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Original article

Circulating microRNAs as Potential Non-invasive Biomarkers for Breast Cancer Detection

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Summary. Breast cancer is one of the most common cancers among women and is the second leading cause of cancer-related deaths. Mammography is currently the gold standard diagnostic tool however it is not without limitations. Over the past decade, research has largely shifted focus from mRNA biomarkers to microRNAs (miRNAs) as a new potential screening biomarker for breast cancer. MiRNAs are 18–25-nucleotides regulatory non-coding RNA molecules that regulate the expressions of a wide variety of genes have crucial roles in many areas from organogenesis to carcinogenesis. This study was conducted to investigate miR 21, miR 27b, miR 125a, miR 155, miR 200c, miR 335, and miR373 in 20 patients with breast cancer patients. 20 healthy women served as controls. microRNAs were assessed using Real Time PCR method. Three microRNAs (miR 21, miR155 and miR125) were found to be significantly more abundant in the plasma of early-stage breast cancer (ESBC) patients compared to controls. Therefore, these 3 microRNAs could represent a promising circulating biomarker candidate for the ESBC diagnosis if the results will be validated in a wider group of patients. (www.actabiomedica.it)

Key words: Breast cancer, Biomarkers, microRNA

Introduction

According to data provided by the World Health Organization, breast cancer is the most common cancer type among women. It affects 2.1 million women each year and causes the highest number of cancer-related deaths. The most important strategy to reduce breast cancer-related mortality is early diagnosis and screening of healthy individuals (1).

Mammography is currently the gold standard diagnostic tool however it is not without limitations, including its use of ionizing radiation and a false positive rate of 8–10% (2). On the other hand, alternative methods such as ultrasound screening has very operator dependent sensitivity and tumor markers such as carbohydrate antigen 15-3 (CA15.3) and carcinoembryonic antigen (CEA) are also non-specific and has limited sensitivity and specificity, and many unnecessary referrals for biopsy evaluation (3-4).

Thus, there is still a pressing need to develop a cost-effective and accurate screening method for this cancer (5). The ideal biomarker should be easily accessible such that it can be sampled relatively noninvasively, sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour-free individuals.

microRNAs (miRNAs) a contemporary class of tiny noncoding endogenous RNA molecules, only 18–25 nucleotides long that regulate the expressions of a wide variety of genes by sequence-specific base

pairing on the 39 untranslated regions of the target mRNA resulting in mRNA degradation or inhibition of translation (6-9). Since their discovery in 1993, these small molecules have been shown to play critical regulatory roles in a wide range of biological and pathological processes. In recent years, miRNAs have been proposed as potential biomarkers for diagnosis, classification, and treatment of different types of cancer, including breast cancer (6-9).

In the present study, the levels of 9 microRNAs (miR 21, miR 27b, miR 125a, miR 155, miR 200c, miR 335, miR373, miR 181, and miR 192) were investigated in the early breast cancer patients and in healthy controls, and their potential use as breast cancer biomarkers was evaluated.

Materials and Methods

Blood samples were obtained from 20 women, after the diagnosis of breast cancer at the Department of Oncology of the Faculty of Medicine of Akdeniz University in Antalya (Turkey). The control group was selected amongst 20 healthy women, age matched, who were followed at the Antalya Genetic Diseases Diagnosis Center of Antalya (Turkey).

Ethics Committee

Approval for the study was obtained from local Ethics Committee of the Faculty of Medicine of Akdeniz University, following the Helsinki Declaration and good clinical practices. A written consent form was signed after informing each individual prior the selection.

Selection of miRNAs

Nine miRNAs dysregulated or with functions in breast cancer were selected as candidates of breast cancer biomarkers (miR 21, miR 27b, miR 125a, miR 155, miR 200c, miR 335, miR373, miR 181, and miR 192). Among them, miR 181 and miR 192 were identified as endogenous controls based on their binding potential and gene expression stability.

5 cc venous blood samples from all patients and healthy individuals were collected in EDTA containing tube. 5 cc of blood was centrifuged for 15 minutes at 2000xg and the plasma was separated. MiRNA isolation using plasma was performed using mirVana™ miRNA Isolation Kit (Invitrogen by Thermo Fisher Scientific). The level of miRNA was measured as ng/µl on the Qubit 3.0 Fluorometer using Qubit™ microRNA Assay Kit (Invitrogen by Thermo Fisher Scientific).

cDNAs were synthesized from the miRNA samples, whose concentration levels were found suitable, using TaqMan Advanced miRNA cDNA Synthesis Kit and Thermal Cycler (Applied Biosystems by Life Technologies).

