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Exploring the miRNA-148b and the caspase-3/Bcl-2/Bax axis as a potential predictive marker in breast cancer

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RESEARCH ARTICLE

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ABSTRACT

Breast cancers (BCs) are the second leading cause of cancer-related deaths among women due to a lack of prediction, diagnosis, and follow-up. MicroRNAs (miRNAs) in liquid biopsies (LBs) are promising tools for the prediction and follow-up of cancer. This study aims to investigate and compare serum miRNA-148b, caspase-3, Bax, Bcl-2, and total antioxidant capacity (TAC) of BC patients with healthy controls. In this study, 300 women were included and divided into four groups of 75 each: Group 1 consisted of healthy controls, Group 2 of early-stage BC patients, Group 3 of chemotherapy-treated BC patients, and Group 4 of mastectomy-treated BC patients. Blood samples were collected for a complete blood count and serum samples were tested for miRNA-148b Bax, caspase-3, Bcl-2, and TAC. RT/PCR, ELISA and spectrophotometric methods were used to determine these parameters. In addition, histopathological examinations were performed on breast tissue samples. The present results indicated that BC patients exhibited elevated miRNA-148b, Bax, and Bcl-2 expressions compared to healthy controls. Importantly, advanced BC stages showed significantly higher miRNA-148b levels than early stages. However, levels of caspase-3 and TAC were reduced in BC patients compared to healthy controls. Histopathological analysis revealed various alterations in breast tissues, including nuclear changes, the presence of giant cells, and inflammation. The present study concluded that miRNA-148b and Bcl-2 are markedly elevated in the serum of BC patients compared to healthy subjects; however, Bax and caspase-3 levels were reduced. These findings underscore that blood miRNA-148b and caspase-3 are promising avenues for the prediction and follow-up of BC patients.

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1. Introduction

Breast cancer (BC) is the most common form of cancer in women worldwide and the leading cause of cancer-related death [1,2]. BC risk factors include genetic, biological, and environmental factors [2,3]. BC genetic risk factors are members of biological factors, including mutations in the breast cancer gene, the breast cancer gene 2, and checkpoint kinase 2 [4]. Moreover, BC's risk factors include obesity, alcohol consumption, smoking, hormone, and radiation exposure [5]. BCs are classified into carcinomas and sarcomas; breast carcinomas are further subcategorized into in situ and invasive carcinomas [6,7]. Understanding the BC subtypes can potentially treat patients with BC [6,7]. BC is a life-threatening disease due to the lack of prediction, diagnosis, and targeted therapy, in addition to BC metastasis and multidrug resistance [6,7]. Despite the applicability of ultrasound diagnostics, magnetic resonance imaging, and other techniques for BC diagnosis, these methods are invasive and unsuitable for BC cancer prediction and monitoring [8]. Therefore, discovering noninvasive and better prediction methods, diagnosis, follow-up, and tumor characterization is a critical issue [8].

Interestingly, liquid biopsies (LB) pinpoint the primary origin of cancer and are promising tools for predicting and monitoring cancer patients [9,10]. LBs are samples from biofluids such as blood, urine, saliva, pleural effusions, and cerebrospinal fluids [9]. LBs have been designated to predict, diagnose, and monitor therapy responses in patients with BC instead of tissue biopsies (TBs) [10]. LBs offer several advantages over TBs, including being easy to obtain, painless, and allowing the measurement of various tumor biomarkers [11]. Thus, biomarkers in LBs apply to the predicting, diagnosing and

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following malignant conditions in BC patients [10]. Therefore, LBs are suggested as alternative noninvasive tools instead of TBs in cancer management [9]. However, TBs and other technologies for BC detection are invasive and have many limitations [12].

In practice, LBs are noninvasive and obtained from tumor tissues in an efficient quantity and quality of biomarkers [9]. Consequently, LBs could resolve the problems of TBs by enabling first tumor detection, prognosis, and recurrence potential [9]. In particular, cancer cells release their signature into body fluids [9]. The cancer signature includes intact cancer cells, extracellular vesicles (EVs), deoxynucleic acids (DNA), ribonucleic acids (RNAs), proteins, lipids, or metabolites [9,10]. These cellular, vesicular, and molecular structures in LBs could provide information on cancer status [9,10]. Ample cancer biomarkers such as tumor cells, tumor-educated platelets, and EVs encapsulated tumor DNA, messenger (mRNAs) or microRNAs (miRNAs) can be measured in LBs, in addition to free cancer DNA, RNA, miRNAs, proteins, or metabolites [9, 10]. Therefore, LBs are applied as noninvasive biomarkers in prediction, diagnosis, screening, prognosis, and patient response to therapy [13,14]. Biomarkers in LBs are suggested to monitor patients with BC [13,14]. However, LBs from BC patients contain tumor cells, oncosomes, proteins, DNA, mRNAs, and miRNAs [13,14].

miRNAs are members of molecular cancer signatures in LBs; architecturally, miRNAs contain about 22-25 nucleotides decoding RNAs. miRNAs are modulators of gene expression at the genome, transcriptome, and proteome levels [15]. Thus, miRNAs are essential for health and disease progression [15]. In this context, miRNAs can affect gene expression by influencing mRNA transcription and translation [16,17]. However, miRNAs could interact with target mRNAs or DNA to influence gene expression [16,17]. Therefore, miRNAs are involved in cell metabolism, growth, proliferation, differentiation, development, and death [16,17]. In this context, miRNA expression is dysregulated in malignant cells. Thus, miRNAs might act as oncogenes or tumor suppressor agents [15]. Consequently, miRNAs play crucial roles in tumorigenesis, metastasis, and apoptosis [17].

