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RESEARCH

The diagnostic significance of circulating miRNAs and metabolite profiling in early prediction of breast cancer in Egyptian women

Safinaz E. El-Toukhy[1] · Sherien M. El-Daly[1,2] · Mahmoud M. Kamel[3] · Heba K. Nabih[1]

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Abstract

Objective Breast cancer (BC) is one of the most commonly diagnosed solid malignancies in women worldwide.

Purpose Finding new non-invasive circulating diagnostic biomarkers will facilitate the early prediction of BC and provide valuable insight into disease progression and response to therapy using a safe and more accessible approach available every inspection time. Therefore, our present study aimed to investigate expression patterns of potentially circulating biomarkers that can differentiate well between benign, malignant, and healthy subjects.

Methods To achieve our target, quantitative analyses were performed for some circulating biomarkers which have a role in the proliferation and tumor growth, as well as, glutamic acid, and human epidermal growth receptor 2 (HER2) in blood samples of BC patients in comparison to healthy controls using qRT-PCR, liquid chromatography/mass spectrometry (LC/MS/MS), and ELISA.

Results Our findings showed that the two miRNAs (miRNA-145, miRNA-382) were expressed at lower levels in BC sera than healthy control group, while miRNA-21 was expressed at higher levels in BC patients than control subjects. Area under ROC curves of BC samples revealed that AUC of miRNA-145, miRNA-382, miRNA-21, and glutamic acid was evaluated to equal 0.99, 1.00, 1.00 and 1.00, respectively. Besides, there was a significantly positive correlation between miRNA-145 and miRNA-382 ($r=0.737$), and a highly significant positive correlation between miRNA-21 and glutamic acid ($r=0.385$).

Conclusion Based on our results, we conclude that the detection of serum miRNA-145, -382 and -21 as a panel along with glutamic acid, and circulating HER2 concentrations could be useful as a non-invasive diagnostic profiling for early prediction of breast cancer in Egyptian patients. It can provide an insight into disease progression, discriminate between malignancy and healthy control, and overcome the use limitations (low sensitivity and specificity, repeated risky exposure, and high cost) of other detecting tools, including mammography, magnetic resonance imaging, and ultrasound.

Keywords HER2/neu · miR-145 · miR-382 · miR-21 · Liquid chromatography/mass spectrometry · Glutamic acid

Introduction

Breast cancer (BC) is the second most common cancer in women worldwide, and one of the leading cancer-causing deaths overall (Ibrahimet al. 2020). According to the World Health Organization (WHO) report in 2020, more than two million women were diagnosed with BC and about 685,000 deaths occur globally (Rakhmina et al. 2021; WHO 2020). BC can be classified as: benign, which is not considered cancerous, not life threatening, grows slowly, and does not invade or spread to other parts of the body; or malignant, which has the potential to be metastatic and life threatening (Wei et al. 2020). Therefore, to minimize the rate of BC death and improve the prognosis of outcome with better therapeutic options, early non-invasive detection of BC

✉ Heba K. Nabih
hk.nabih@nrc.sci.eg; dr_heba_kamal@hotmail.com

1 Medical Biochemistry Department, Medicine and Clinical Studies Research Institute, National Research Centre, 33 El-Bohouth st., Dokki, P.O. 12622, Giza, Egypt

2 Cancer Biology and Genetics Laboratory, Centre of Excellence for Advanced Sciences, National Research Centre, Giza, Egypt

3 Laboratory Department, Baheya Hospital for Early Detection and Treatment of Breast Cancer, National Cancer Institute, Cairo University, Giza, Egypt

would be a promising approach that facilities the diagnosis and discrimination of BC for personalized medications (Li et al. 2020; Mar-Aguilar et al. 2013). Liquid biopsy represents a rich source of multiple circulating biomarkers that could be considered as non-invasive promising diagnostic factors, which discriminate between healthy, benign, and malignant candidates for early detection of breast cancer (Underwoodet al. 2020; Marrugo-Ramírez et al. 2018).

MicroRNAs (miRNAs) are a set of small sequence (18–25 nucleotides) non-coding RNAs that regulate gene expressions at the transcriptional or post-transcriptional level by sequence-specific binding to the 3′ untranslated regions (3′ UTR) of the target gene, leading to the degradation or translation inhibition of the gene (Lv et al. 2019). Thus, miRNAs have an important role in controlling many biological processes such as cell growth, proliferation, invasion, differentiation, adhesion, apoptosis, and cellular metabolic pathways (Orlandella et al. 2021; El-Daly et al., 2020; Nabih 2020; Pedroza-Torres et al. 2019; Yanwirasti et al. 2017; Pinweha et al. 2016). Abnormal alterations of miRNAs expression patterns are frequently observed in various cancers, including BC. Because of their distinct expression pattern in cancer and noticeable stability in blood, miRNAs are considered to be highly promising biomarkers for BC early diagnosis (Rakhminaet al. 2021; Mar-Aguilar et al. 2013).

MiRNA-145 was reported to be located in a fragile region of chromosome 5 (5q32-33) and played a profound role in the inhibition of cell growth, proliferation, angiogenesis, invasion, and migration of human BC through targeting multiple genes such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), human epidermal growth factor receptor (HER3), cmyc, hypoxia-inducible factor 2 alpha (HIF2a), sex-determining region Y-box2 (SOX2), rho-associated coiled-coil kinase (ROCK1), RTKN (rhotekin), octamer-binding transcription factor 4 (Oct4), and transforming growth factor-β1 (TGF-β1) (Ibrahim et al. 2020; Ye et al. 2019; Zeinali et al. 2019; Ding et al. 2017; Hu et al. 2012; Kim et al. 2011; Wang et al. 2009). Accordingly, the down-regulation of the tumor suppressor miRNA-145 contributes to the metastasis and progression of the tumor and is considered to be a potential biomarker for BC diagnosis, screening, and prognosis (Lv et al. 2020; XU et al. 2019; Gonzalez-Villasana et al. 2019; Quan et al. 2018).

MiRNA-382 locates on the 14q32 locus and its expression level in BC is critically associated with cell viability, proliferation, migration, invasion, and survival (Gonzalez-Villasanaet al. 2019; Feng et al. 2017; Tao and Wu 2016). The function of miRNA-382 was detected to be mediated via its counteracting genes, which have a role in the promotion of proliferation and metastasis in breast cancer cells (Zhang et al. 2019; Lv et al. 2019). Additionally, it could potentially be used as a reported non-invasive biomarker that can differentiate between BC patients and healthy candidates (Fu et al. 2016; Mar-Aguilar et al. 2013).

