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Deregulated Expression of Candidate MicroRNAs and BRCA Mutations Frequency in Breast Cancer Patients

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Abstract

Circulating microRNAs (miRs) have attracted a great deal of attention as promising novel markers for various diseases. Our study aimed at evaluating the clinical utility of specific types of plasma miRs as diagnostic biomarkers in early-stage breast cancer (BC) patients and to study their relation with breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2) mutations in BC patients and high-risk females. The study included 45 early-stage BC patients (30 non-familial and 15 familial), 15 high-risk subjects and 20 clinically healthy females as control. Using quantitative real-time polymerase chain reaction (RT-PCR), the relative expression levels of some plasma miRs (10b, 21, 155, 145 and let-7c) were determined in breast cancer patients and high-risk, compared to controls. Also, multiplex PCR was applied for the detection of 185delAG and 5382insC mutations in BRCA1 gene, and 6174delT mutation in BRCA2 gene using multiplex PCR and mutations were confirmed using High Resolution Melt (HRM) technique. Plasma miR-10b and miR-21 levels were significantly up-regulated, while miR-155 and miR-let-7c levels were significantly down-regulated in BC patients and high-risk subjects, compared to controls. MiR-21 was significantly up-regulated, whereas miR-155 level was significantly down-regulated in patients with lymphatic invasion. MiR-21 was showed a significant association with mutation in exon 2 of BRCA 1 in high-risk individuals. Deregulated expression of miR-10b, miR-21, miR-155, and let-7c serves as a potential non-invasive diagnostic marker in early-stage II BC, and surveillance biomarkers for individuals at a higher-expected risk of developing BC. Also, high-risk individuals harbor classical mutations in BRCA1 gene, considering it a high priority for these individuals to have a strict follow-up.

Keywords: Breast cancer, miRNAs, mutations, BRCA1, BRCA2

1. Introduction

Breast cancer (BC), the second most typical kind of cancer within the world, is the second chief reason for gynecological cancer mortality [1-2]. In Egypt, 35.1% of overall female cancer patients (23.081/65,693) suffered from BC [3]. Approximately, 85% to 90% of breast cancers do not seem to be related to heritable mutations (sporadic in nature) that result from the build-up of gained and uncorrected genetic modifications in somatic genes, with none germline mutation [4-5]. By contrast, women with robust case history of BC could inherit genetic variations that alter their danger of disease, where a case history of BC can boost a woman's hazard for acquiring the disease two- to threefold [6-7]. BRCA1 and BRCA2 represent the 2 most often mutated genes causing inherited BC, where transformations in BRCA1/2 genes are accountable for about 16-20% of the chance for familial BC [8-9].

MicroRNAs (miRs) are small single-stranded RNAs that play a vital regulatory role in the cell. They function as post-translational monitors of mRNA expression resulting in inhibition of translation or mRNA degradation [10-11]. Several miRs exert oncogenic or tumor-suppressive functions. They were shown to be deregulated in cancers since quite 50% of miR genes are located at fragile sites and regions of deletion or amplification that are altered in human cancers [11-12]. MiRs had important advantages including their stability in plasma/serum and, therefore, offer a vital diagnostic tool in cancer screening, detection and repeated measurement in a non-invasive manner [13-15].

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MiR-10b is encoded by a highly preserved genomic region situated near the homeobox D (HOXD) cluster on chromosome 2. Its overexpression promotes cell migration and invasion [16]. It has been shown that raised expression of the transcription factor Twist in BC cells enhances the transcription of miR-10b. Also, miR-10b lessens HOXD10 protein synthesis, a negative controller of BC progression, allowing expression of pro-metastatic gene products, like Rho C, urokinase plasminogen activator receptor (uPAR), α3-integrin, and membrane type-1 matrix metalloproteinase (MT1-MMP), which esteems tumor cell migration, invasion and metastasis [17].

MiR-21, located on chromosome 17 (17q.23.1) within the 11th intron of the transmembrane protein 49 (TMEM49) gene, is a well-known tumor-promoting miR [18]. It is up-regulated in several cancers and related to progressive clinical phases of tumor metastasis and poor prognosis [19-20]. MiR-21 targets numerous genes related to tumor growth and metastasis, like tumor suppressor tropomyosin1 (TPM1), programmed cell death 4 (PDCD4), tissue inhibitor of metalloproteinases-3 (TIMP-3) and phosphatase and tensin homolog (PTEN) [21]. Moreover, current proof reveals that transforming growth factor-beta (TGF-β) stimulation enhances miR-21 expression in tumor cells, which consequently accelerates the progression of epithelial-mesenchymal transition (EMT), resulting in tumor metastasis and invasion [22]. On the other hand, demolition miR-21 expression could attenuate its contribution to the cancer process [23].

MiR-155 is a multifunctional oncogenic miR that's encoded by chromosome 21. It performs an important role in BC metastasis because of its implication in the acquirement of EMT and augmented ability for invasion and metastasis [24]. The ectopic expression of miR-155 impacts cancer cell existence and chemosensitivity through suppressing forkhead box O3 (FOXO3), while knocking down the expression of miR-155 can augment cell chemosensitivity and facilitate apoptosis [25].

MiR-145 is found on chromosome 5 (5q32-33) and performs its tumor-suppressive role by silencing target genes in stage-specific actions [26]. The protein expression of Rhotekin (RTKN) is reduced by miR-145 within MCF-7 breast cancer cell line, leading to the suppression of cell growth and induction of apoptosis [27]. Also, miR-145 can exactly target the transmembrane glycoprotein mucin 1 (MUC1), a metastasis gene, resulting in the decrease of cadherin 11 and β-catenin, conferring repressed cell invasion and lung metastasis [28]. The EMT of BC cells has been reported to be inhibited by miR-145 via barring the expression of octamer-binding transcription factor 4 (Oct4) [29]. Let-7c, which is encoded by chromosome 21, operates as a tumor suppressor by controlling cancer-related genes, like C-MYC, N-RAS, PBX2, MMP1 and PBX3 [30-31].