At the functional level, some miRNAs are oncogenes and mediate cancer proliferation, metastasis, and multidrug resistance [18]. On the contrary, other miRNAs are tumor suppressors that mediate apoptosis to inhibit the growth and development of cancer cells [18]. Furthermore, miRNAs influence autophagy and the epithelial-mesenchymal transition [19]. Furthermore, miRNAs regulate cancer cells angiogenesis, migration, invasion, and metastasis [17,20]. In this regard, miRNAs induce contradictive effects in breast cancer cell lines [19]. Therefore, expression miRNAs display specific patterns in BC subtypes [18]. Consequently, miRNAs could be used as biomarkers for BC prediction, diagnosis, and typing of BC [18]. Frequently, miRNAs are liberated in free form or packaged into EVs in biofluids. In this regard, abundant studies have demonstrated the presence of miRNAs in biofluids at easily measurable levels [21,22]. For example, blood miRNAs can be easily collected and measured as non-invasive diagnostic biomarkers. Several studies have suggested miRNAs as innovative biomarkers for predicting and detecting BCs [23]. Specifically, miRNA-148 is overexpressed in many cancers, including BCs [15]. More studies are needed to address this issue. Certainly, BCs are associated with altering apoptosis mechanisms; thus, malignant transformed breast cells overexpressed pro-survival proteins with down expression of pro-apoptotic proteins [24]. Hence, Bcl2/Bax equilibrium disruption is connected with breast cell tumorigenesis [25]. Bax is a pro-apoptotic protein that plays a vital role in cell apoptosis [25]. Bax increases the permeability of the mitochondrial membrane to release cytochrome C [25]. The release of mitochondrial cytochrome C provokes redox imbalance and oxidative stress that mediate DNA damage and apoptotic cell death. Caspases are also activated in response to apoptotic signals [25]. On the contrary, the B-cell lymphoma two gene (Bcl2) antagonizes the Bax action and induces an antiapoptotic effect to provoke oncogenesis [25]. Thus, Bcl2 is commonly upregulated in several types of tumors to encourage cancer development [24]. Furthermore, the overexpression of Bcl2 is mediated by oxidative stress, microbial infection, exposure to xenobiotics, and nutrient deprivation [24]. These factors act together to induce the alteration of the Bcl2/Bax balance [24]. Furthermore, Bcl2 expression is triggered by the nuclear factor kappa B family, Janus kinase, and signal transduction activators of transcription and various cytokines [24]. Higher or lower levels of apoptotic parameters could be leveraged as biomarkers for the predicting, diagnosing, or monitoring of BC [26]. Furthermore, variations in the activity of apoptotic parameters were demonstrated in response to cancer therapy. This may provide insights into treatment effectiveness [26]. Consequently, posttreatment elevation of caspases indicates induction of apoptosis in malignant cells with better survival rates in cancer patients [26].

Despite the success of conventional technologies, and TBs for the predicting and detecting BC, they are invasive methods and have several limitations [12]. Therefore, discovering noninvasive and novel biomarkers is critical for the early detection and control of BC [23]. In this context, miRNAs in LBs are suggested as promising noninvasive biomarkers for BC management [18,27]. Consequently, this study aimed to investigate miRNA-148b in patients with BC as a non-invasive biomarker for the prediction and follow-up of BC. Consequently, miRNA-148b, caspase-3, Bax, Bcl-2, and total antioxidant capacity (TAC) were investigated in female BC patients.

2. Experimental

2.1. Study design and ethical approval

This is a comparative control study that enrolled 300 female patients. This study was conducted in the outpatient clinic of Damanhour Oncology Center from December 2021 to February 2023. The Research Ethics Committee of the Faculty of Pharmacy, Damanhour University, Egypt, approved the study (Ref. No. 620PB20). The study adhered to the Helsinki Declaration of ethical principles for research involving human subjects.

2.2. Subjects

The goals and procedures of this study were clarified in verbal and written consent for each of the study participants. Information including age, family history of BC, hypertension, diabetes, other malignancies, and menopausal symptoms was obtained from the medical records of the patients. Furthermore, the tumor size of all participants was reported. The female enrolled in this study does not have diabetes, connective tissue diseases, autoimmune diseases, other malignancies, cardiac, respiratory, renal diseases, or other diseases. The 300 females were classified into four groups: Group I (control): included 75 healthy volunteers. Group II (early stage): this group included 75 early-stage BC patients. Group III (chemotherapy): This group included 75 patients with BC receiving chemotherapy (doxorubicin and cyclophosphamide (60 and 600 mg/m^2) IV every 3 weeks/4 cycles, respectively [28]. Group IV (surgery): included 75 patients with BC who were treated surgically (mastectomy involves the removal of the entire breast, including tissue and skin). Figure 1 shows the studied groups.

2.3. Blood sample collection

Blood samples (6 mL) were collected from the peripheral veins of the participants.

Table 1. The primers sequence of miRNA-148b, Caspase-3, Bcl2 Bax, and U6.