MiRNA-21oncogene is located on the chromosome 17q23.2 region which is frequently amplified in breast cancer. The up-regulation of miRNA-21 results in the promotion of cancer growth, proliferation, invasion, angiogenesis, and metastasis via targeting of many genes involved in apoptosis and tumor suppression, including programmed cell death protein 4 (PDCD4), tropomyosin 1 (TPM1), RAS p21 protein activator (RASA1), phosphatase tensin and homolog (PTEN), MASPIN, P53, B cell lymphoma 2 (Bcl2), signal transducer and activator of transcription 3 (STAT3), and leucine zipper transcription factor-like 1 (LZTFL1). Hence, miRNA-21 is a useful early diagnostic biomarker that discriminates between malignant breast cancer, benign breast cancer, and healthy control (Wu 2020; Savitri et al. 2020; Elzoghby et al. 2019; Wang et al. 2019; Zhang et al. 2016a, b; Gao et al. 2013; Yan et al. 2008).

Along with the enormous challenge of early diagnosis of BC, which is widely accredited as the key to successful therapy, mass spectrometry-based metabolomics is a promising approach for detecting the concentration of endogenous metabolic biomarkers produced by cancerous cells during their growth, such as alanine, aspartate, and glutamate that have a vital role in BC development (Yang et al. 2020). The accumulation of glutamic acid in the body might lead to the down-regulation of its derivative glutamine; consequently, this would promote the incidence of BC by activating mammary epithelial cells proliferation by enhancing the production of ATP and nucleotides biosynthesis (Pietkiewicz et al. 2021; El Ansari et al. 2018; Coloff et al. 2016). Additionally, elevated levels of glutamic acid via glutaminolysis could maintain the Krebs cycle, which is the main source of energy in the cells (Dowling et al. 2015). Up-regulated glutamic acid levels were suggested to distinguish between healthy, benign, and BC patients, which could be helpful as an early diagnostic biomarker in breast carcinoma (Wang et al. 2018).

It is well known that breast cancer cells expressing human epidermal growth factor receptor 2 (HER2) have a poor prognosis and survival associated with an increased rate of proliferation, invasion, angiogenesis, metastasis, and recurrence, as compared to HER2-negative tumors. HER2/neu is a proto-oncogene located on chromosome 17q21 and encodes for p185, which is a transmembrane glycoprotein with an intrinsic tyrosine kinase activity domain that regulates cellular growth and differentiation (Iqbal and Iqbal 2014; Ferretti et al. 2007). HER2 could be used as circulating diagnostic biomarkers with the potential to provide a valuable prediction of disease progression and response to therapy (Wu and Chu 2022).

Since the main aim of this research is to explore circulating biomarkers that would predict the onset of breast

cancer, the expression levels of miRNAs-145, -382, and -21 panel were assessed using qRT-PCR, besides HER2/neu by ELISA, as well as metabolomics (amino acids profiling) by LC/MS/MS in the blood samples of healthy, benign, and breast cancer candidates.

Subject and methods

Participant selection criteria

In the current research study, serum samples from 80 women were collected. Among them were 30 newly diagnosed breast carcinoma patients (stage I, II, and III), 30 women with benign breast disease, and 20 age-matched healthy women as control. The mean age range of the enrolled cases was 39–62 years for early-diagnosed breast cancer cases, 27–58 years for benign breast patients, and 41–53 for healthy controls. Patients included in our study were recruited from October 2020 to February 2021from Baheya Hospital, Giza, Egypt, for early detection and treatment of breast cancer. Each subject's diagnostic medical report, including data such as age, diagnosis (benign or early-stage breast cancer), type, grade, estrogen, and progesterone status (if cancer), was available. The breast cancer diagnosis was confirmed by physical, radiological, and histopathological examinations. All samples were collected before surgery or any chemotherapy and radiotherapy intervention. None of the included patients were suffering from any chronic disease, diabetes, hypertension, or any other type of cancer. Additionally, none of the healthy controls had a history of malignancy or chronic hepatitis, or liver cirrhosis and was verified to be healthy by physical examination. Exclusion criteria included those with late stage or metastatic cancer or had undergone modified radical mastectomy or breast-conserving surgery, chemotherapy, targeted therapy, adjuvant radiotherapy, or endocrine therapy. All patients had signed an informed consent to be enrolled in the study. This study was approved by the Bioethical Committee of the National Research Centre (Ethical Clearance Document Registry Number 19382).

Methods

Blood samples

Blood samples (3 ml) were withdrawn and divided into two parts: 0.5 ml on EDTA for the preparation of dried blood spot (DBS) for liquid chromatography/mass spectrometry (LC/MS/MS) technique; and the rest on a gel vacutainer tube, centrifuged to separate serum for the assessment of tumor diagnostic biomarkers by qRT-PCR and ELISA assays. Blood samples were first centrifuged at 1600 rpm for 10 min at 4 °C for serum separation. A second centrifugation step for serum samples was conducted to remove residual platelets for efficient extraction of cell-free RNA.

RNA isolation and qRT-PCR quantification of microRNAs

As recommended by Link et al. (2019), all procedures related to RNA isolation or quantification was conducted under RNAse-free conditions. Isolation of cell-free RNA, including miRNAs, from serum samples, was performed using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. Following the extraction steps, total RNA including miRNAs was eluted from the miRNeasy column using 15 µl of RNase-free water. The quantity and purity of eluted RNA were assessed by NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Scientific™) directly upon isolation. RNA was reverse transcribed using the miRCURY LNA Reverse Transcription Kit (Qiagen, Cat#339340) following the guidelines of the provided kit. The cDNA was kept at −20 °C till further processing by real-time PCR. Expression analysis of miRNAs was evaluated by real-time PCR using miRCURY LNA SYBR Green PCR Kit (Cat# 339346) and miRCURY LNA miRNA PCR assays for miR-21-5p, miR-145-5p, miR-328-5p, and miR-16-5p. The PCR cycling conditions were set as follows: PCR initial heat activation for 2 min at 95 °C, followed by two-step cycling of denaturation for 10 s at 95 °C, and a combined annealing/extension step at 60 s for 56 °C. A total of 40 cycles were performed, followed by a melting curve analysis. For each miRNA expression analysis, each sample was run in triplicate. The expression levels were normalized to miR-16, as suggested by several reports on the validity of miR-16 as an internal reference in circulating miRNA analysis (El-Daly et al. 2019; Lange et al. 2017; Witwer 2015). ΔCt was calculated for each sample by normalizing the Ct value of the miRNA of interest to the Ct value of the miR-16 (normalizer). ΔΔCt was then calculated by subtracting the ΔCt of the test sample from the mean ΔCt values of control samples. Data were presented as relative mRNA expression (fold change).