Although core biopsy and mammography are detection tools for BC, they are not sensitive for examinations [32]. Accordingly, the progress of a blood-based diagnostic means could be of a great benefit because it would allow screening to start at a earlier age and will possibly diagnose tumors that are not measurable by mammography. Therefore, the expression of some circulating miRs, like miR-10b, miR-21, miR-145, miR-155, and let-7c, for the early detection of stage II BC patients as non-invasive molecular biomarkers was analyzed. In addition, the current study aimed at testing the profiles of three founder mutations, including 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2, which are frequently reported in breast cancer patients from various ethnic backgrounds, that reflect the risk of BC in Egyptian women at high risk due to strong family history.

2. Patients and methods

2.1. Patients

The study comprised 45 newly diagnosed female BC (early stage II) patients selected from the Outpatient Clinics and admitted to Surgery and Pathology Departments (National Cancer Institute, Cairo University, Egypt). BC patients were divided into two groups; patients without a family history of BC (sporadic or non-familial, n=30) and patients with a family history of BC (familial, n=15). BC women that obtained chemotherapy before breast resection were excluded. Diagnosis of all patients depended on standard clinical criteria [33]. Clinicopathological characteristics of non-familial and familial BC patients at initial diagnosis are represented in Table 1. In addition, 15 subjects at a higher-expected risk of developing BC (high-risk; clinically healthy with a positive family history) and 20 healthy female volunteers were recruited. None of the high-risk or healthy controls had been previously diagnosed with any malignancies.

2.2. Ethics approval and consent to participate

Ethical approval was permitted by the Institutional Review Board of the Egyptian National Cancer Institute (Approval No: 2010014066.3) and informed written consents were acquired from all participants.

2.3. Study design

The change in the expression level of selected miRs (10b, 21, 155, 145 and let-7c) was evaluated in the plasma using quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, screening for BRCA1 (exon 2 and 20) and BRCA2 (exon 9)

mutations using multiplex PCR was investigated and confirmed using the HRM technique.

Table 1. Clinicopathological data of BC patients included in the study.

Clinical Features		Groups	
		Non-familial n=30 (%)	Familial n=15 (%)
Age	≤45	13 (43)	2 (13)
	>45	17 (57)	13 (87)
Menopausal	Pre	16 (53)	1 (7)
	Post	14 (47)	14 (93)
Tumor size	<5	21(70)	7 (47)
	>5	9 (30)	8 (53)
Lymph nodes involvement	(+ve)	19 (63)	11 (73)
	(-ve)	11 (37)	4 (27)
Tumor stage	II	30 (100)	15 (100)
Grade	IDC	26 (87)	8 (54)
	ILC	4 (13)	7 (46)
Subtype	Luminal A (ER +ve; PR +ve; HER2/neu -ve)	30 (100)	15 (100)

ER: Estrogen receptor; PR: Progesterone receptor; HER2/neu: Human epidermal growth receptor 2.

2.4. Blood sample collection and storage

The blood was collected into sterile EDTA-containing vacutainer labeled tubes, centrifuged at 4°C for 10 min at 1,900×g and the plasma was then removed into a sterile tube that was further centrifuged for 10 min at 1,600×g at 4°C. The separated plasma was stored at -80°C for the analysis of candidate miRs.

2.5. RNA extraction, cDNA synthesis and qRT-PCR analysis of selected miRs

Extraction of total RNA, including miRs, cDNA synthesis, and quantitative real-time polymerase chain reaction of candidate miRs was performed as previously mentioned elsewhere [33]. The primer sequences (Qiagen, Hilden, Germany) are listed in Table 2 and the relative quantification of the expression of candidate miRs was calculated by the comparative threshold cycle (ΔΔCT) method [34].

2.6. Detection of BRCA 1 and BRCA 2 mutations using Multiplex PCR

DNA was extracted from peripheral blood samples of healthy control and high-risk subjects using the EZ-10 Spin Column Genomic DNA Miniprep kit (BIO BASIC, Canada). In addition, paraffin sections of breast tumor specimens from non-familial and familial BC patients were obtained from the Pathology Department at the National Cancer Institute, and DNA was extracted according to the method of Wang et al. [35]. The concentration and degree of purity of extracted DNA were estimated by measuring the absorbances at 260 and 280 nm wavelengths by Nanodrop 2000 Spectrophotometer 9 (Isogen Life Science, Netherlands). Multiplex PCR was performed in Perkin-Elmer/Cetus DNA Thermal Cycler 450 (Waltham, MA, USA) to detect 185delAG and 5382insC mutations in exon 2 and 20 of the BRCA1 gene, respectively, and 6174delT mutation in exon 9 of the BRCA2 using sets of the allele-specific primers previously published by Chan et al. [36] and provided by Qiagen (Hilden, Germany). For each allelic mutation, three primers were used (one common, one specific for the wild type allele, and one specific for the mutant allele). The amplification was done in a final volume of 25 µl containing 100 ng of genomic DNA, 1×PCR master mix (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM dNTPs and 2U Taq DNA polymerase) and 2 µM for P1 and P3, 0.4 µM for P2, 0.12 µM for P4, P5 and P6, 0.3 µM for P7 and P9, and 0.24 µM for P8 primers (Table 2). The reaction mixtures were heated at 94°C for 5 min followed by 35 cycles of amplification, each consisting of 40 sec of denaturation at 94°C, 40 sec of annealing at 55°C, and 60 sec of extension at 72°C, followed by final extension for 5 min at 72°C. The amplified products, assessed by judgement with a 100 bp DNA ladder marker (Amersham, UK), were detected by electrophoresis on 3% ethidium bromide-stained agarose gel and visualized using Photo Doc Analyzer System under UV transillumination and photographed (Figure 1 a-c).