The cDNAs were kept at -20°C by collecting sufficient number of either 30 or 50 samples to study. The gene expression levels were measured using StepOnePlus™ Real-Time PCR system (Catalog No: 4376598, ThermoFisher) in a total volume of 20 µl with the kit components and cDNA in each well.

Threshold cycle (Cт) values were automatically exported from the system to an excel file. The mean Cт values of the duplicated samples were calculated and the Ct values of endogenous controls miR 181 and miR 192 were considered when defining ΔCт values.

The ΔCт values of the individuals with breast cancer were compared to the ΔCт values of the healthy control group.

Statistical evaluation

All data was evaluated with SPSS (Statistical Package for the Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) program. Descriptive findings are presented with number, percentage, mean ± standard deviation and median. Shapiro-Wilk test and skewness/kurtosis values were used to evaluate whether the data represented normal distribution. Independent samples "t" test was used if the data conformed to the normal distribution and Mann-Whitney U test was used if the data was not normally distributed. Comparisons were made between breast cancer group and healthy women group. A p value $p < 0.05$ was considered statistically significant.

Receiver Operating Characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity and diagnostic efficacy of miRNAs among the investigated groups (10).

Results

The age range of 20 patients included in the study was 33-72 years, and the mean age was 51.45 ± 18.4 years. The age range of the control group was 30-68 years, and the mean age was 48.3 ± 17.8 years.
The medical history of the breast cancer group showed that 10% of the patients had a history of cancer, in their first-degree relatives, and were tobacco smokers. Breast cancer stages and pathology results indicating the type of cancer are summarized in Table 1.
Tumor Node Metastasis (TNM) system was used for staging of the breast cancer. Of the 20 breast cancer patients; 6 were Stage I, 7 were Stage II, 2 were Stage III, and 3 were Stage IV.

Invasive ductal carcinoma was diagnosed in 16 patients, medullary carcinoma in 1 patient, invasive micropapillary carcinoma in 1 patient, invasive mucinous carcinoma in 1 patient and invasive lobular carcinoma in 1 patient were identified.

Receptor characteristics of breast cancer patients determined by immunohistochemistry are summarized in Table 2. Estrogen receptors were positive in 16 patients (80%), negative in 2 patients (10%) negative, and unidentified in 2 patients (10%). Progesterone receptors were positive in 16 patients (80%), negative in 2 patients (10%), and unidentified in 2 patients (10%). HER-2 was positive in 4 patients (20%), negative in 14 patients (70%), and unidentified in 2 patients (10%).

$DeltaC_T$ values of MiRNAs according to endogenous miR 181 in breast cancer cases were compared to the healthy subjects. The mean and median values of miR125 ($p < 0.001$), miR21 ($p = 0.032$) and miR155 ($p < 0.001$) in breast cancer patients were significantly higher than those in healthy controls (Table 3).

However, when $DeltaC_T$ values of miRNAs were calculated based on the endogenous control miR 192, only the mean and median values of mir155 were significantly higher than those in healthy controls ($p = 0.006$) (Table 4).

ROC Analysis

ROC curve analysis was performed to evaluate the diagnostic value of three miRNAs. The closer the value of area under the curve (AUC) was to 1.00, the more important was the miRNA that reflected the significant difference between breast cancer and healthy controls. In the ROC analysis curve, Delta181CTmir155, Delta181CTmir125a, Delta192CTmir155 and Delta181CTmir21, were significantly high AUCs, which were respectively 0.856, 0.846, 0.765, and 0.699 (Table 5). The sensitivity and specificity values of Delta181CTmir155, with an optimal cut-off value of 1.03185558350, were respectively 83.3% and 82.4%. The sensitivity and specificity values of Delta181CTmir125a, with an optimal cut-off value of

Table 1. Tumor Node Metastasis (TNM) stages and pathology types of breast cancer patients

Stage	n	%	Pathological type	n	%
Stage 1	5	25	Invasive ductal carcinoma	16	80
Stage 2	7	35	Medullary carcinoma	1	5
Stage 3	2	10	Invasive micropapillary carcinoma	1	5
Stage 4	3	15	Invasive mucinous carcinoma	1	5
Unidentified	3	15	Invasive lobular carcinoma	1	5

Table 2. Receptor status of breast cancer

Estrogen Receptor (ER)	n	%	Progesterone Receptor (PR)	n	%	HER-2	n	%
Positive	16	80	Positive	16	80	Positive	4	20
Negative	2	10	Negative	2	10	Negative	14	70
Unidentified	2	10	Unidentified	2	10	Unidentified	2	10