Analysis of L-amino acids by liquid chromatography/mass spectrometry (LC/MS/MS)

Sample preparation As described by Wang et al. (2016), a 3 mm (diameter) disc was punched from each DBS paper. The discs were placed into the Millipore Multi Screen HV 96-well plate (Millipore, Billerica, MA, USA) for metabolite extraction. Briefly, for each well containing a DBS disc, 100 µl working solution was added. Then, the plates were centrifuged at 1500×*g* for 2 min after gentle shaking for 20 min. By using new flat-bottom 96-well plates, the filtrate was collected. For each plate, four randomly selected blank

wells were added with two low-level and two high-level QC control solutions individually. Pure nitrogen gas was used to dry the QC and filtrate samples at 50 °C. Further, 60 µl of acetyl chloride/1-butanol mixture (10:90 v/v) was added to the dried samples at 65 °C for 20 min for derivatization. The derivatized samples were dried again with nitrogen gas at 50 °C. For metabolomic analysis, each dried sample was dissolved in 100 µl fresh mobile phase solution.

Metabolomic analysis The direct injection MS metabolomic analysis was conducted by using an AB Sciex 4000 QTrap system (AB Sciex, Framingham, MA, USA). The equipped ion source was an electrospray ionization source. A 20 µl sample was injected for each run. The mobile phase was 80% acetonitrile aqueous solution. The initial flow rate was 0.2 ml/min. Afterward, the flow rate was set to 0.01 ml/min within 0.08 min, kept constant until 90 s, returned to 0.2 ml/min within 0.01 min, and held constant for another 30 s. The ion spray voltage was 4.5 kV. The curtain gas pressure was set at 20 psi. A 35 psi pressure was applied to the ion source gas 1 and gas 2. The auxiliary gas temperature was maintained at 350 °C. Analyst v1.6.0 software (AB Sciex) was used for system control and data collection. ChemoView 2.0.2 (AB Sciex) was used for data preprocessing. Partial least squares discriminant analysis (PLS-DA) was performed by using SIMCA-P v12.0 (Umetrics, Umeå, Sweden).

Quantification of HER2/neu by ELISA assay

Human HER2/neu was estimated by enzyme-linked immunosorbent assay (ELISA) in sera of all collected samples, according to the method of Shukla et al. (2016) with some modifications. The ELISA kit (sunLong Biotech Co., China) uses the sandwich ELISA technique as the principle of the working methodology. The 96-well ELISA strip plate provided in the kit had been pre-coated with a primary antibody specific to HER2. Standards or samples were added to the appropriate wells and combined with the specific antibody. Then, horseradish peroxidase (HRP)-conjugated secondary antibody specific for the primary HER2 was added to each well and incubated. Free unconjugated components were washed away through repeated washing steps. Finally, 3,3′,5,5′- tetramethylbenzidine (TMB) substrate solution was added to each well. Only those wells that contain the detected antigen and the corresponding HRP-conjugated antibody would appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) was measured by microplate ELISA reader at a wavelength of 450 nm (Tristartlb 942 microplate reader; Berthold, Germany). The OD values are proportional to the concentration of the detected antigen. The concentration of HER2/neu in each sample was calculated from a standard curve.

Statistical analysis

All data were represented as mean ± standard error of the mean (SEM) with statistically significant consideration at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and **** $P < 0.0001$ using a two-sided independent Student's t test. All experiments were repeated in triplicate. The potential of biomarkers and their diagnostic efficacies were evaluated by receiver operating characteristic (ROC) analysis. Moreover, correlations between estimated biomarkers were analyzed using the Pearson correlation coefficient (r). The statistical analyses and calculations were performed using Statistical Package for the Social Sciences (SPSS, Inc., Chicago USA) version 16.0 software. Graph Pad Prism 8.0.1 (Graph Pad Software Inc., USA) was used for the figures' output, correlation coefficient, and ROC analysis.

Results

Clinicopathological features and medical recorded data for the enrolled breast cancer patients

According to the diagnostic medical sheet of each included breast carcinoma patient ($n = 30$), the type of malignancy was differentiated as follows: 83.33% (25/30) of cases were diagnosed as invasive ductal carcinoma (IDC), 10% (3/30) of patients were characterized as ductal carcinoma in situ (DCIS), and 6.66% of patients (2/30) were diagnosed with multifocal carcinoma. The grade of tumor was distributed among patients as 13.33% with grade I (1/30), 70% with grade II (21/30), and 26.66% with grade III (8/30). Concerning endocrine receptor expression in enrolled cases, there were 76.66% (23/30) and 90% (27/30) positively expressed estrogen receptor (ER) and progesterone receptor (PR), respectively, while there were 23.33% (7/30) and 10% (3/30) negatively expressed ER and PR, respectively. HER2/neu was found to be positively expressed in 13.33% (4/30) of cases and negatively expressed in 86.66% (26/30) of breast cancer patients (Table 1).

Expression levels of the circulating microRNAs among the investigated groups

As shown in Table 2 and Fig. 1, the median levels of the detected miRNAs −145, and −382 were recorded to be highly significantly decreased ($P \leq 0.001$) in both benign and malignant groups as compared to control individuals. On the other hand, our data revealed a significant increase in the expression level of miRNA-21 in patients with breast

Table 1 Clinicopathological characterization of early-diagnosed breast cancer patients

Parameter	Percentage (%)
Age	39–62 years
Clinical tumor grade	Stage I 3.33% (1/30)
	Stage II 70% (21/30)
	Stage III 26.66% (8/30)
Pathological type	Invasive ductal carcinoma (IDC) 83.33% (25/30)
	Ductal carcinoma in situ (DCIS) 10% (3/30)
	Multifocal carcinoma 6.66% (2/30)
Estrogen receptor (ER) status	Positive 76.66% (23/30)
	Negative 23.33% (7/30)
progesterone receptor (PR) status	Positive 90% (27/30)
	Negative 10% (3/30)
HER2/neu status	Positive 13.33% (4/30)
	Negative 86.66% (26/30)

Table 2 The mean of the fold change of microRNA expression levels

MicroRNA	Control	Benign	Malignant
miRNA-145	1.032 ± 0.1241	0.3907 ± 0.05218[a] ($P \leq 0.001$)	0.4202 ± 0.0746[a] ($P \leq 0.001$)
miRNA-382	1.014 ± 0.06464	0.1731 ± 0.04228[a] ($P \leq 0.001$)	0.2244 ± 0.0302[a] ($P \leq 0.001$)
miRNA-21	0.9017 ± 0.05714	1.594 ± 0.2169[a] ($P \leq 0.05$)	2.103 ± 0.239[a] ($P \leq 0.01$)

[a]Significant as compared to control patients

lesions (benign) ($P \leq 0.05$) and a highly significant increase in patients with breast cancer ($P \leq 0.01$).