Primer name	Sequences
Primer of miRNA-148b	
Tm = 59.3	F: 5′CATCGCCCTGTGGATGACTG3′
Tm = 58.9	R: 5′GGCCATATAGTTCCACAAAGGC3′
Primer of caspase-3	
Tm = 60.6	F: 5′ TGTCCTGGGACACCGGTTAT3′
Tm = 60.1	R: 5′ TCTGTTGCCACCTTTCGGTT3′
Primer of Bcl-2	
Tm = 57.1	F: 5′AAATACAACATCACAGAGAAGT3′
Tm = 60.1	R: 5′ TCCCGGTTATCGTACCCTGT3′
Primer of Bax	
Tm = 60.02	F: 5′GTCTCCGGCGAATTGGAGAT3′
Tm = 59.83	R: 5′ACCCGGAAGAAGACCTCTCG3′
Primer of U6	
Tm = 60.5	F: 5′TTGTGGGCAAGGATGTGGTC3′
Tm = 60.4	R: 5′CCGCATCCTGTAGCAACTGT3′

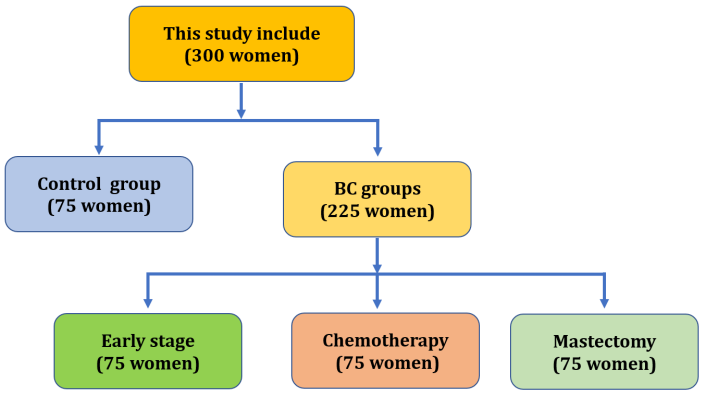

Figure 1. Study design.

A 1 mL was collected using a complete aseptic technique into an ethylenediaminetetraacetic acid (EDTA) tube for a complete blood count (CBC) assessment. The remaining 5 mL was collected in a tube containing separated gel, followed by centrifugation at 5000 rpm for 10 minutes to separate the serum. The serum collected was stored at -80 °C to evaluate the miRNA-148b and Bax genes using the quantitative real-time PCR technique (qRT-PCR). However, the caspase-3 and Bcl-2 proteins were measured by the ELISA technique. Spectrophotometry was used to determine the TAC. CBC was evaluated with the ADVIA-2120 hematology analyzer® (Siemens Healthcare, Diagnostics, USA), according to the manufacturer's protocol.

2.4. Gene expression study

The miRNA-148b, caspase-3, Bcl2 and Bax genes were analyzed using qRT-PCR. Firstly, the total RNA was extracted from the serum of all samples [29]. The samples were processed using miRNA extraction kit # 217004 (QIAGEN, Germany) according to the manufacturer's protocol. The extracted RNA concentration and purity were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) by measuring the absorbent wavelengths from 260 to 280 nm. The concentration of isolated RNA was expressed as µg/m. An aliquot of extracted total RNA (1 µg of RNA) was reverse transscribed into cDNA using the RT / PCR preMix kit # 122020-96.

The expression of the miRNA-148b, Caspase-3, Bcl2 and Bax genes was analyzed using qRT-PCR, SYBR Green and Master Mix (Thermo Fisher, Scientific, Inc., Waltham, MA). Expression cycles proceed as follows: activation step at 95 °C for 10 minutes; denaturation at 95 °C for 15 seconds repeated for 40 cycles; annealing at 56 °C for 15 seconds; extension at 72 °C for 30 seconds; and final extension at 72 °C for 10 minutes. The experiments were repeated three times (triplet). The relative expressions of miRNA-148b, Caspase-3, Bcl2 and Bax were presented as fold changes relative to U6, and the results were calculated using the $2^{-\Delta\Delta CT}$ method [30,31]. Table 1 displays the primer sequence of miRNA-148b, Casp-3, Bcl2, Bax, and U6.

2.5. Assessment of caspase-3

Caspase-3 activity was evaluated using the ELISA kit #E-EL-H0017 (Elabscience Biotechnology Company, USA). Briefly, 100 µL of standard, blank and sample was added and incubated for 90 minutes at 37 °C. Then 100 µL of biotinylated detection Ab working solution was added and incubated for 10 minutes at 37 °C. Then 350 µL of wash buffer was added to each well, soaked for 1 minute, and the solution was aspirated from each well. After that, 100 µL of HRP conjugate working solution was added and incubated for 30 minutes at 37 °C. Substrate reagent (90 µL) was added and incubated for 15 minutes at 37 °C. A 50 µL stop solution was added and the optical density was measured using a microplate reader at 450 nm. Caspase-3 concentration was calculated using the standard curve.

2.6. Assessment of Bcl-2

The Bcl-2 ELISA kit #CS0520 was used to determine serum Bcl-2 levels. Standard diluent standards samples (100 µL) were added to the appropriate wells, and 100 µL of anti-Bcl-2 detection antibodies were added and incubated for 1 hour at room temperature. Firstly, 100 µL of anti-rabbit IgG-HRP working solution was incubated for 30 minutes at room temperature. 100 µL of stabilized coloring agents were then added and incubated for 30 minutes at room temperature. Finally, 100 µL of the stop solution was added and mixed well. The optical density was recorded at 450 nm. Bcl-2 concentrations were determined by interpolation from the standard curve.

Table 2. Clinical data on age, tumor size, history of BC, systolic blood pressure, FBG, and PPG of the subjects studied *.