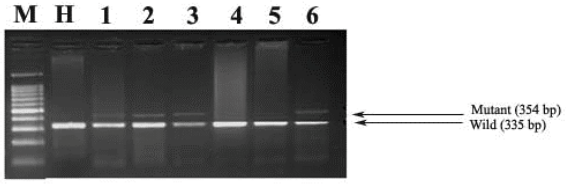

Figure (1-a). A representative photo showing the 185delAG mutation in BRCA1 exon 2 in non-familial breast cancer patients using multiplex PCR, the mutant and wild type amplicons are 354 and 335 bp, respectively. Lane M: ladder marker. Lane H: a healthy control showing one band (wild). Lanes 1, 2, 3, and 6 showed 185delAG mutation in BRCA1 exon 2 (two bands; wild and mutant alleles). Lanes 4 and 5 show absence of mutation (one band, wild).

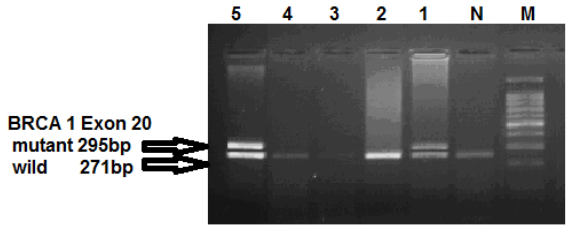

Figure (1-b). A representative photo showing the 5382insC mutation in BRCA1 exon 20 in familial breast cancer patients using multiplex PCR, the mutant and wild type amplicons are 295 and 271 bp, respectively. Lane M: ladder marker (100 bp). Lane N: normal, Lanes 1 and 5 showed two bands (mutant alleles). Lanes 2, 3 and 4 showed one band (absence of mutant alleles).

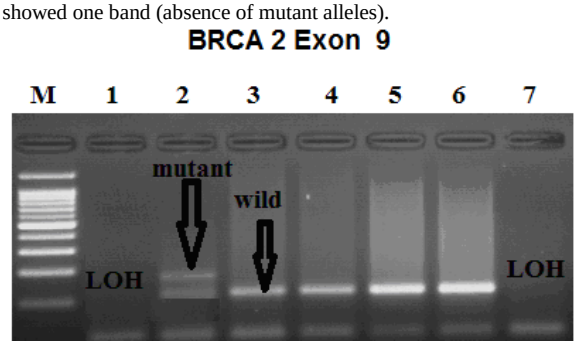

Figure (1-c). A representative photo showing the 6174delT 5382insC mutation in BRCA2 exon 9 in familial breast cancer patients using multiplex PCR, the mutant and wild type amplicons are 171 and 151 bp, respectively. Lane M: ladder marker (100 bp). Lanes 3, 4, 5, and 6 showed absence of any mutant alleles. Lane 2 showed two bands representing mutant alleles. Lane 1 and 7 showed loss of heterozygosity (LOH).

2.7. High resolution melt (HRM) assay as a confirmatory test for detection of BRCA 1 and 2 genes mutation

Mutations detected by Multiplex PCR were further confirmed by the High Resolution Melt curve (HRM) technique. The PCR assay was performed in AB 7500 Fast Real-Time PCR (Applied Biosystems (USA) to amplify BRCA 1 exons 2 and 20 and BRCA 2 exon 9 using sets of primers previously published by de Leener et al. [37] and provided by Qiagen (Hilden, Germany) (Table 2). The PCR and HRM were implemented in a single run in a reaction mix containing MeltDoctor™ HRM Master Mix (Applied Biosystems, USA), 200 nM of each PCR primer and 20 ng of genomic DNA in 20 µl PCR reactions. The reaction conditions included an activation step at 95ºC for 10 min followed by 55 cycles of 95ºC for 10 sec, a touch down of 65ºC to 55ºC for 10 sec (1ºC/cycle) and 72ºC for 30 sec. Before the high-resolution melting step, the products were heated at 95ºC for 1 min. The HRM was carried out over the range from 72ºC to 95ºC rising at 1ºC per sec with 30 acquisitions per degree. Analysis of the obtained curves was performed using Applied Biosystems® HRM v2.0.2 software. During the HRM stage of the melt (dissociation) curve, double-stranded amplicons slowly denature, releasing bound dsDNA-binding dye. The decrease in the fluorescent signal is measured by the real-time PCR instrument, and the HRM software plots fluorescence signals over temperature (Figure 2 a-c).

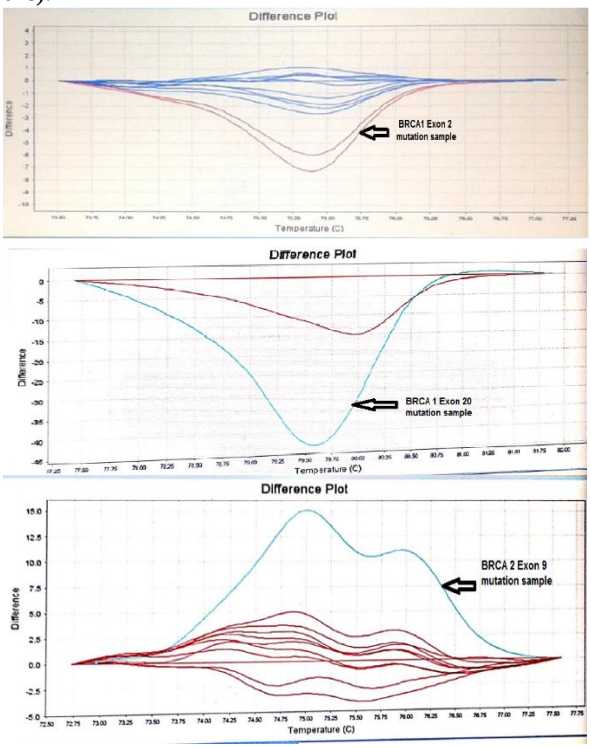

Figure 2. HRM Results: (**a**) Aligned melt curves of BRCA1 gene exon 2 by HRM. Dark blue: wild type, red: mutant type, (**b**) Aligned melt curves of BRCA1 gene exon 20 by HRM. Red: wild type, mutant type blue (**c**) Aligned melt curves of BRCA2 gene exon 9 by HRM. Red: wild type, mutant type blue.