Estimation of glutamic acid concentration by LC/MS/MS

Using the dried blood spot (DBS)-based mass spectrometry metabolomics analysis technique, breast tumor metabolite markers, amino acids and acylcarnitine profiles were screened (attached as Supplementary file). In light of this, a DBS-based metabolomic study was performed by using direct LC/MS/MS analysis of BC and the control samples in this study focusing on the detection of glutamic acid concentrations. As shown in Table 3 and Fig. 2, there was a highly significant difference between the benign and control groups. Furthermore, similar statistical significance was noticed between malignant and both benign and control groups.

Serum concentration levels of HER2/neu

The evaluated mean concentration of HER2/neu in control healthy candidates was 6.1 ± 0.455 µg/L, while in patients with breast lesions (benign) and malignancy, it was calculated to be 8.4 ± 1.39 and 9.6 ± 1.8 µg/L, respectively. This slightly increase of HER2/neu in sera of malignant patients might be due to the presence of only four enrolled patients (4/30, 13.33%) who were recorded to be positively expressed HER2/neu in their provided medical reports.

Pearson correlation coefficient (r) between all investigated parameters in all studied groups

As indicated in Table 4 and Fig. 3, our data revealed a strong positive correlation between the expression pattern of miR-145 and miR-382 ($r = 0.737$) and a mild positive correlation between the expression of miR-21 and glutamic acid ($r = 0.385$). On the other hand, our data analysis revealed a negative correlation between the expression of miR-21with both miR-145 ($r = -0.359$) and miR-382 ($r = -0.486$). Also, a negative correlation was recorded between glutamic acid with miR-145 ($r = -0.466$) and miR-382 ($r = -0.524$) ($P < 0.0001$). Interestingly no significant correlations were detected between HER2/neu expression and the rest of the parameters.

Receiver operating characteristic (ROC) curve analysis

The most prominent approaches for evaluating the diagnostic accuracy of biomarkers are through assessing the area under the curve (AUC), in addition to sensitivity and specificity percentages as determined from ROC curves analysis.

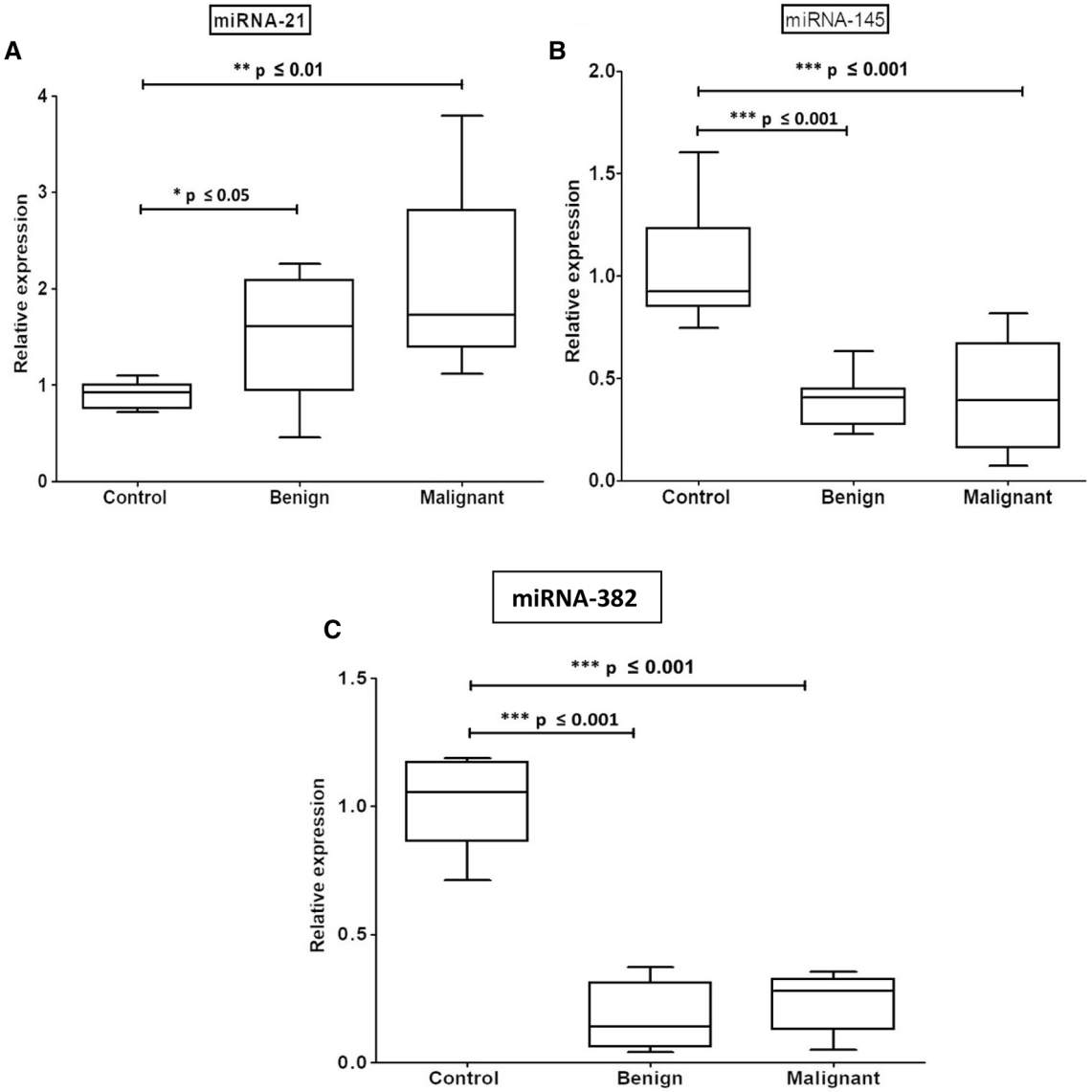

Fig. 1 Whisker boxplot displays the relative expression patterns of serum miRNA-21 (**A**), -145 (**B**), and -382 (**C**) among the investigated study groups. Significant differences between the compared groups are also shown

Table 3 Detected mean values of glutamic acid concentrations for each studied group