Parameters	Control	Early-stage	Chemotherapy	Surgery
Age	47.88±5.42	48.24±7.46	48.70±3.72	49.40±6.07
Tumor size (cm^2)	-	1.826±0.89	1.520±0.51	15.54±4.01
History of BC	No	No	No	No
Hypertension	117.30±8.32	126.90±7.85	109.50±6.14	113.70±4.89
FBG	91.16±6.74	87.04±4.15	93.77±4.41	83.06±5.18
PPG	134.26±3.02	137.63±5.19	125.87±5.74	114.86±4.19

A one-way analysis of variance (ANOVA) followed by Tukey post hoc test was used in data analysis. Data were expressed as mean±SD, N = 75. $p \leq 0.05$ was used to compare the significance between the groups studied.

Table 3. Complete blood count parameters in terms of Hb level and lymphocytes and platelet numbers of the studied subjects *.

Parameters	Control	Early-stage	Chemotherapy	Surgery
Hb (g/dL)	12.67±0.68	12.42±0.94	11.07±1.18	11.64±1.03
Lymphocytes	29.47±4.35	56.30±6.24	58.40±8.94	50.48±5.81
Platelets ($10^3/cm^2$)	183.40±14.8	130.30±8.69	96.99±10.06	118.00±8.10

* One-way ANOVA followed by Tukey post-hoc test was used in data analysis. Data were expressed as mean±SD, N = 75. $p \leq 0.05$ was used to compare the significance between the studied groups.

Table 4. Summary and correlation of the CBC parameters, as well as miRNA148b, Bax, caspase-3, Bcl2, and TAC of the studied subjects

Parameters	Control	Early-stage	Chemotherapy	Surgery
Hb	Normal	Normal	Decreased*	Decreased*
Lymphocytes	Normal	Increased**	Increased**	Increased*
Platelets	Normal	Decreased*	Decreased***	Decreased**
MiRNA-148b	Normal	Increased*	Increased**	Increased***
Bax	Normal	Increased***	Increased**	Increased*
Caspase-3	Normal	Decreased*	Decreased***	Decreased**
Bcl-2	Normal	Increased*	Increased***	Increased**
TAC	Normal	Decreased*	Decreased**	Decreased***

* There is a positive correlation between miRNA-148b, Bcl2, and Bax. However, a negative correlation exists between miRNA-148b, Bcl2, and Bax compared to Caspase-3 and TAC. On the contrary, there is a positive correlation of caspase-3 and TAC. Mild changes
** Moderate changes
*** Sever changes. Abbreviations: Hb; Hemoglobin, TAC; Total antioxidant capacity.

2.7. Measurement of TAC levels

Serum TAC was determined using TAC Assay Kit #MAK 187. Trolox standard solution (1 nmol/µL) (0, 4, 8, 12, 16, and 20 µL) and 50 µL samples were added to water to bring the volume to 100 µL. 100 µL of cupric ions (Cu^{2+}) working solution was then added to all standards. The samples were mixed well and incubated for 90 minutes at room temperature. The optical was measured at 570 nm. The Trolox standards values were used to plot a standard curve.

2.8. Histopathological investigations

After excision, human breast tissue was washed with a 0.9% sterile saline solution and immediately fixed in a 10% formalin solution. Formalin-fixed tissue specimens were transferred to 70% ethanol and embedded in paraffin. They were cut into 5 µm thick sections and mounted on positively charged glass slides. The tissue sections were stained with hematoxylin and eosin (HE) and examined under an optical microscope to detect pathological changes [32].

2.9. Statistical analysis

Data were investigated using GraphPad InStat software, version 4 (GraphPad, ISI Software Inc., La Jolla, CA, USA). The results were compared using a one-way analysis of variance. Data were expressed as mean±SD and p-value < 0.05 was used as a significance criterion.

3. Results

3.1. Demographic and clinical characteristics

Table 2 presents the demographic data and clinical characteristics of the participants. All patients and controls were females. The results showed that the surgery-treated group had significantly larger tumors than those of the other groups (15.54±4.01 cm^2). Tumor size was reported to increase between early-stage and chemotherapy-treated groups (1.826±0.89 and 1.520±0.51 cm^2, respectively) compared to the control.

3.2. Blood count

Table 3 presents the CBC parameters. Hemoglobin levels were found to be significantly decreased in both chemotherapy and surgery groups (11.07±1.18 and 11.64±1.03 g/dL; $p < 0.001$), respectively, compared with the control group, and no significant differences were observed in hemoglobin levels of the early stage group compared with the control group (12.42±0.9 g/dL; $p > 0.05$).

The levels of white blood cells (WBCs) and lymphocytes were significantly increased between patients in early-stage, chemotherapy, and surgery groups (56.30±6.24, 58.40±8.94, and 50.48±0.81; $p < 0.001$), respectively. Platelet count was significantly decreased in patients in the early stage, chemotherapy and surgery group patients (130.3±8.69, 96.99±10.06, and 118±8.10 * $10^3/cm^2$; $p < 0.001$), respectively.