2.8. Statistical analysis

All data were shown to be non-parametric ($p>0.05$) by using the Shapiro-Wilks test for normality. The non-parametric test (Mann-Whitney U) was used to compare two independent groups. Spearman's correlation was applied and ROC curves were computed, where AUC and cut-off values were computed to assess the diagnostic accuracy of the markers. Cross-tabulation analysis was used, where the significance (χ^2) and the likelihood ratio (LR) were computed from the selected cut-off values. All statistics were investigated using SPSS Statistical Software version 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Data represented in Table 3 show a significant up-regulation in the relative expression of plasma miR-10b and miR-21 levels, whereas a significant down-regulation was demonstrated in the expression of plasma miR-155 in high-risk individuals, as well as BC patients (familial and non-familial), compared to

healthy controls. In addition, a significant down-regulation was shown in the expression of let-7c in the plasma of BC patients, compared to healthy controls.

ROC curve analysis revealed significant AUC values for plasma miR-10b, miR-21 and miR-155 in high-risk individuals (0.75, 0.87 and 0.76, respectively), compared to healthy controls. At the optimal cut-off values for each marker, the sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy percent were calculated and presented in Table 4. Among the analyzed miRs, absolute specificity (100%) value and highest diagnostic accuracy were recorded for miR-21. When comparing the expression of candidate miRs between BC patients and healthy individuals, significant AUC values for plasma miR-10b, miR-21, miR-155 and let-7c were obtained (0.935, 0.984, 0.836 and 0.699, respectively). Absolute specificity (100%) values were recorded for both miR-10b and miR-21, whereas the highest diagnostic accuracy was recorded for miR-21(Table 4).

Table 2. Nucleotide sequence of the primer sets used for qRT-PCR amplifications of selected miRs, the detection of BRCA1 and BRCA2 mutations by multiplex PCR and HRM methods (Qiagen, Hilden, Germany).

Method	miR	Code		Sequence	
qRT-PCR	miR-10b	hsa-miR-10b-3p		ACAGAUUCGAUUCUAGGGGAAU	
	miR-21	hsa-miR-21-3p		CAACACCAGUCGAUGGGCUGU	
	miR-155	hsa-miR-155-3p		CUCCUACAUAUUAGCAUUAACA	
	miR-145	hsa-miR-145-3p		GGAUUCCUGGAAA- UACUGUUCU	
	let-7c	hsa-let-7c-3p		CUGUACAACCUUCUAGCUUUCC	
	miR-16	hsa-miR-16-3p		CCAGUAUUAACUGUGCUGCUGA (housekeeping miR)	
Method	**Gene**	**Exon**	**Primers**	**Sequence**	**Amplicon size**
Multiplex PCR	BRCA1	Exon 2 185delAG	Common Forward (P1)	5'-GGTTGGCAGCAATATGTGAA-3'	Wild: 335 bp Mutant: 354 bp
			Wild-type Reverse (P2)	5'-GCTGACTTACCAGATGGGACTCTC-3'	
			Mutant Reverse (P3)	5'-CCCAAATTAATACACTCTTGTCGTGACTTACCAGATGGGACAGTA-3'	
		Exon 20 5382insC	Common Reverse (P4)	5'-GACGGGAATCCAAATTACACAG-3'	Wild: 271 bp Mutant: 295 bp
			Wild-type Forward (P5)	5'-AAAGCGAGCAAGAGAATCGCA-3'	
			Mutant Forward (P6)	5'-AATCGAAGAAACCACCAAAGTCCTTAGCGAGCAAGAGAATCACC-3'	
	BRCA2	Exon 9 6174delT	Common Reverse (P7)	5'-AGCTGGTCTGAATGTTCGTTACT-3'	Wild: 151 bp Mutant: 171 bp
			Wild-type Forward (P8)	5'-GTGGGATTTTTAGCACAGCTAGT-3'	
			Mutant Forward (P9)	5'-CAGTCTCATCTGCAAATACTTCAGGGATTTTTAGCACAGCATGG-3'	
HRM analysis	BRCA1	Exon 2	Forward	5'-CTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT-3'	173 bp
			Reverse	5'-TCCCAAATTAATACACTCTTGTGCTGA-3'	
		Exon 20	Forward	5'-CTTTCTCTTATCCTGATGGGTTGTG-3'	170 bp
			Reverse	5'-GAGTGGTGGGGTGAGATTTTTGTC-3'	
	BRCA2	Exon 9	Forward	5'-ATAAGGGGGGACTACTACTATATGTG-3'	220 bp
			Reverse	5'-CAAAAAAACCTGTAGTTCAACTAAACAGAG-3'	

Table 3. Statistical significance of the relative quantification (RQ) of candidate plasma miRs in high-risk individuals, all BC patients, non-familial and familial BC patients, compared to control subjects.