Table 3. Comparison of miRNA in breast cancer (ca) and healthy controls (Delta181CT)

miRNA	Mean ± Standard Deviation	Median	P value
mir125			
Breast ca (n=20)	0.94373002050±2.393207166077	0.98937177650	**< 0.001***
Controls (n=19)	-2.43345320853±2.627056151939	-2.78703117400	
mir21			
Breast ca (n=20)	-1.66752185820±3.346716227004	-1.29511690100	**0.032***
Controls (n=19)	-3.75930996947±2.391387065653	-3.90891838100	
mir155			
Breast ca (n=18)	3.87487157183±3.324017758150	3.21931839000	**< 0.001†**
Control s (n=17)	0.04327908682±1.606969131983	0.65145111100	
mir335			
Breast ca (n=16)	-1.73451858756±2.986015580539	-0.60250520750	0.052*
Controls (n=19)	-3.60843758826±2.504742945574	-3.58927345300	
mir27b5p			
Breast ca (n=14)	-7.02835486508±3.739053080008	-6.53322410600	0.381*
Controls (n=19)	-8.27098565447±3.971230704023	-8.97427368200	
mir200c			
Breast cancer (n=12)	-4.91058476775±2.327751440005	-5.15111017250	0.723*
Controls (n=12)	-4.43942181267±3.889838833217	-4.72640895850	
mir373			
Breast ca (n=2)	3.74489450463±4.777039907303	5.07454586050	1.000†
Controls (n=8)	4.91632747650**±0.**228309140564	4.91632747650	

Legend:*Independent samples t test;†Mann-Whitney U test

-1.52255821200, were respectively 83.3% and 64.2%. The sensitivity and specificity values of Delta192CT-mir155 with an optimal cut-off value of 7,00602817550 were respectively 77.8% and 64.7%. The sensitivity and specificity values of Delta181CTmir21, with an optimal cut-off value of -3.62148714050, were respectively 72.2% and 64.7%. As a result, miR 21, miR 125a and miR 155 were found to be specific and sensitive in patients with breast cancer among the 9 candidate miRNA biomarkers.

Discussion

The miRNA expression profile is a critical regulator in the developmental stages of the mammary gland. Therefore, there is an association between miRNA levels and breast development, lactation or neoplasia. MiRNAs including miR-126, miR-150 and miR-145 have been shown to play a role in lipid metabolism during lactation. While miR-206, miR-34a, miR-17-5p and miR-125 a/b have tumor suppressor features; miR-21, miR-10b and miR-155 act as oncogenes. As a diagnostic biomarker, oncogenic miRNAs including miR-21, miR-221 and miR-210 have been reported to be overexpressed in triple receptor negative breast cancer cases (11).

In our study, miR 21, miR 27b, miR 125a, miR 155, miR 200c, miR 335, and miR373 were selected from the current literature as candidates of breast cancer biomarkers, and their expression levels were evaluated in 20 breast cancer patients and 20 healthy individuals. As a result, the values (mean/median) of miR 21, miR 125a and miR 155 were significantly high in breast cancer cases according to endogenous control miR 181. However, only miR 155 was found signifi-

Table 4. Comparison of miRNA in breast cancer (ca) and healthy controls (Delta 192CT)

miRNA	Mean ± Standard Deviation	Median	P value
mir125			
Breast ca (n=20)	5.72041964545±2.805935380779	5.40960073450	0.103†
Controls (n=19)	3.29374855447±4.027696025838	5.01218032800	
mir21			
Breast ca (n=20)	3.10916776660±2.704336095889	2.94862747150	0.238†
Controls (n=19)	1.96789179353±2.166708854763	2.71998214700	
mir155			
Breast ca (n=18)	8.47366661522±1.832332817426	8.44516563450	**0.006***
Controls (n=17)	6.11715406541±2.874619414342	6.63515853900	
mir335			
Breast ca (n=16)	2.91358882175±2.153924466686	2.88386583300	0.337†
Controls (n=19)	2.11876417463±1.768649822511	2.71636581400	
mir27b5p			
Breast ca (n=14)	-2.36461456000±1.485626477841	-2.52472877500	0.697*
Controls (n=19)	-2.54378389053±0.808368678397	-2.52287864700	
mir200c			
Breast ca (n=12)	-0.10264539700±3.007035627931	0.42615127600	0.204†
Controls (n=12)	0.77745135617±2.021266850004	1.35782718650	
mir373			
Breast ca (n=2)	7.88395690863±2.456283519221	8.26941013350	0.068†
Controls (n=8)	11.18940163000±1.210953808933	11.18940163000	

Legend:*Independent samples t test; †Mann-Whitney U test

Table 5. Sensitivity and Specificity of miRNAs by ROC analysis.

miRNAs	AUCs	Sensitivity (%)	Specificity (%)	P value
Delta181CTmir155	0.856	83.3	82.4	< 0.001
Delta181CTmir125a	0.846	83.3	64.7	< 0.001
Delta192CTmir155	0.765	77.8	64.7	0.008
Delta181CTmir21	0.699	72.2	64.7	0.044

cantly high according to endogenous control miR 192. The elevated levels of oncomiRs, miR21 and 155, (and tumor suppressor miR 125a) were also detected in our study, similarly to previous studies (11).