3.3. miRNA-148b, caspase-3, Bcl-2 and Bax expression

The expression of miRNA-148b was significantly increased in the early stage, chemotherapeutic and surgery-treated groups (3.684±1.10, 5.260±0.81, and 6.165± 1.00), respectively, compared with the control group. The surgery-treated group resulted in a more significant up-regulation of miRNA-148b expression than the control group (Figure 2a). Regarding the expression of caspase-3, there is a substantial decrease in the early-stage, chemotherapeutic, and surgery treated groups (0.681±0.039, 0.456±0.026, and 0.554±0.052), respectively, compared with the control group (Figure 2b). Similarly, there was a significant increase in Bcl-2 expression in early-stage, chemotherapeutic and surgery treated groups (2.427±0.104, 2.419±0.07, and 2.819±0.09), respectively, compared to the control group (Figure 2c). Furthermore, Bax expression was significantly decreased in the early-stage, chemotherapeutic,

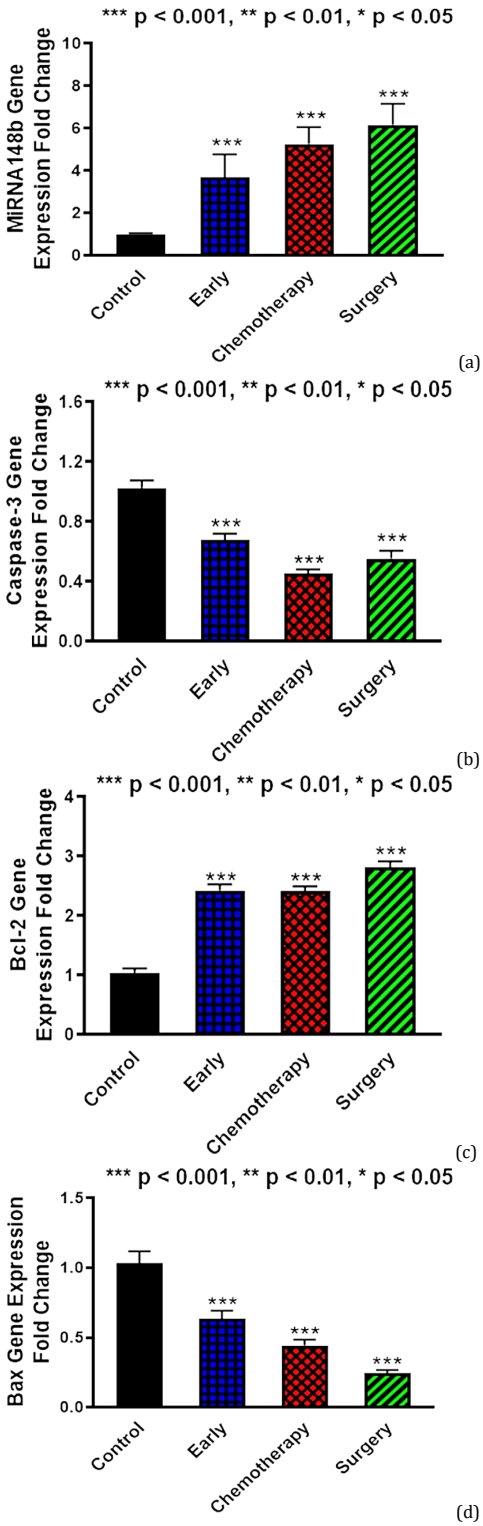

Figure 2. (a) Expression of miRNA-148b, (b) Expression of caspase-3, (c) Expression of Bcl-2, (d) Expression of Bax in all study groups. The expression of miRNA-148b, caspase-3, Bcl-2, and Bax was normalized with U6. Data were expressed as mean±SD (n = 75). In data analysis, one-way ANOVA followed by Tukey post hoc test at $p < 0.05$ was used. * Significant difference from the control group.

and surgery-treated groups (0.638±0.06, 0.445±0.04, and 0.245±0.02) compared to the control group (Figure 2d).

3.4. Caspase-3 and Bcl-2

Serum levels of caspase-3, Bcl-2, and TAC are presented in Figure 3. There was a significant decrease in caspase-3 levels in both chemotherapy and surgery-treated groups (2.302±0.75 and 2.773±0.84) compared to the control group (6.016±0.6672),

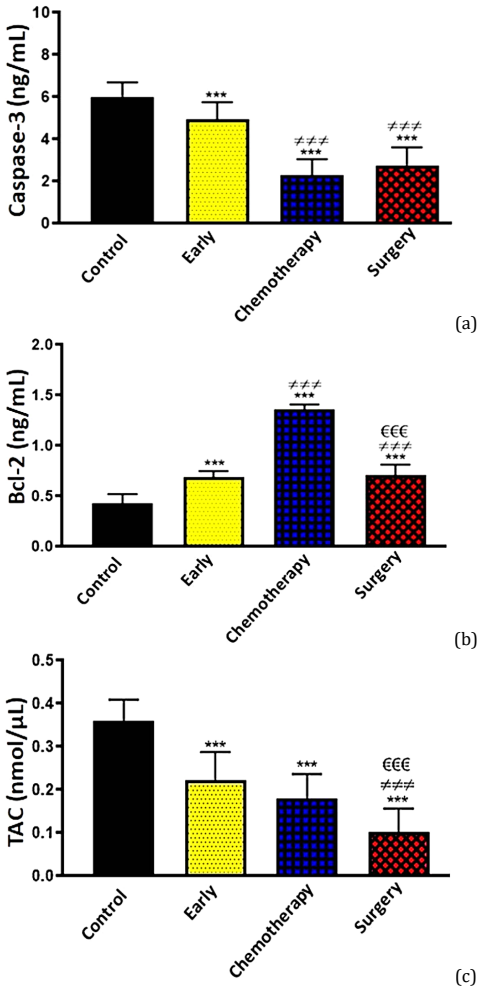

Figure 3. (a) Caspase-3 levels, (b) Bcl-2 levels in all study groups using the ELISA technique with specific antibodies, and (c) levels of TAC in all study groups using a spectrophotometry assay. One-way ANOVA followed by Tukey posthoc test was used for data analysis; the data were expressed as mean±SD (n= 75). $p < 0.05$ was used as a criterion of significance. * Significant difference from the control group. ≠ Significant difference from the early stage group. € Significant difference from the chemotherapy group.