Subjects	Mean (µmol/L) ± SEM
Control	84.05 ± 2.79
Benign	146.6 ± 7.39[**a]
Malignant	241.9 ± 13.52[**a,b]

**$P \leq 0.01$

[a]As compared to the control group

[b]as compared to the benign group

In the present study, we performed ROC analysis to each measured biomarker for data collected from control and benign samples (Table 5, Fig. 4A). The highest AUC, sensitivity, and specificity values were reported for circulating miRNA-145 and miRNA-382 (AUC = 1, sensitivity 100%, and specificity 100%). Moreover, the ROC curve analysis was carried out to compare between malignant and control subjects (Table 6, Fig. 4B). We observed highly significant AUC, sensitivity, and specificity values for all detected circulating parameters. Interestingly, when we conducted ROC curve analysis on results collected from benign and malignant samples (Table 7, Fig. 4C), the AUC, sensitivity, and specificity values were recorded to be lower. However, glutamic acid showed a reasonable AUC value, with moderate sensitivity % and specificity% (AUC = 0.91, sensitivity% = 86.67, specificity% = 83.33). In our analysis, we did

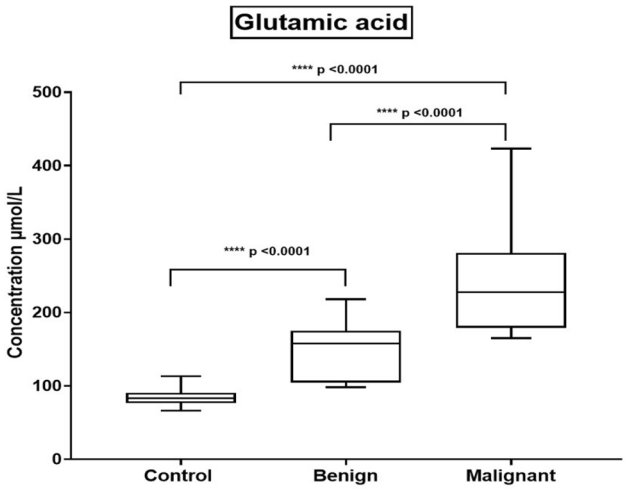

Fig. 2 Whisker boxplot illustrates the mean values of the glutamic acid concentrations among the investigated study groups. Significant differences between the compared groups are also shown

not include the HER2 data, since only four subjects were HER2 positive in our study.

Discussion

Breast cancer represents the most commonly diagnosed cancer and the fifth cause of cancer related-death all over the world, with an estimated 2.3 million cases (24.5% of all cancer cases) and 685,000 deaths (15.5% of cancer deaths) in 2020. Well-diagnosed cases are predicted to reach about 4.4 million in 2070. The incidence and mortality of BC vary among countries (Lei et al. 2021). Although various treatment options for BC are available, its effective management is still difficult because of the deficiency of sensitive and specific biomarkers for early detection and disease monitoring. Accumulating evidence in the last years has highlighted the prospective use of peripheral blood circulating miRNAs in BC diagnosis, prognosis, and monitoring the response to therapeutic agents. Because of their structural stability and ease of isolation procedure, miRNAs are increasingly suggested as promising non-invasive biomarkers for BC early diagnosis (El-Daly et al. 2022; Escuin et al. 2021; Jang et al. 2021).

In the present study, we observed a highly significant decrease ($P \leq 0.001$) in the detected expression level of miRNA-145 in both benign and malignant cases compared to controls. Additionally, the AUC value for miRNA-145 was estimated to equal 0.99, suggesting the potential use of miRNA-145 for early diagnosis to discriminate between breast tumor patients and normal controls.

Ng and the co-workers were the first to demonstrate the reduction of miRNA-145 level in the plasma of BC patients, but according to their result and similar to our finding, the aberrant expression of miR-145 was not significant enough to differentiate between benign and invasive breast cancer cases (Ng et al. 2013).

Our finding was also comparable with the study by Ibrahim et al. 2020, which reported that the plasma expression level of miRNA-145 (AUC = 0.70) was recorded to be significantly decreased ($P < 0.01$) at the initial BC diagnosis, as compared to healthy individuals. Also, Iorioet al. (2005) identified miRNA-145 to be down-regulated in breast cancer tissues as compared to normal tissues (Nakhaie et al. 2020; Tsai et al. 2018; Quan et al. 2018). This detected reduction in the expression level of miRNA-145 in BC cases was suggested to be as a result of the methylation of its promoter (Liu et al. 2017). According to the above studies, miR-145 functions by inhibiting proliferation, angiogenesis, and metastasis.

In contrast to our results, the tumor suppressor miRNA-145 was found to be significantly higher in the serum samples of BC patients from the Mexican population ($p < 0.001$), with AUC value of 0.9777 (Mar-Aguilar et al. 2013). The same pattern of elevation in the expression of circulating miRNA-145-5p in BC patients was also reported for HER-2-positive Kazakh patients (Ashirbekov et al. 2020). Ashirbekov et al. suggested that the measured expression level of miRNA-145 in serum/plasma/tissue of BC patients might vary according to the ethnicity of the studied population. In Lebanese patients, Itani et al. (2021) reported high expression of miRNA-145 in plasma of BC patients as compared to healthy subjects. Also, Gonzalez-Villasana et al. (2019) concluded that the levels of miRNA-145 packed in

Table 4 Pearson correlation coefficient (r) between the measured parameters in the different study groups

	miRNA-21	miRNA-145	miRNA-382	Glutamic acid	HER2/neu
miRNA-21	1	−0.359**	-0.486****	0.385***	0.154
miRNA-145	−0.359**	1	0.737****	−0.466****	−0.06
miRNA-382	−0.486****	0.737****	1	−0.524****	−0.125
Glutamic acid	0.385**	−0.466****	−0.524****	1	0.023
HER2/neu	0.154	−0.06	−0.125	0.023	1

**Pearson correlation (r) with statistically significant consideration at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and **** $P < 0.0001$ (2-tailed)

