR receptors compared to those with positive PR receptors (12.40 ± 1.46 vs. 8.95 ± 0.91 ng/mL). No significant differences were observed in the CYFRA 21-1 in the patients with positive ER or HER2 receptors compared to those with negative receptors (Table 4).

Serum CEA association with metastatic characteristics in MBC

Elevated CEA marker levels in MBC patients

In patients with advanced cancer or metastatic cancer, a higher level of CEA has been observed compared to those with localized diseases (23). The findings of this study revealed that serum CEA levels were significantly higher in patients with MBC (9.47 ± 0.5 ng/mL) than in non-MBC patients (7.32 ± 0.4 ng/mL), as shown in Fig. 1D.

Serum CEA and clinicopathological features related to metastasis

As shown in Table 3, serum CEA concentration varied significantly across different stages. Patients in stages III–IV exhibited higher CEA levels (9.58 ± 0.5 ng/mL) compared to those in stages I–II (7.52 ± 0.12 ng/mL). Also, patients with tumors larger than 2 cm had higher CEA levels (9.43 ± 0.48 ng/mL) compared to those with tumors smaller than 2 cm (7.68 ± 0.31 ng/mL). CEA concentration was also higher in patients with positive Ki67 (7.81 ± 0.18 ng/mL) compared to those with negative Ki67 (9.66 ± 0.58 ng/mL). Serum concentration of CEA was not significantly correlated with clinicopathological characteristics, including distance metastasis, lymph node metastasis, age, grade, lymph vascular invasion, and histology subtypes across the studied groups.

Serum CEA levels associated with ER, PR, and HER2 receptors in BC patients

The expression of CEA was significantly higher in patients with negative HER2 receptor compared to those with HER2 positive receptor (9.58 ± 0.52 vs. 7.82 ± 0.33 ng/mL). No significant differences in CEA levels were observed between positive ER/PR receptors and ER/PR negative receptors (Table 4).

Sensitivity and specificity of plasma/serum cfDNA, miR-182, CYFRA21-1, and CEA in MBC and non-MBC patients

Sensitivity and specificity are critical indicators of a diagnostic test's accuracy and play a crucial role in determining its clinical utility (24). Sensitivity and specificity values of plasma/serum cfDNA, miR-182, CYFRA21-1, and CEA in distinguishing between MBC and non-MBC cases were compared. Table 5 shows that among the individual biomarkers, cfDNA demonstrated the highest sensitivity at 90%, followed by miR-182 (87%), CYFRA21-1 (82%), and CEA (80%). In terms of specificity, miR-182 exhibited the highest value at 80%, followed by cfDNA (79%), CYFRA21-1 (61%), and CEA (58%). Receiver operating characteristic (ROC) curve analysis was also performed to assess the diagnostic value of these tumor markers in the diagnosis of MBC, and the area under the curve (AUC) analysis was performed. According to Table 3 and Fig. 2, each tumor marker demonstrated significant diagnostic potential for MBC (AUC > 0.5).

Table 5. Sensitivity, specificity, and cutoff of single plasma cfDNA, miR-182, and serum CYFRA21-1 and CEA levels in metastatic breast cancer.

Tumor marker	Sensitivity (%)	Specificity (%)	Cutoff
cfDNA	90	79	934.7
miR-182	87	80	2.130
CYFRA21-1	82	61	11.21
CEA	80	58	8.77

cfDNA, Circulating cell-free DNA; miR-182, micro-RNA 182; CYFRA21-1, cytokeratin fragment antigen 21.1; CEA, carcinoembryonic antigen.