Group / miRNA		Control (20)	High-risk (15)	p<	Breast cancer (45)	p<	Non-familial breast cancer (30)	p<	Familial breast cancer (15)	p<
miR-10b	Median	0.72	1.45	0.05	6.68	0.001	6.68	0.001	7.16	0.001
	IQR	(0.49 - 0.78)	(0.48-4.72)		(5.06-11.24)		(4.98-10.85)		(5.06-14.32)	
miR-21	Median	0.97	1.87	0.001	17.16	0.001	17.77	0.001	17.15	0.001
	IQR	(0.57-1.43)	(1.40-4.45)		(9.85-25.99)		(9.86-25.99)		(8.57-27.86)	
miR-145	Median	1.22	0.75	NS	0.93	NS	0.83	NS	1.06	NS
	IQR	(0.36-2.54)	(0.26-2.79)		(0.43-1.79)		(0.43-1.67)		(0.43-2.28)	
miR-155	Median	1.26	0.47	0.01	0.33	0.001	0.59	0.001	0.13	0.001
	IQR	(0.59-1.5)	(0.23-0.76)		(0.13-0.84)		(0.14-0.92)		(0.09-0.50)	
let-7c	Median	0.84	0.59	NS	0.36	0.05	0.48	0.05	0.24	0.01
	IQR	(0.28 - 1.46)	(0.22-1.44)		(0.20-0.72)		(0.22-0.75)		(0.16-0.63)	

- Mann-Whitney *U* test for non-parametric data was applied.

Table 4. Diagnostic values (sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy %) of significant biomarkers.

		AUC	p<	Cut-off	Sens.%	Spec.%	PPV	NPV	Acc.%
High-risk vs Control	miR-10b	0.75	0.05	0.87	73	85	78.6	81	80
	miR-21	0.87	0.001	1.53	73	100	100	83.3	88.6
	miR-155	0.76	0.01	0.945	70	80	66.7	82.4	74.3
BC vs Control	miR-10b	0.935	0.001	1.76	89	100	100	80	92.3
	miR-21	0.984	0.001	1.5	96	100	100	90.9	96.9
	miR-155	0.836	0.001	1.11	70	91	87.2	77.8	84.6
	let-7c	0.699	0.05	0.92	50	89	80	66.7	76.9

AUC: Area under curve; Sens.%: Sensitivity percent; Spec.%: Specificity percent; PPV; Positive predictive value; NPV: Negative predictive value; Acc.%: Diagnostic accuracy percent.

Table 5 illustrates significant correlations among different miR profiles in high-risk individuals as well as familial and non-familial BC patients. MiR-155 shows a significant positive correlation with let-7c (r=0.54) in high-risk individuals, while miR-21 has a significant negative correlation with let-7c (r=0.672) and miR-145 (r=0.444) in familial and non-familial BC patients, respectively (Table 5).

Using the chosen cut-off values of candidate miRs, it was possible to significantly sort out 91.1% (41/45) and 88.9% (40/45) of BC patients with up-regulated miR-21 and down-regulated miR-155 and let-7c expressions, respectively. Also, it was possible with down-regulated miR-155 and let-7c expressions (Table 6).

A significant relation (p=0.002) was reported between tumor size and BRCA1-exon 20 mutation in BC patients, where patients with tumor size <5 have a wild exon 20 (24 patients, 53.3%) or a mutant exon 20 (4 patients, 8.9%), while patients with tumor size >5 have a wild exon 20 (7 patients, 15.6%) or a mutant exon 20 (10, 22.2%). Also, a significant relation was shown between lymph nodes involvement and miR-21 (p=0.041) or miR-155 (p=0.003) expressions in BC patients. Thirty BC patients (66.7%) with positive lymph nodes involvement have up-regulated miR-21 expression, while 13 patients (28.9%) with negative lymph nodes involvement have up-regulated miR-21 expression. By contrast, thirty BC patients (66.7%) with positive lymph nodes involvement have down-regulated miR-155 expression, while 11 patients (24.4%) with negative lymph nodes involvement have down-regulated miR-21 expression (Table 8).

Table 5. Correlations among candidate miRs in different groups.

			miR-10b	miR-21	miR-145	miR-155	let-7c
High-risk individuals (n=15)	**miR-10b**	**Correlation Coefficient**	1.000	0.163	0.157	-0.410	-0.428
		p<	--	0.561	0.576	0.129	0.111
	miR-21	**Correlation Coefficient**	0.163	1.000	-0.098	0.073	-0.021
		p<	0.561	--	0.727	0.795	0.940
	miR-145	**Correlation Coefficient**	0.157	-0.098	1.000	-0.016	-0.105
		p<	0.576	0.727	--	0.955	0.708
	miR-155	**Correlation Coefficient**	-0.410	0.073	-0.016	1.000	**0.540**
		p<	0.129	0.795	0.955	--	**0.038***
	let-7c	**Correlation Coefficient**	-0.428	-0.021	-0.105	**0.540**	1.000
		p<	0.111	0.940	0.708	**0.038***	--
Familial BC (n=15)	**miR-10b**	**Correlation Coefficient**	1.000	-0.115	-0.102	0.138	0.479
		p<	--	0.684	0.717	0.623	0.071
	miR-21	**Correlation Coefficient**	-0.115	1.000	-0.019	-0.350	**-0.672**
		p<	0.684	--	0.947	0.201	**0.006****
	miR-145	**Correlation Coefficient**	-0.102	-0.019	1.000	-0.048	0.175
		p<	0.717	0.947	--	0.866	0.532
	miR-155	**Correlation Coefficient**	0.138	-0.350	-0.048	1.000	0.189
		p<	0.623	0.201	0.866	--	0.501
	let-7c	**Correlation Coefficient**	0.479	**-0.672**	0.175	0.189	1.000
		p<	0.071	**0.006****	0.532	0.501	--
Non-familial BC (n=30)	**miR10b**	**Correlation Coefficient**	1.000	-0.244	-0.044	-0.133	0.062
		p<	--	0.194	0.819	0.485	0.744
	miR-21	**Correlation Coefficient**	-0.244	1.000	**-0.444**	0.119	0.093
		p<	0.194	--	**0.014***	0.530	0.624
	miR-145	**Correlation Coefficient**	-0.044	**-0.444**	1.000	0.025	-0.033
		p<	0.819	**0.014***	--	0.894	0.863
	miR-155	**Correlation Coefficient**	-0.133	0.119	0.025	1.000	0.279
		p<	0.485	0.530	0.894	--	0.135
	let-7c	**Correlation Coefficient**	0.062	0.093	-0.033	0.279	1.000
		p<	0.744	0.624	0.863	0.135	--

Spearman correlation for non-parametric data was applied.

