Original Article

Study the Expression of miRNA let 7 Gene Among Iraqi Breast Cancer Patients

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Abstract

Background: MicroRNAs (miRNAs) are short non-coding RNA sequences (approximately 22 nucleotides) that play an important role in gene regulation. **Objective:** The aim of this study was to evaluate the expression of serum (miRlet-7) in the Iraqi pupation as important biomarkers among the Iraqi women. **Materials and Methods:** This study included 50 female breast cancer patients. Patient's peripheral blood was obtained, and apparently healthy female volunteers acted as controls. Each individual had 5 mL of venous blood draw under aseptic circumstances, which was directly placed in Trizol preservation for RNA extraction. Circulating serum miRNAlet-7 expression was measured using RT-qPCR. **Result:** There were significant increases in groups (41–50) years old (P = 0.016) and over 60 years old (P = 0.02) compared to control groups. Of these 50 female patients with breast cancer, 28 (46%) were under 50 years old and 22 (44%) were over 50 years old. Approximately 37 (74%) of these hadestrogen-positive receptors (ER) and 36 (72%) had progesterone-positive receptors (PR). Also 20 of 50 cases (20%) had positive and 30 (60%) had HER-2 expression. According to histopathological type 96% were invasive ductal carcinoma (IDC), and 4% were invasive lobular carcinoma (ILC) of all breast cancer patients. **Conclusion:** Based on the results of this investigation, we find that miRlet-7 expression is significantly higher (P = 0.003) in breast cancer patients than in apparently healthy controls; therefore, it is correlated with breast cancer. Also, this study found that among Iraqi women with breast cancer, the occurrence of the Ductal subtype is more significant than that of the lobular subtype.

Keywords: Breast cancer, estrogen and progesterone, gene expression, let-7, lobular carcinoma, miRNA

Introduction

Cancer is a major public health problem worldwide and is the second leading cause of death in the world.[1] Breast cancer (BC) is a disease in which cells in the breast grow out of control. There are different kinds of breast cancer. The kind of breast cancer depends on which cells in the breast turn into cancer.[2] BC can begin in different parts of the breast. A breast is made up of three main parts: lobules, ducts, and connective tissue.[3] Most breast cancers begin in the ductsi or lobules.[4]

MiRNAs are 21–25 nucleotides noncoding RNAs that may be related to cell proliferation, differentiation, and apoptosis.[5] regulate the expression of several genes and proteins by negatively controlling gene expression. In general, miRNAs bind to complementary sequence sites of a target mRNA and induce translational silencing or degradation..[6,7] MiRNAs are liberated into the circulation after radiation-induced tumor tissue destruction, so they can serve as biomarkers to directly and dynamically monitor response to RT by analyzing blood samples.[7] Over the last few years, miRNAs have gained major attention in breast cancer research due to their imperative role in tumor initiation and progression which has created the new prospect for early cancer diagnosis and therapies.[8] The growing number of research demonstrating the presence of miRNAs in circulating serum/plasma raises the possibility of employing miRNAs as a biomarker for cancer and other

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disorders.[9] Recent studies have reported the upregulation of a tumor suppressor (TS) miRNAs, namely let-7 family miRNAs.[10]

Several types of cancer rely on the 1et-7 family of miRNAs (1et-7a, 1et-7b, 1et-7c, 1et-7d, 1et-7e, 1et-7f, 1et-7g, and 1et-7i) as a tumor suppressor. Multiple species' sequences and functions for the let-7 family are quite similar.[11] In many cancers, compared to normal tissue and as tumors advance, the expression of let-7 family members is down-regulated. It appears that downregulation of 1et-7 family members contributes to the development of various cancers.[12]

Materials and Methods

Participants were enrolled in the study between November 2022 and March 2023 at the Genetic Engineering and Biotechnology Institute at Baghdad University. Breast cancer patients who sought care at Baghdad's Al-Andalus Specialist Oncology Hospital or the Center for Cancer Treatment at Al-Fallujah Hospital provided the samples.