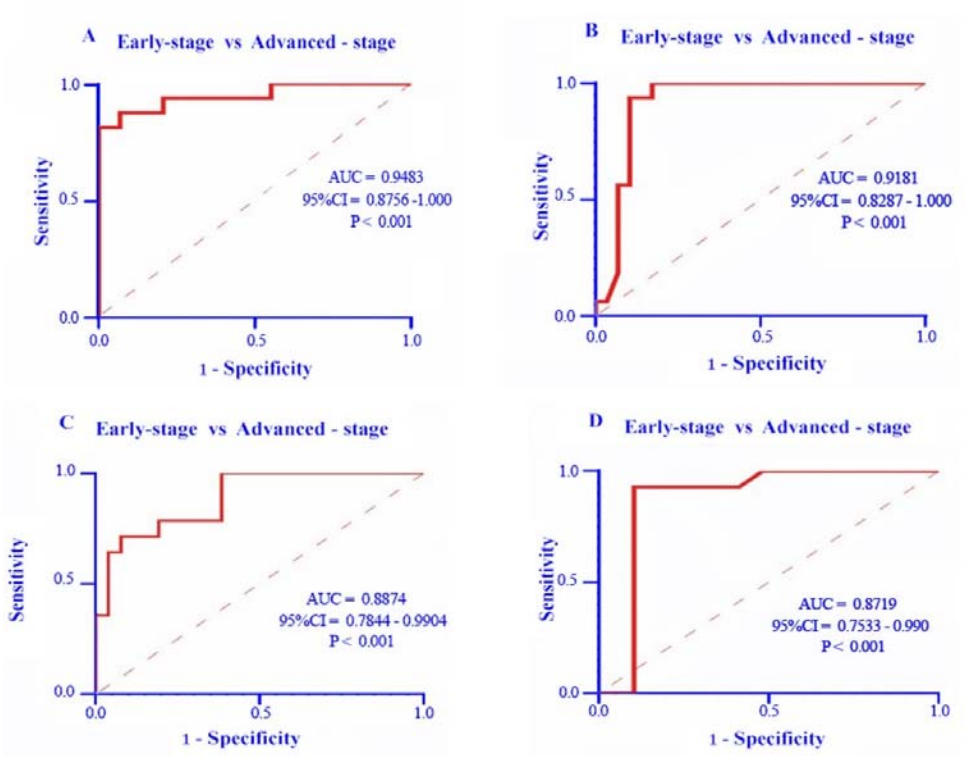

Fig. 2. ROC curve of (A) cfDNA, (B) miR-182, (C) CYFRA21-1, and (D) CEA. cfDNA, Circulating cell-free DNA; miR-182, micro-RNA 182; CYFRA21-1, cytokeratin fragment antigen 21.1; CEA, carcinoembryonic antigen.

For cfDNA, the area under the curve (AUC) was 0.94 (95% CI: 0.8756 - 1.000, $P < 0.001$), with a cutoff value of > 934.7 ng/mL. For miR-182, the AUC was 0.91 (95% CI: 0.8287-1.000, $P < 0.001$), with a cutoff value of > 2.130 ng/mL. For CYFRA21-1, the AUC was 0.88 (95% CI: 0.7844 - 0.9904, $P < 0.001$), with a cutoff value of > 11.21 ng/mL. Lastly, for CEA, the AUC was 0.87 (95% CI: 0.7533 - 0.9905, $P < 0.001$), with a cutoff value of > 8.77 ng/mL. cfDNA (AUC = 0.94) showed the highest AUC in single detection, followed by miR-182 (AUC = 0.91), CYFRA21-1 (AUC = 0.88), and CEA (AUC = 0.87).

DISCUSSION

Plasma and serum tumor markers are widely utilized in the clinical diagnosis and treatment of malignant tumors due to their role as important indicators of disease outcome monitoring, convenient, rapid, and cost-effective. In this study, tumor markers, such as cfDNA, miR-182, CYFRA 21-1, and CEA, were evaluated for their roles in cancer diagnosis, metastasis monitoring, and recurrence detection. However, large-scale clinical analysis and validation of these markers are still insufficient.

cfDNA is increasingly recognized for its clinical relevance in diverse cancer types. a study by Coombes *et al.* reported that plasma ctDNA achieved 89% sensitivity in detecting relapse among 16 out of 18 patients with MBC (25). Another study found that BC patients with higher levels of cfDNA reflected tumor characteristics (17). Furthermore, the reduction of cfDNA after chemotherapy was shown to aid in monitoring disease progression. Several studies have consistently found elevated levels of cfDNA in individuals with advanced gastric cancer compared to healthy individuals (26, 27). Similar associations were observed in thyroid cancer patients, where higher cfDNA levels were linked to tumor size (28). Advanced stages of ovarian cancer (29) and metastatic prostate cancer (30) also exhibited increased cfDNA levels and highlighted the potential of cfDNA as an early predictive marker. In addition, numerous studies reported significantly higher cfDNA levels in non-small cell lung cancer patients (31), pancreatic cancer patients (32), and colorectal cancer patients (16). Our result supports existing evidence that cfDNA levels vary according to disease stage and aggressiveness. These findings suggest that cfDNA has the potential to be a sensitive and specific diagnostic biomarker, as confirmed by ROC curve analysis showing high sensitivity and specificity.