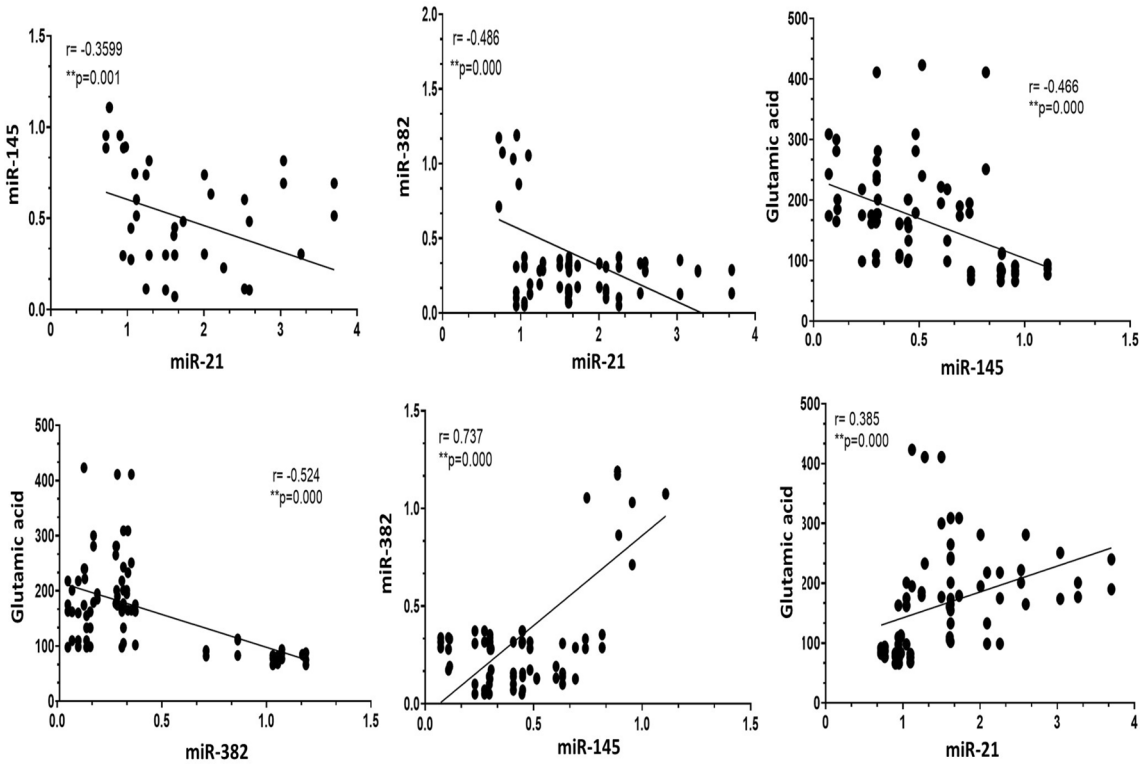

Fig. 3 Pearson correlation coefficient (r) between the measured parameters in the different study groups

Table 5 Receiver operating characteristic (ROC) analysis of all measured parameters in the benign samples, as compared to control subjects

	AUC	95% confidence interval (CI)	*P* value	Cutoff value	Sensitivity %	Specificity %
miR-21	0.9	0.8166–0.9834	< 0.0001	> 1.011	86.67	85.00
miR-145	1	1.000–1.000	< 0.0001	< 0.6893	100.0	100.0
miR-382	1	1.000–1.000	< 0.0001	< 0.5425	100.0	100.0
Glutamic acid	0.9633	0.9108–1.000	< 0.0001	> 96.00	100.0	90.00

circulating exosomes were similar to those in healthy individuals with an increased concentration of exosomes isolated from BC sera than those of healthy controls, which reflects their role in BC progression.

Another miRNA, whose pattern of expression we evaluated is miRNA-382. Our results revealed that the expression level of miRNA-382 was significantly decreased ($P \leq 0.001$) in subjects with benign or malignant tumors as compared to normal control. The AUC value was estimated to equal 1.00, so miRNA-382 could be a good diagnostic biomarker. In addition, our investigation recorded a strong significant positive correlation ($r = 0.737$) between miRNA-145 and miRNA-382. Our results agree with those of Tao and Wu (2016), who found that the expression level of miRNA-382 was significantly lower in tissue samples of Chinese BC patients compared with that in normal tissue. Moreover, a study performed by Li et al. (2013) showed that miR-382-5p expression was significantly decreased in tissues of patients with ductal carcinoma in situ (DCIS).

On the contrary, miRNA-382-3p was observed to be significantly up-regulated in the serum of breast cancer patients with AUC ranging from 0.740 to 0.9666, as compared to non-cancerous control (Zhang et al. 2019; Fu et al. 2016; Mar-Aguilar et al. 2013). The study by Gonzalez-Villasana et al. (2019) stated that miRNA-382 was detected in both exosomes isolated from sera of BC patients and healthy individuals, but with more elevated concentrations in BC cases. Generally, the oncogenic or tumor suppressive functions of miRNA-382 would be predicted depending on the targeting genes of miRNA-382 (Zhang et al. 2019; Lv et al. 2019; Feng et al. 2017).

Oncomir-21 in the current research was quantified by qRT-PCR and evaluated to be expressed in BC patients' sera with significantly higher levels ($P \leq 0.01$) than in benign