Figure 3a. In addition, serum caspase-3 in the early-stage group decreased but with less degree (4.933±0.82) compared with the control group; see Figure 3a. On the contrary, the serum Bcl-2 level was significantly increased in the chemotherapy and surgery treated groups (1.36±0.05 and 0.71±0.11) compared to the control group (0.43±0.09). Similarly, in the early-stage group, levels of Bcl-2 increased in serum (0.69±0.06); these findings were displayed in Figure 3b.

3.5. TAC

The present results showed that serum TAC levels decreased significantly in the early-stage group (0.22±0.06), chemotherapy (0.18±0.08), and surgery-treated groups (0.11±0.05) compared to the control group (0.36±0.05) as presented in Figure 3c. The current investigation shows a positive correlation between miRNA-148b, Bcl2, and Bax*. However, a negative correlation exists between miRNA-148b, Bcl2 and Bax compared to Caspase-3 and TAC. On the contrary, there is a positive correlation of caspase-3 and TAC. Table 4. Summary and correlation of the CBC parameters and miRNA148b, Bax, caspase-3, Bcl2, and TAC.

3.6. Histopathological findings

The histopathological result showed typical structures of luminal and myoepithelial cells. Moreover, regular, small, rounded hyperchromatic nuclei in the control group, see Figure 4a. On the contrary, breast tissues obtained from early-stage BC patients showed histopathological changes regarding heterogeneous cells and granulomatous inflammation, Figure 4b. Furthermore, the tissue from the chemotherapy-treated group showed changes in nuclear features, size, and shape (Figure 4c); similarly, the tissue from the surgery group showed giant cells, polymorphonuclear cells, and central inflammation (Figure 4d).

3. Discussion

BC is a complicated health problem due to a lack of prediction, relapse, metastasis, and drug resistance [33]. miRNAs are dual-edged cancer inhibition, oncogenesis, metastasis, and drug resistance of BC [34]. In this regard, miRNA-26a, -26b, -125a, -125b, -145, -486-5p, -497, -874, and -99a are proposed as pro-apoptotic miRNAs and are down-regulated in BC patients [35]. However, miRNA-21, -155, -96, -182, -196a, and -210 are proposed as antiapoptotic miRNAs upregulated in BC patients [35]. Thus, dysregulation of miRNAs is an aspect of the oncogenesis and development of BC [19]. Despite the success of classical tools in predicting, diagnosing, and prognostic BC, most of these methods are invasive and still have abundant limitations [36].

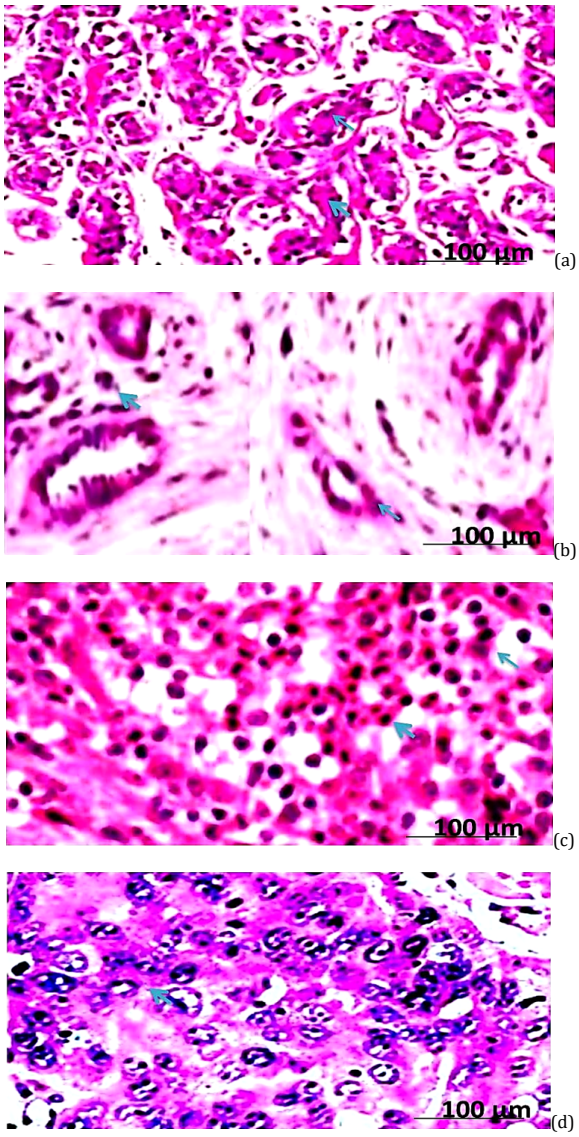

Figure 4. Histopathological changes: (a) The control group shows normal structures of luminal and myoepithelial cells. (b) Early-stage BC group showing heterogeneous changes in breast tissues (arrow). (c) The chemotherapy-treated group showed changes in nuclear features (size and shape) (arrow). (d) The surgery group showed two giant cells and central inflammation (arrow). Slides were examined using H&E stain with magnification ×40 and scale bar= 100 µm.

Several studies reported that LBs miRNAs act as novel non-invasive biomarkers for BC diagnosis. However, miRNA correlated with positive estrogen receptors (ER) and negative ER BC cells [37]. Thus, miRNA measurement in LBs could be a non-invasive tool to predict BC [36,37]. Furthermore, a previous study indicated that miRNAs are overexpressed in BC cells [36]. Several miRNAs such as miRNA-148b-3b, miRMA-190b, miRNA-429, and other miRNAs have also been reported as a signature for the diagnosing BC [36]. Specifically, miRNA-148b shows a significant relationship with the survival time of BC patients [36]. Thus, miRNAs are suggested as a non-invasive biomarker for BC prediction, diagnosis, and improved BC patient survival [38].