Furthermore, another marker examined in this study is miR-182, which was found in higher concentrations among patients with MBC compared to non-MBC patients. It is noteworthy to mention that miR-182 plays diverse roles across various cancer types, functioning both as an oncogene and an anti-oncogene. It has also been identified as a biomarker in multiple cancers, such as prostate cancer (33), bladder cancer (34), urothelial carcinoma (35), and colorectal cancer (36). In BC, higher levels of miR-182 are associated with increased cell proliferation and migration, suggesting its potential as a diagnostic biomarker (37). Its overexpression may interfere with DNA recombination pathways, contributing to BC development. Another study found that the expression of miR-182-5p in the blood plasma of prostate cancer patients was significantly higher than in individuals with benign prostate hyperplasia (38). These results strengthened that miR-182 has the potential to be a valuable diagnostic index for MBC, especially when used in conjunction with other markers. ROC curve analysis also confirmed its high sensitivity and specificity after cfDNA.

CYFRA 21-1, derived from CK19, is a polypeptide released after cell death and serves as a distinctive marker for cancer diagnosis. It is also associated with tumor size, clinical stage, and lymph node involvement (39). Multiple studies have shown that MBC patients have higher levels of serum CYFRA21-1 (40). In cases of differentiated thyroid cancer, favorable outcomes characterized by complete thyroglobulin response and low levels of CYFRA21-1 were observed, while high levels of CYFRA21-1 increased the risk of recurrences and mortality rates (41). Elevated levels of CYFRA21-1 contribute to invasion and metastasis. BC patients showed a higher presence of atypical CYFRA 21-1 (9). Another study revealed significant differences in CYFRA21-1 levels across lung cancer stages I-III, as well as between stages III and IV (42).

Our study indicates that serum CYFRA21-1 can be used as a biological indicator for assessing BC occurrence, progression, and prognosis, irrespective of treatment modality. ROC curve analysis also showed its high sensitivity and specificity after cfDNA and miR-182.

CEA can be detected in various types of cancer tissues in adults and has been utilized for tasks such as assisting in diagnosing, monitoring treatment effectiveness, predicting prognosis, and anticipating cancer recurrence. Numerous studies have also reported elevated serum levels of CEA in MBC patients (43).

However, CEA alone lacks sufficient specificity for diagnosing BC, as reported by Kabel *et al.* (23). However, the prognostic significance of CEA levels varies by BC subtypes. For instance, elevated preoperative CEA levels are considered a significant prognostic factor in luminal BC, but don't hold the same predictive value in triple-negative breast cancer (44).

A decline in the CEA concentration could serve as a reliable indicator of immunotherapy efficacy in patients with non-small cell lung

cancer. On the other hand, a study reported that the median serum CEA values for lung fibrosis, pancreatic cancer, uremia, and chronic obstructive pulmonary disease were higher than those in rectal and colon cancers (45).

CONCLUSIONS

Our findings revealed significantly higher concentrations of cfDNA, miR-182, CYFRA21-1, and CEA in women with MBC compared to those without MBC. Higher levels of these markers were linked to advanced clinical stage and larger tumor size. Some showed correlations with the breast receptors, which play key roles in metastasis. Increased cfDNA, miR-182, and CYFRA21-1 levels were identified as risk factors for lymphatic metastasis, while elevated cfDNA and CYFRA21-1 levels were associated with distant metastasis. To our knowledge, the analysis of ROC curves revealed that the combination assessment of cfDNA, miR-182, CYFRA21-1, and CEA enhances the diagnostic accuracy and sensitivity of MBC.

Acknowledgements

The authors would also like to acknowledge the Iran National Tumor Bank, Cancer Institute of Tehran University of Medical Sciences, for their contribution of the samples.

The content of this paper was extracted from the Ph.D thesis submitted by Z. Salimi, which was financially supported by the Vice-Chancellery for Research of Isfahan University of Medical Sciences, Isfahan, Iran, through Grant No. 54945.

This work is also funded by the Iran National Science Foundation (INSF) under project number 4003196.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

Z. Salimi carried out all the experiments under the supervision of M. Aghaei and M. Panjehpour. Z. Salimi wrote the manuscript under the supervision of M. Panjehpour, who conceived the study and was the principal investigator in the management and leadership of the research project. R. Fatehifard contributed to patient and sample identification. All authors read and approved the final manuscript.

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