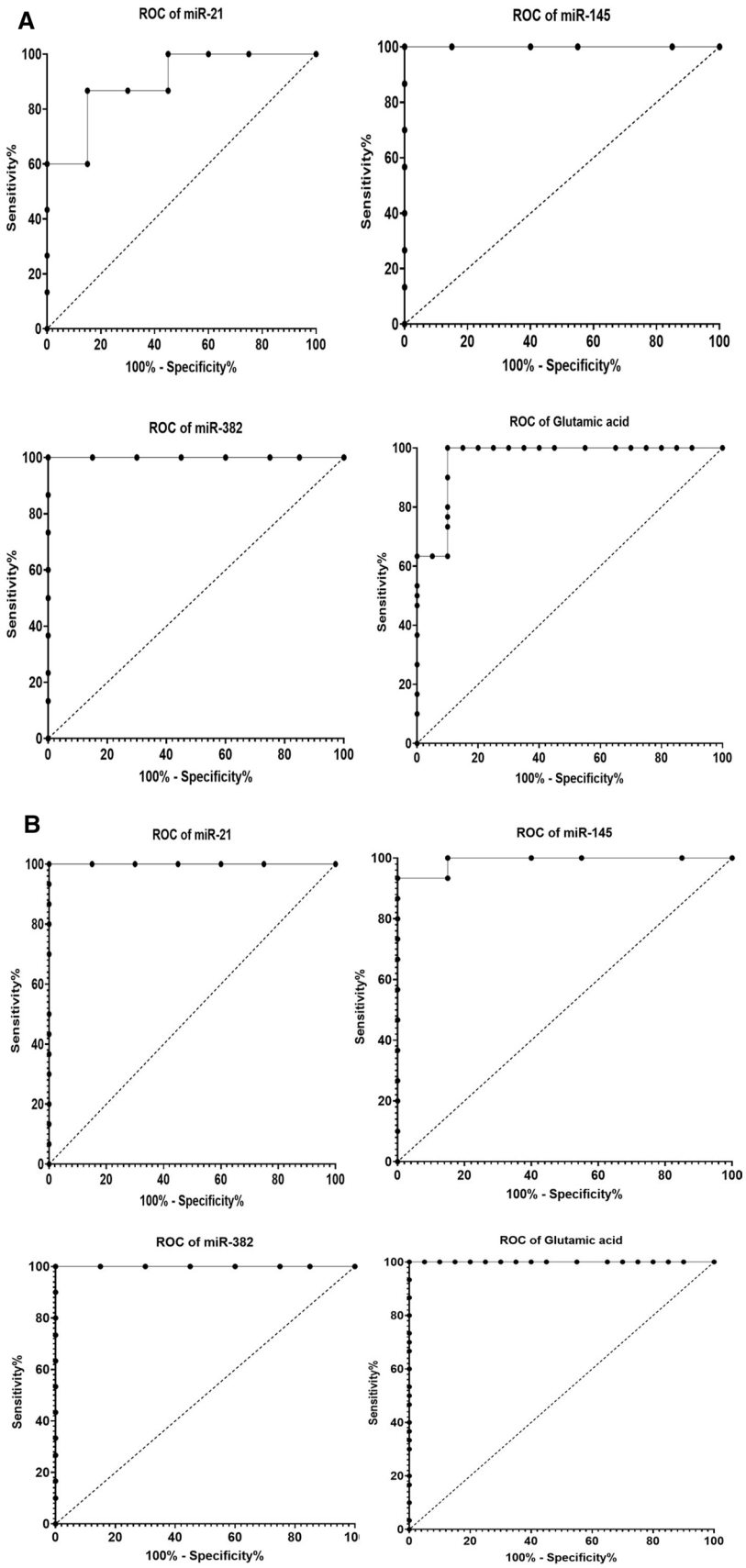

Fig. 4 **A** Receiver operating characteristic (ROC) analysis of the measured parameters individually in benign samples, as compared to the control. **B** Receiver operating characteristic (ROC) analysis of the measured parameters individually in malignant patient samples, as compared to healthy subjects. **C** Receiver operating characteristic (ROC) analysis of measured parameters individually in malignant patient samples, as compared to benign cases

Fig. 4 (continued)

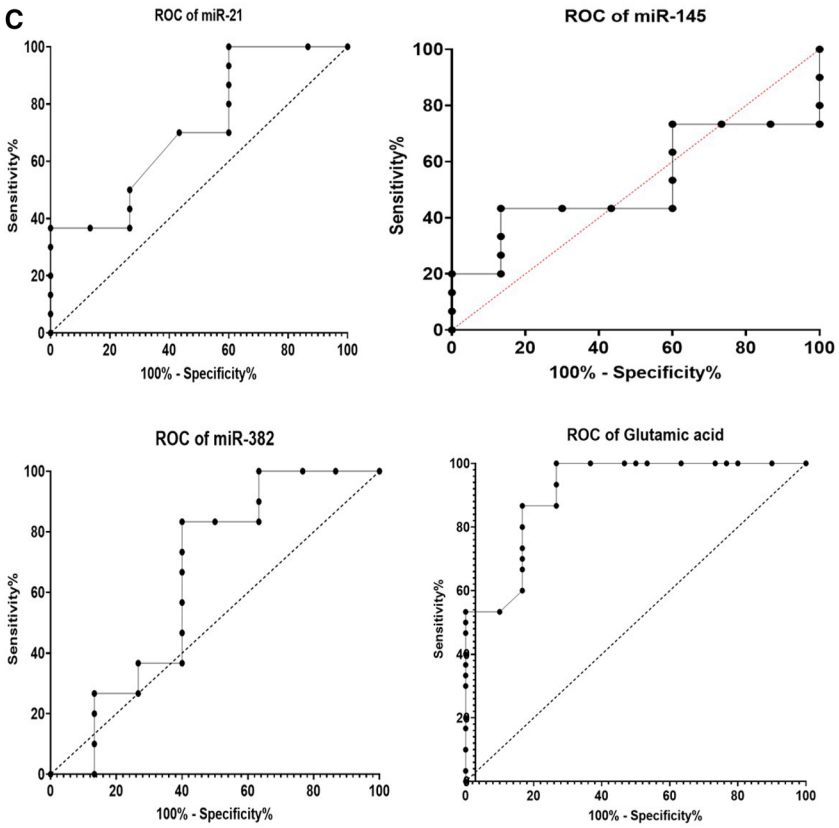

Table 6 Receiver operating characteristic (ROC) analysis of all measured parameters in malignant patients, as compared to healthy control samples

	AUC	95% Confidence interval (CI)	*P* value	Cutoff value	Sensitivity %	Specificity %
miR-21	1.00	1.000–1.000	< 0.0001	> 1.108	100	100
miR-145	0.99	0.9716–1.000	< 0.0001	< 0.8512	100	85
miR-382	1.00	1.000–1.000	< 0.0001	< 0.5329	100	100
Glutamic acid	1.00	1.000–1.000	< 0.0001	> 139.0	100	100

Table 7 Receiver operating characteristic (ROC) analysis of all measured parameters in malignant patient samples, as a comparison with benign cases

	AUC	95% Confidence interval (CI)	*P* value	Cutoff value	Sensitivity %	Specificity %
miR-21	0.7144	0.5849–0.8440	0.0043	> 1.612	70	56.67
miR-145	0.5222	0.3671–0.6774	0.7675	< 0.2971	73.33	40
miR-382	0.6456	0.4984–0.7927	0.0528	< 0.1653	83.33	60.00
Glutamic acid	0.9111	0.8397–0.9825	< 0.0001	> 176.0	86.67	83.33

and healthy women. The AUC was calculated to be 1.00 for miRNA-21 individually in BC patients as a comparison with control subjects. The underlying explanation for such elevation of miRNA-21 levels in BC could be related to its location on the region of chromosome 17q23.2 that is frequently amplified in breast tumors, or related to the hypomethylation of CpG island region that is located upstream of the mature miRNA-21 sequence (Elzoghby et al. 2019). Our data synchronize with the findings of Elzoghby et al. (2019), Swellam et al. (2018), Motawi et al. (2016), and Toraih et al. (2015), who showed that early diagnostic miRNA-21 expression levels were mostly up-regulated in the serum of Egyptian BC patients, followed by benign then healthy subjects, with AUC equal to 0.861. Additional harmonized investigations were found to agree with our results in the sera of studied ethnically diverse candidates (Salim