Table 6. Relation between candidate miRs in BC patients (n=45).

		miRs					
		miR-21		**miR-155**		**let-7c**	
		No	**Up**	**No**	**Down**	**No**	**Down**
miR-10b	**No**	0 (0)	5 (11.1)	1 (2.2)	4 (8.9)	0 (0)	5 (11.1)
	Up	2 (4.4)	38 (84.4)	3 (6.7)	37 (82.2)	5 (11.1)	35 (77.8)
	p<	0.609		0.354		0.402	
miR-21	**No**	--	--	2 (4.4)	0 (0)	2 (4.4)	0 (0)
	Up	--	--	2 (4.4)	41 (91.1)	3 (6.7)	40 (88.9)
	p<	--		**0.001***		**0.001***	
miR-155	**No**	--	--	--	--	2 (4.4)	2 (4.4)
	Down	--	--	--	--	3 (6.7)	38 (84.4)
	p<	--		--		**0.01***	

No: No change; Up: Up-regulated expression; Down: Down-regulated expression.

Table 7. Frequency of BRCA1 and BRCA2 mutations in high-risk individuals, familial and non-familial BC patients.

	BRCA1				**BRCA2**	
	Exon 2		**Exon 20**		**Exon 9**	
	Wild	**Mutant**	**Wild**	**Mutant**	**Wild**	**Mutant**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
High-risk individuals (n=15)	**11 (73%)**	**4 (27%)**	**13 (87%)**	**2 (13%)**	**13 (87%)**	**2 (13%)**
Familial BC (n=15)	**9 (60%)**	**6 (40%)**	**9 (60%)**	**6 (40%)**	**10 (67%)**	**5 (33%)**
Non-familial BC (n=30)	**20 (67%)**	**10 (33%)**	**22 (73%)**	**8 (27%)**	**23 (77%)**	**7 (23%)**

Table 8. Relation between BRCA mutations and candidate miRs with clinicopathological data of BC patients.

		BRCA1				**BRCA2**		**miRs**							
		Exon 2		**Exon 20**		**Exon 9**		**miR-10b**		**miR-21**		**miR-155**		**let-7c**	
		Wild	**Mutant**	**Wild**	**Mutant**	**Wild**	**Mutant**	**No**	**Up**	**No**	**Up**	**No**	**Down**	**No**	**Down**
		N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Age	**≤45**	9 (20)	6 (13.3)	12 (26.7)	3 (6.7)	10 (22.2)	5 (11.1)	3 (6.7)	12 (26.7)	0 (0)	15 (33.3)	1 (2.2)	14 (31.1)	0 (0)	15 (33.3)
	>45	20 (44.4)	10 (22.2)	19 (42.2)	11 (24.4)	23 (51.1)	7 (15.6)	2 (4.4)	28 (62.2)	2 (4.4)	28 (62.2)	3 (6.7)	27 (60)	5 (11.1)	25 (55.6)
	p<	0.66		0.255		0.475		0.18		0.306		0.711		0.094	
Menopause	**Pre**	10 (22.2)	7 (15.6)	14 (31.1)	3 (6.7)	10 (22.2)	7 (15.6)	3 (6.7)	14 (31.1)	0 (0)	17 (37.8)	1 (2.2)	16 (35.6)	0 (0)	17 (37.8)
	Post	19 (42.2)	9 (20)	17 (37.8)	11 (24.4)	23 (51.1)	5 (11.1)	2 (4.4)	26 (57.8)	2 (4.4)	26 (57.8)	3 (6.7)	25 (55.6)	5 (11.1)	23 (51.1)
	p<	0.539		0.128		0.086		0.277		0.26		0.581		0.065	
Tumor size	**<5**	15 (33.3)	13 (28.9)	**24 (53.3)**	**4 (8.9)**	18 (40)	10 (22.2)	4 (8.9)	24 (53.3)	2 (4.4)	26 (57.8)	4 (8.9)	24 (53.3)	3 (6.7)	25 (55.6)
	>5	14 (31.1)	3 (6.7)	**7 (15.6)**	**10 (22.2)**	15 (33.3)	2 (4.4)	1 (2.2)	16 (35.6)	0 (0)	17 (37.8)	0 (0)	17 (37.8)	2 (4.4)	15 (33.3)
	p<	0.051		**0.002***		0.078		0.384		0.26		0.103		0.913	
Lymph nodes involvement	**Pos**	19 (42.2)	11 (24.4)	20 (44.4)	10 (22.2)	22 (48.9)	8 (17.8)	3 (6.7)	27 (60)	**0 (0)**	**30 (66.7)**	**0 (0)**	**30 (66.7)**	2 (4.4)	28 (62.2)
	Neg	10 (22.2)	5 (11.1)	11 (24.4)	4 (8.9)	11 (24.4)	4 (8.9)	2 (4.4)	13 (28.9)	**2 (4.4)**	**13 (28.9)**	**4 (8.9)**	**11 (24.4)**	3 (6.7)	12 (26.7)
	p<	0.826		0.649		1.00		0.737		**0.041***		**0.003***		0.18	
Grade	**ILC**	8 (17.8)	3 (6.7)	6 (13.3)	5 (11.1)	7 (15.6)	4 (8.9)	0 (0)	11 (24.4)	0 (0)	11 (24.4)	0 (0)	11 (24.4)	0 (0)	11 (24.4)
	IDC	21 (46.7)	13 (28.9)	25 (55.6)	9 (20)	26 (57.8)	8 (17.8)	5 (11.1)	29 (64.4)	2 (4.4)	32 (71.7)	4 (8.9)	30 (66.7)	5 (11.1)	29 (64.4)
	p<	0.509		0.237		0.403		0.177		0.411		0.233		0.177	

No: No change; Up: Up-regulated expression; Down: Down-regulated expression.