In the present study, miRNA-148b transcriptomes were significantly overexpressed in the surgery group compared to the early-stage and chemotherapy groups. Furthermore, there was a significant overexpression of miRNA-148b in the chemotherapy group compared to the early-stage group ($p < 0.05$). These results are in conjunction with several studies that proposed miRNAs as noninvasive biomarkers for predicting BC [37]. This indicated that miRNA-148b expression was higher in the advanced BC stage than in the early stage [39,40]. Consequently, miRNA-148b could be considered a biomarker prediction and prognosis of BC [39-41]. The results of the present work are in sync with the results of Cuk et al., who confirmed the overexpression of miRNA-148b in the plasma of patients with BC [42]. In the same context, the observation of Nassar et al. is in parallel with the present study's finding regarding the upregulation of miRNA-148b in BC. [43]. The current results support the suggestion of miR-148b up-regulation in cancer. A previous study demonstrated that miRNA-148b is overexpressed in BC [36]. Likewise, miRNA-148b has been reported to be elaborate in worsening some types of cancers [44]. The present findings are align with several miRNAs investigated in biofluids as non-invasive biomarkers for cancer prediction [38]. Specifically, the plasma level of miRNAs was suggested as a non-invasive cancer biomarker [38]. In this regard, plasma miRNA-30b-5p was indicated as a biomarker for BC. Plasma miRNA-99a-5p was also measured as a biomarker for numerous cancers [38].

The imbalance of pro-survival, such as Bcl-2, and pro-apoptotic proteins, such as Bax, is correlated with the development of various cancers and poor prognosis [24]. Therefore, the study of Bcl-2, Bax, and caspase-3 indicates cell

apoptosis and the decreased Bcl-2/Bax ratio means the initiation of apoptosis [45]. Upregulation of the Bcl-2 gene renders tumor cells grow and resistant to anticancer agents. Therefore, Bcl-2 could be a valuable indicator for cancer detection [24]. In the current study, Bax was significantly overexpressed in the early-stage group compared to the chemotherapy and surgery groups. Furthermore, Bax was significantly overexpressed in the chemotherapy group compared to the surgery group. The current results are agree with the results published by Pluta *et al*., which confirmed that Bax is one of the mechanisms of evasion of apoptosis by tumor cells [45]. The increase of the Bax gene designated the initiation of apoptosis at the early stages of BC compared to the surgery and chemotherapy groups. Similarly, the present results confirmed that Bcl-2 increased significantly in chemotherapy-treated, surgery-treated, and early stage groups compared with the control group. These findings are consistent with ample studies that found overexpression of Bcl-2 in patients with BC [46,47]. In this regard, Bcl-2 expression is upregulated in the early stage of BC with ER-positive cancer [48,49]. Elevation of Bcl-2 mediates an antiapoptotic effect implicated in carcinogenesis [50].

In addition, caspases play a crucial role in apoptosis; caspases are cysteine proteases that have critical functions in apoptosis [51]. Therefore, unusual caspase expression has been implicated in various cancers. Therefore, the loss of caspases encourages tumorigenesis [51]. Specifically, caspase-3 is an extensive effector in the process of apoptosis. Caspase-3 activation triggers the protease cascade to induce programmed cell death [51]. Thus, caspase-3 activation directs the cells toward apoptosis [51]. In BCs, low caspase-3 expression is closely related to the low expression of apoptosis-associated proteins with poor BC prognosis [51]. In the current study, caspase-3 was significantly lower in the early-stage, chemotherapy-treated and surgery-treated groups than the healthy control group. The same observations were obtained by Devarajan *et al.,* who found that 75% of BC cells lacked the caspase-3 transcriptome and proteomes as mechanisms of oncogenesis and drug resistance [52]. Likewise, decreased expression of caspase-3 has been reported to be associated with malignant transformation and tumor progression of BC [53].

The imbalances between free radicals production and antioxidant defense disturb the ratio of total oxidant status to TAC in BC patients' blood [54]. This resulted in oxidative stress that alters many cellular processes, such as metabolism, signaling pathways, gene expression, cell proliferation, and apoptosis [54]. This is attributed to altering the function of significant proteins, lipids, and nucleic acids as the body's major biomolecules. This might lead to tissue damage and oncogenesis [54]. In the present work, serum TAC levels, and current results indicated that TAC levels were significantly decreased in the chemotherapy-treated, surgery-treated, and early-stage groups compared to healthy subjects. The results were in harmony with those obtained by Abbasalizad *et al*., who reported that the decrease in TAC levels was correlated with BC risk. However, proper levels of TAC decreased the risk of BC [55]. Similarly, Rashad *et al.* observed significantly lower TAC levels in cancer patients than in healthy subjects [56]. Conversely, ample studies have documented increase serum TAC levels in BC situations as a compensatory mechanism for maintaining redox balance [54]. MiRNA-148b inhibition has been documented to improve antioxidant power with enhanced cell survival by suppressing apoptosis and activating the Wnt/β-catenin signaling pathway [57]. The present study reported a negative correlation between miRNA148b and TAC levels. However, up-regulation of the miRNA-148b transcriptome is associated with a lower TAC ability [56]. Furthermore, the present results confirmed hematological and TAC appears to be positively and negatively correlated with miR-NA-148b, caspase-3, Bax, Bcl2 and TAC. Therefore, the present findings suggest the use of these parameters to predict, detect, and monitor BC.