et al. 2020; Han et al. 2017; Zhang et al. 2016a, b; Wang et al. 2015; Si et al. 2013; Gao et al. 2013). Many studies are in accordance with our results in confirming the increased expression of miRNA-21 in breast cancer tissue compared to the corresponding adjacent normal one, and recognizing miRNA-21 as one of the most important diagnostic biomarkers implicated in early detection of breast malignancy, since its expression is gradually up-regulated with the severity of the disease (Amirfallahet al. 2021; Wu 2020; Soleimanpour et al. 2019;Yanwirasti et al. 2017; Zhang et al. 2016a, b; Wang et al. 2015; Qian et al. 2009; Yan et al. 2008). Supportively, expression of circulating miRNA-21 was reported to be significantly elevated in plasma of breast cancer patients, with good to fair AUC values, compared to healthy individuals, and these amplified levels of miRNA-21 expression was observed to be reduced after completion of surgery and chemotherapy (Ibrahim et al. 2020; Savitri et al. 2020; Anwar et al. 2019; Soleimanpouret al. 2019; Jurkovicova et al. 2017).

However, Mar-Aguilar et al. (2013) and Chan et al. (2013) found comparable levels of miRNA-21 expression in BC patients and controls, and miR-21 was not identified as the most important diagnostic marker.

Mass spectrometry (MS)-based platforms have become an essential approach for rapidly screening and qualifying many biomarkers for further development and validation of BC. However, there is a persisting need to search for reliable biomarkers that can detect BC constantly at an early stage (Ducret et al. 2019). As tumor cells have distinct metabolic pathways and metabolites that are similar to their counterparts, liquid chromatography/mass spectrometry (LC/MS/MS) was used in the current study to investigate the amino acid profiles in dried blood spots (DBS) of BC patients, women with benign lesions, and control group. Amino acids are the basic building blocks for almost all cell types and specific amino acids were reported to be changed in plasma of numerous cancer patients, including breast and lung (Cascino et a. 1995). In the context of cancer metabolomics, many amino acids have been demonstrated to provide valuable clues for studying pathogenesis and to act as potential indicators for diverse malignancies (Kimura et al. 2009).

Our LC/MS/MS results elucidated gradual elevated levels of glutamic acid concentration among the studied groups. The mean concentration values were 84.05, 146.6, and 241.9 µmol/L for control, benign, and malignant subjects, respectively. High statistical significance ($P \leq 0.001$) was observed between malignant and non-cancerous groups. Besides, AUC of malignant group as compared to control was detected to be 1.00 with associated high sensitivity and specificity percentages (100%). This gradual elevation of glutamic acid concentration among the studied groups indicates this amino acid to be a recent diagnostic biomarker that can discriminate between BC and healthy subjects. Wang et al. (2018) confirmed our results by using HPLC–MS/MS to evaluate the concentrations of free amino acids in serum of BC, benign, and healthy control groups. They reported a highly significant increase ($P \leq 0.001$) in the mean glutamic acid concentration in the serum of BC patients (287.95 µmol/L) as compared to the benign (206.61 µmol/L) and control population (158.79 µmol/L). Additionally, they suggested that BC patients with higher level of taurine, glutamic acid, and lower ethylmalonic acid were likely to be diagnosed with BC. Accumulation of glutamic acid in the body causes low production of glutamine, enhancing proliferation and progression of breast carcinoma, and maintaining the Krebs cycle which is the main source of energy in the cells (Pietkiewicz et al. 2021; El Ansari et al. 2018; Wang et al. 2018; Cala et al. 2018a, b; Coloff et al. 2016). Similarly, additional spectroscopy-based metabolomics investigations support our findings (Cala et al. 2018a, b; More et al. 2018; Torata et al. 2018).

In contrast to our results, recent mass spectrometry-based studies conducted in plasma, serum and urine of BC patients to discover novel circulating diagnostic biomarkers for early detection of breast malignancy stated that the level of glutamic acid metabolite was found to be decreased in BC individuals in comparison with healthy control. The down-regulation of glutamic acid could be related to the high demand of amino acids in tumor metabolism (Yuan et al. 2019; Eniu et al. 2019; Cala et al. 2018a, b).

Overexpression of human epidermal growth factor receptor 2 (HER2) was found to be associated with clinically aggressive breast cancers with an elevation of metastasis, recurrence, and drug resistance. Amplification of HER2 occurs in about 15–30% of breast malignancies (Iqbal and Iqbal 2014; Ferretti et al. 2007). The function of nuclear HER2 could be directed by STAT3, which recruits HER2 to activate the expression of miRNA-21 that in turn down-regulates the expression of the metastasis suppressor protein programmed cell death 4 (PDCD4) in breast carcinoma. A negative correlation was recognized between circulating miRNA-21 and overall survival in HER2-positive breast cancers treated with neoadjuvant chemotherapy and trastuzumab or lapatinib (Amirfallahet al. 2021; Feng and Tsao 2016; Özgün et al. 2013). As our research was conducted in newly early-diagnosed women with non-invasive BC, the expression of HER2 was found to be positive in only 13.33% (4/30) patients. Thus, it is not a surprise to perceive the mean concentration of HER2 in BC sera (9.6 µg/L) to be slightly, but significantly higher than that in non-cancerous groups.

Totally, although current standard early diagnostic tools for detection of breast cancer are available, there is an urgent need for new minimally invasive diagnostic approaches to improve the screening rate of breast cancers and overcome the limitations of the current mammogram screening. Blood represents the primary source for discovery of potential

breast cancer diagnostic biomarkers, which include miRNA-145, miRNA-382, miRNA-21, glutamic acid, and HER2/neu. All of these biomarkers have a role in the promotion of proliferation and progression of breast malignancy. Therefore, measuring the expression levels of these set of circulating diagnostic biomarkers will be valuable for early prediction of breast cancer and differntiate malignant patients from healthy subjects.

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Author contributions SEElT: project administration and work design, ELISA, LC/MS/MS analyses, and review. SMElD: molecular investigations, statistical analyses, and produced figures, and review. MMK: collection of the enrolled blood samples and medical data. HKN: writing, review—editing, interpretation of data, ELISA analysis, and isolation of sera and RNA. All authors read and approved the final manuscript for submission.

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Data availability All data are available in the current research paper.

Declarations

Conflict of interest All authors declare no competing interests.

Ethical approval All patients had signed an informed consent to be enrolled in this study. This study was approved by the Bioethical Committee of the National Research Centre (Ethical Clearance Document Registry Number 19382).

Consent to participate The present human study has been examined by the appropriate ethics committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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