Table 9. Relation between BRCA mutations and candidate miRs in BC patients.

			BRCA1				BRCA2	
			Exon 2		Exon 20		Exon 9	
			Wild	Mutant	Wild	Mutant	Wild	Mutant
			N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
miR-10b		**No**	5 (11.1)	0 (0)	4 (8.9)	1 (2.2)	4 (8.9)	1 (2.2)
		Up	24 (53.3)	16 (35.6)	27 (60)	13 (28.9)	29 (64.4)	11 (24.4)
		p<	0.078		0.569		0.721	
miR-21		**No**	2 (4.4)	0 (0)	2 (4.4)	0 (0)	1 (2.2)	1 (2.2)
		Up	27 (60)	16 (35.6)	29 (64.4)	14 (31.1)	32 (71.1)	11 (24.4)
		p<	0.283		0.331		0.445	
miR-155		**No**	3 (6.7)	1 (2.2)	4 (8.9)	0 (0)	3 (6.7)	1 (2.2)
		Down	26 (57.8)	15 (33.3)	27 (60)	14 (31.1)	30 (66.7)	11 (24.4)
		p<	0.644		0.159		0.937	
let-7c		**No**	4 (8.9)	1 (2.2)	4 (8.9)	1 (2.2)	4 (8.9)	1 (2.2)
		Down	25 (55.6)	15 (33.3)	27 (60)	13 (28.9)	29 (64.4)	11 (24.4)
		p<	0.441		0.569		0.721	
BRCA1	**Exon 2**	**Wild**	--	--	**16 (35.6)**	**13 (28.9)**	20 (44.4)	9 (20)
		Mutant	--	--	**15 (33.3)**	**1 (2.2)**	13 (28.9)	3 (6.7)
		p<	--		**0.007***		0.372	
	Exon 20	**Wild**	--	--	--	--	**20 (44.4)**	**11 (24.4)**
		Mutant	--	--	--	--	**13 (28.9)**	**1 (2.2)**
		p<	--				**0.047***	

No: No change; Up: Up-regulated expression; Down: Down-regulated expression.

Table 10. Relation between BRCA mutations and candidate miRs in high-risk individuals.

			BRCA1				BRCA2	
			Exon 2		Exon 20		Exon 9	
			Wild	Mutant	Wild	Mutant	Wild	Mutant
			N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
miR-10b		**No**	**2 (13.3)**	**2 (13.3)**	**4 (26.7)**	**0 (0)**	**4 (26.7)**	**0 (0)**
		Up	**9 (60)**	**2 (13.3)**	**9 (60)**	**2 (13.3)**	**9 (60)**	**2 (13.3)**
		p<	**0.218**		**0.36**		**0.36**	
miR-21		**No**	**1 (6.7)**	**3 (20)**	**4 (26.7)**	**0 (0)**	**3 (20)**	**1 (6.7)**
		Up	**10 (66.7)**	**1 (6.7)**	**9 (60)**	**2 (13.3)**	**10 (66.7)**	**1 (6.7)**
		p<	**0.011***		**0.36**		**0.423**	
miR-155		**No**	**2 (13.3)**	**1 (6.7)**	**3 (20)**	**0 (0)**	**2 (13.3)**	**1 (6.7)**
		Down	**9 (60)**	**3 (20)**	**10 (66.7)**	**2 (13.3)**	**11 (73.3)**	**1 (6.7)**
		p<	**0.77**		**0.448**		**0.255**	
BRCA1	**Exon 2**	**Wild**	--	--	**9 (60)**	**2 (13.3)**	**10 (66.7)**	**1 (6.7)**
		Mutant	--	--	**4 (26.7)**	**0 (0)**	**3 (20)**	**1 (6.7)**
		p<	--		**0.36**		**0.423**	
	Exon 20	**Wild**	--	--	--	--	**11 (73.3)**	**2 (13.3)**
		Mutant	--	--	--	--	**2 (13.3)**	**0 (0)**
		p<	--				**0.551**	

No: No change; Up: Up-regulated expression; Down: Down-regulated expression.

A significant relation (p=0.007) was established between BRCA1 exon 2 and 20 mutations in BC patients, where 16 patients (35.6%) harbor wild BRCA1 gene, 1 patient (2.2%) has mutations in both exons, 13 patients (28.9%) have wild-type exon 2 and mutant exon 20, 15 patients (33.3%) have mutant exon 2 and wild-type exon 20. On the other hand, a significant relation (p=0.047) was recorded between BRCA1 exon 20 and BRCA2 exon 9 mutations in BC patients, where 20 patients (44.4%) harbor wild BRCA1/2 genes, 1 patient (2.2%) has mutation in both exons, 11 patients (24.4%) have wild-type BRCA1 exon 20 and mutant BRCA2 exon 9, 13 patients (28.9%) have mutant BRCA1 exon 2 and wild-type BRCA2exon 9 (Table 9).

As for high-risk individuals, a significant relation was shown between BRCA1 exon 2 mutation and miR-21 expression, where 10 patients (66.7%) with up-regulated miR-21 expression have no mutations in BRCA1 exon 2, while one patient (6.7%)

with up-regulated miR-21 expression has a mutant BRCA1 exon 2. On the other hand, 3 patients (20%) that have mutant BRCA1 exon 2 show miR-21 expression below cut-off values (Table 10).