Histopathological examination is an essential method for the investigation of new biomarkers of diseases [58]. In the present study, there are a series of histopathological changes and heterogeneous breast cells in tissues obtained from earlystage BC, as well as chemotherapy and surgerytreated groups. Furthermore, typical cytological characteristics and changes in nuclear characters, including size, shape, and membrane irregularity, were observed in BC patients. These results are consistent with a previous study that demonstrated significant abnormalities in nuclear characteristics in BC patients [59]. The nuclear morphological changes are vigorous for understanding and confirming molecular function [59]. Therefore, the findings of the histopathological studies confirmed the biochemical and molecular biology findings on BCs in the present study.

Regarding hematological parameters, the control and patients enrolled in the present study were investigated for CBC. The current results indicated that hemoglobin levels in the chemotherapy and surgery groups were significantly lower than in the healthy control subjects. However, there was no significant decrease in hemoglobin levels in the early stage group compared with the control group. These findings were consistent with Raza et al., who investigated post-treatment hematological variations in BC patients [60]. Their results showed that patients who received surgery, radiation therapy, or chemotherapy had a statistically significant decrease in hemoglobin and CBC after treatment [60].

CBC is a mirror for the cellular immune response in patients with malignancy. Therefore, the changes in hematological parameters reflect tumor development [54]. Specific CBC indices are valuable in predicting results in patients with BC [54]. Furthermore, the hematological profile of patients not only fluctuates dramatically after treatment, but is also influenced by the treatment pattern and stage of the disease [60]. Equally, similar observations were documented by another study that evaluated the connection of CBC to the survival of BC patients [61]. The current study confirmed the CBC indices as an indicator for the development of BCs and the influence of different therapeutic modalities. However, several clinical trials have shown that patients with BC had adverse hematological outcomes [62,63]. Similarly, females with BC have been reported to show disturbed CBC compared to healthy people [54]. Furthermore, post-treatment anemia has been linked to chemotherapeutic drugs such as platinum salts, anthracycline, and gemcitabine [62,63]. This is attributed to suppression of hematopoiesis and the reduction of erythropoietin levels due to renal impairment [62,63].

In addition, most chemotherapy drugs induced myelosuppression and progression of anemia with each cycle of chemotherapy [62,63]. Furthermore, compared to the control group, there was a significant increase in WBC and lymphocyte levels among patients in the early stages, chemotherapy, and surgery groups. These findings are in conjunction with several studies that reported a higher elevation of WBC counts in BC patients than in healthy subjects [63]. In contrast, the results obtained by Raza et al. were incompatible with the WBC results of the present study [60]. Regarding platelet count, it was significantly lower in patients in the early stage, chemotherapy, and surgery groups compared to healthy control subjects. Similarly, another study showed that deaths in BC patients from chemotherapy have decreased platelet count as one of the complications of diseases and side effects of chemotherapy [63,64].

This work has limitations; however, it is cross-sectional work, which does not allow causal inferences or understanding of temporal relationships between miRNA-148b levels, along with Bax, caspase-3, Bcl-2 and TAC levels and treatment outcomes. The preliminary data of this work, the low sample

size, may be inadequate to draw generalizable assumptions. Studies with larger sample sizes must approve the present results and their clinical importance. The study only evaluated miRNA-148b levels and Bax, caspase-3, Bcl-2, and TAC levels simultaneously, this study touched on their prognostic value and response to therapy. Therefore, longitudinal studies are vital to the management of BC patients. The characteristics of the control group should be carefully considered, as differences in demographics could influence measured miRNA-148b levels, along with Bax, caspase-3, Bcl-2, and TAC as BC biomarkers.

4. Conclusions

This study concludes that miRNA-148b and apoptosis markers in the blood are encouraged non-invasive biomarkers for the prediction, diagnosis, monitoring, assessment of the anticancer response and general follow-up of BC patients. Therefore, measuring circulating blood miRNA-148b and caspase-3 could enhance the management of BC. Despite the promising findings of this study, it has several limitations, such as a small sample size that can affect the generalizability of the results. Moreover, this study's focus on the homogeneous population (Egyptian females) limits applicability to other demographics. Moreover, the lack of longitudinal and multicenter approach limits the applicability of the findings.

Disclosure statement

Conflict of interest: The authors declare that they have no conflicts of interest to report on the present study.
Ethical approval: This study was conducted at the outpatient clinic of Damanhour Oncology Center from December 2021 to February 2023. The Research Ethics Committee of the Faculty of Pharmacy, Damanhour University, Egypt, approved the study (Ref. No. 620PB20). The study adhered to the Helsinki Declaration of ethical principles for research involving human subjects.
Data availability: All data are included in the manuscript.
Consent to participate: Informed consent was obtained from all participants.

CRediT authorship contribution statement

Conceptualization: Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Methodology: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran, Mohamed Noureldin; Software: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Validation: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Formal Analysis: Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Investigation: Tarek Mahmoud Okda, Gamal Abd El-Hay Omran, Ahmed Noreldin; Resources: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Data Curation: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Writing - Original Draft: Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Writing - Review and Editing: Gamaleldin Ibrahim Harisa, Samiyah Alshehri, Sulthan Al Rashid; Visualization: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Funding acquisition: Gamaleldin Ibrahim Harisa; Supervision: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran; Project Administration: Gamaleldin Ibrahim Harisa, Tarek Mahmoud Okda, Gamal Abd El-Hay Omran.

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