In breast cancer, BRCA1 and BRCA2 proteins are well-defined tumor suppressors, which are of interest for treatment. It has been shown that BRCA1 is the target of about one hundred miRNA and it can directly suppress the miR-155 activity (12). In this study, miR155, which has a potential role in breast cancer was found different between patients and healthy controls using both miR181 and miR192 endogenous controls, and the relation between miR155 and BRCA1 supports the potential oncomiR activity of miR155.

Swellam et al. (13) aimed to investigate the relation between the expression levels of three oncomiRNAs (miRNA-21, miRNA-222 and miRNA-373) and clinical findings and pathological features for early

detection of breast cancer. For this purpose, patients with primary breast cancer (n = 137), benign breast lesion (n = 60) and healthy individuals (n = 38) were included in the study. MiRNA expression levels were evaluated using real-time quantitative polymerase chain reaction (RT-qPCR) in serum samples from three groups. MiRNA-373 reported to have the highest diagnostic efficacy in comparison to miRNA-21 and miRNA-222. MiRNA levels were determined to differ significantly depending on clinical stages and histological characteristic of the breast cancer. MiRNA-21 and miRNA-373 levels were found to be statistically higher in invasive channel carcinoma (IDC) than non-IDCs**.** In the this study, IDC was found in 16 out of 20 breast cancer cases, but we could not interpret the association between pathological results and miRNA levels since the number of patients with other types of breast cancer was not adequate to generate statistics.

Han et al. (14) investigated whether the serum levels of 5 miRNAs, including miR-21, miR-125b, miR-145, miR-155, and miR-365 could distinguish breast cancer patients from healthy controls. MiRNA levels were measured in 99 breast cancer patients and 21 healthy controls. Furthermore, miRNA levels were evaluated in 20 breast cancer patients after surgical resection. The level of miR-155 in stages I and II was reported to be significantly higher compared to stage III. In addition, the levels of miR-21 and miR-155 were shown to be significantly decreased after surgical resection. ROC curve analysis was performed to evaluate the sensitivity and specificity of miRNAs as diagnostic biomarkers.

They concluded that the combination of serum miR-21, miR-155 and miR-365 might act as sensitive and specific biomarker that potentially distinguished breast cancer patients from healthy people**.**

In our study, the distribution of the 20 breast cancer patients by clinical stages were: 5 Stage I, 7 Stage II, 2 Stage III, 3 Stage IV and 3 unidentified. ROC curve analysis revealed that miR 21, miR 125a and miR 155 could be sensitive and specific miRNAs in breast cancer, but we couldn't evaluate sensitive miRNAs for each cancer stage.

According to the status of hormone receptors positivity (ER+, PR+, HER2 +), breast cancers are classified into four sub-molecular subtypes as Luminal A, Luminal B, HER2 Positive and Triple Negative Breast Cancer (TNBC). Hormone receptor status is of great value when deciding to follow the anti-estrogenic adjuvant therapy approach for treatment (15,16).

MiR551b-3p expression is reported to be increased in TNBC patients (17). Similarly, impaired expression of miR-10b, miR-21, miR-29, miR-145, miR-200, miR-203, miR-221/222 has been reported in TNBC patients. Therefore, specific miRNA clusters may act in TNBC biology and understanding them will help in disease prognosis and treatment (18). In this study, ER and PR were positive in 80% of patients, while HER-2 was positive in 20%. We could not find any association between the receptors and miR 21, miR155 and miR125.

In conclusion, miRNAs represent a class of potential biomarkers for breast cancer diagnosis and prediction of treatment. Among 9 microRNAs, selected from the literature, 3 microRNAs (miR 21, miR155 and miR125) were found to be significantly more abundant in the plasma of early-stage breast cancer (ESBC) patients compared to controls. Therefore, these 3 micro RNAs could represent a promising circulating biomarker candidates for the ESBC diagnosis if the results will be validated in a wider group of patients.

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