4. Discussion

BC epigenetic mechanisms are not wholly clarified, but it is now undisputable that miRs play a vital role in BC pathogenesis and development, and being able to exactly modify several kinds of crucial genes with oncogenic, tumor-suppressive properties, or also acting in DNA fixing mechanisms [38]. In addition, a high percentage of altered miRs in genetic tumors is frequently found in earlier studies with non-familial breast cancers, signifying that there are miRs that probably control significant oncogenes and tumor suppressor genes involved in both familial and sporadic cancer development, irrespective of their genetic background or molecular subtypes [39-40]. Hereditary alterations, particularly in BRCA1 and 2 genes meaningfully surge the hazard of BC development [41].

In the current study, we profiled 5 miRs; two were up-regulated (miR-21 and miR-10b) in BC patients (familial and sporadic) and high-risk individuals, while miR-155 was down-regulated in BC patients and high-risk individuals, and let7c was down-regulated in BC patients. By contrast, miR-145 expression was not changed in all groups, compared to controls. Zhu et al. [13] reported that the expression of serum miR-145 was not altered in breast cancer patients, compared to normal samples. The authors recommended the determination of circulating miR-145 levels over the period of cancer growth and progression.

Like BC patients, the significant up-regulation in miR-21 and miR-10b, along with the significant down-regulation in miR-155 expressions in high-risk subjects (those with a positive family history), agrees with the previous study of O'Day and Lal [42] who proved that the down-regulation of miR-155 in early-stage breast cancer is associated with cancer invasiveness. We therefore suggest the importance of using these biomarkers to further predict which individuals require extreme follow-up than others, especially those at a high-risk for developing BC. Also, both miR-21 and miR-10b have the highest diagnostic accuracy to distinguish BC patients or high-risk individuals from healthy controls, which suggests considering their expression values as non-invasive diagnostic molecular biomarkers for BC. We reported similar findings for the diagnosis of locally advanced BC patients [33].

MiR-21 is one of the oncogenic miRs that is required for the initiation, progression and metastasis of breast carcinomas by targeting several significant genes [43-45]. Regarding the association between miRs expression and clinicopathological data, we established a significant correlation between lymph nodes involvement and up-regulated miR-21 expression in BC patients (30 out of 45; 66.7%), which supports the involvement of miR-21 in cancer metastasis. Also, our study indicates that miR-155 levels were significantly lower in BC patients with lymphatic invasion than in those without invasion ($p=0.003$), which agrees with the finding of Kong et al. [46] who reported that miR-155 seems to play a crucial part the metastasis of breast cancer due to its involvement in EMT gaining and increased probability for invasion and metastasis.

A large number of different mutations in BRCA1 and 2 genes have been described globally, however, little is recognized about their frequency and patterns in Egyptian populations. In the current study, we found that 185delAG mutation in exon 2 BRCA1 was present in 27, 40, and 33%, whereas the 5382insC mutation in exon 20 BRCA1 we detected in 13, 40, 27%, whereas the 6174delT mutation of exon 9 BRCA2 was detected in 13, 33, and 27%, in high-risk individuals, familial, and non-familial breast cancer patients, respectively (Table 7).

The high frequency of BRCA 1 and BRCA 2 genes comes from the strict selection for studied groups and also a specific selection for specific types of founder mutations. A similar study shows a higher expression of the 185delAG founder mutation in Egyptian BC patients [47]. In addition, numerous studies reported these founder transformations in other populations [48-50]. Also, the result of the current study agrees with that performed on Ashkenazi Jewish breast cancer patients, which showed the high prevalence of the 185delAG mutation in the BRCA1 gene [51].

The current study investigated the role of these two susceptibility genes in breast cancer and their relations with alterations in specific miRs in the development of breast cancer. BRCA1 and BRCA2 proteins are well-defined tumor suppressors. Approximately 100 miRs target BRCA1 and the latter represses miR-155 activity. Furthermore, miR-21-targeted therapy might be beneficial for the treatment of BRCA2 mutation carriers [37]. Notably, a huge number of miRs (151) proved substantial differential expression between BRCA tumors and other mutation-free breast tumors, establishing a signature of "BRCAness" [52-53].

In our results, statistical associations were observed regarding changes in the expression of miRs (10b, 155, and 21) and BRCA genes mutation. Similarly, Chang et al. [54] reported that the temporary up-regulation of BRCA1 protein expression lessens miR-155 expression. The mechanism of

BRCA1 regulation of miR-155 is via straight binding of BRCA1 protein to the miR-155 promoter, which recruits histone deacetylase (HDAC) to suppress B-cell integration cluster (BIC) expression and thus miR-155. This close link with the breast cancer susceptibility gene strengthens the importance of miR-155 in breast cancer. Therefore, to investigate the correlation between miR-155 and BRCA-mutant tumors needs a larger sample size.

Our results show a significant association between BRCA1 exon 20 mutation and larger tumor size (> 5 cm) in BC patients. Also, only one BC patient (2.2%) had both BRCA1 exon 2 and 20 mutations, and one BC patient (2.2%) had both BRCA1 exon 20 and BRCA2 exon 9 mutations. Although we could not establish a relation between BRCA mutations and candidate miRs in BC patients, however, the high-risk individuals recorded a significant correlation between up-regulated miR-10b expression and BRCA1 exon 2 mutations.

In conclusion, the data obtained from our study demonstrated the deregulated expression of miR-10b, miR-21, miR-155, and let-7c in early-stage II BC patients, serving as potential non-invasive diagnostic markers in BC, and surveillance biomarkers for individuals at a higher-expected risk of developing BC. Also, high-risk individuals harbor classical mutations in the BRCA1 gene, considering it a high priority for these individuals to have had a strict follow-up. However, additional large prospective clinical researches are needed to approve our initial findings.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and have approved its submission.

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Dedication

To the memory of late Professor Dr Hala M. El-Desouki.

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