Fifty female patients with pathologically proven breast cancer from Iraq were sampled, and 50 female controls were also collected. Patients and healthy controls each had 5 mL of venous blood drawn; those with a diagnosis of BC had their blood deposited in gel tubes and centrifuged for 15 min at room temperature to separate their serum for molecular testing.

Protocol of microRNA extraction

According to the TRIzol reagent extraction methodology, total RNA was isolated from the sample; 0.2 mL of chloroform was added to the aqueous phase containing the RNA; 0.5 mL of isopropano1 was used to precipitate the RNA as a white gel-like pellet; and 0.5 mL of 70% ethanol was used to wash the pellet. To conclude, pellet was rehydrated in 50 L of nuclease-free water and then heated in a water bath at 55–60°C for 10–15 min.

Reverse transcription for complementary DNA (c DNA) synthesis

Four microliters of RNA material was combined with 1 µL of miRlet-7-5p stem-loop RT primers. In this investigation, primers were designed and synthesized using the NCBI Genei Bank data base and miRBASE. The primers used in this study were selected from those in the Macrogene (South Korea) primer set [Table 1]. The bonds in the hairpin loop structures seen in Table 2 were broken by incubating the mixture for 5 min at 70 C and then 10 min at 4 C.

Quantitative real-time polymerase chain reaction (RT-q PCR)

RT-qPCR was performed using SYBR Green PCR Kit (Synthol/Russia) following the manufacturer's instructions. The quantitative reaction was performed as 10 µL total reaction volume, containing 5 µL of SYBR Green Master Mix, 0.5 µL of specific miRNA primer, forward and reverse, 2 µL of RNase-free water and 2 µL of cDNA, and performed using a real-time PCR system (Synthol/Russia). The cycling conditions were set as follows: 95°C for 1 min (1 cycle), 45 cycles of 95°Cfor 20 s, 55°C for 20 s and 72 for 20 s. MiRNA expression levels were presented in terms of fold change normalized by the house keeping gene *miR-16-1* using the formula: $2^{-\Delta\Delta CT}$.[13]

Table 1: Primer sequence for miRlet-7-5p and miRNA-16 gene expression

Primer name	Sequence
miRlet-7 RT	5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAG
CCAACAACTATA -3′	
miRlet-7 F	5′- GGTTTTTTTTGAGGTAGTAGGTTG -3′
Universal Reverse	5′ - GTGCAGGGTCCGAGGT -3′
MiR-16-1 RT	5-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCA
ACCGCCAAT-3′	
MiR-16 -1 F	5′- GGTTTTTTTTAGCAGCACGTAAAT -3′

Table 2 Thermal cycler program for first reaction

Steps	°C	min:sec	Cycle
Priming annealing	70	05:00	1
Hold	4	10:00	
Cool in ice and spin			

Ethical approval

This work was approved by the Ethical Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq according to the Ref number (EC/2991) dated November 28, 2022. The samples were collected after the approval of the patients.

Statistical analysis

Analysis of data was carried out using International Business Machine Statistical package for the Social Sciences (IBM SPSS, version 28).The results were analyzed statistically, and the values were expressed as mean ± SE. *t* test test was used to significant compare between two samples. Chi-square test was used to significant compare between percentages. Statistical significance was considered whenever the P value was equal or less than 0.05.

Results

This study involved 100 females, who were enrolled in the study consisting of 50 females with BC of diagnosis during chemotherapy, along with 50 healthy females to serve as a control group. All BC samples were divided into five groups according to age; these groups ranged from ≤ 30-year-old to more than 60 years.

The age distribution of females involved in the control group showed a mean of about 49.1 ± 10.6 years. The mean of female's age with BC was 35.9 ± 11.9 years, that significant increase in grouped (41-50) years old (P = 0.016) and significant in grouped upper than 60 years old (P = 0.02) as compared to apparently healthy control groups.. Among these 50 Breast cancer females 28 sample were aged less than 50 years, and 22 sample were upper than 50 year [Table 3].

The results demonstrated a substantial as compared to the control group, with 28 (55.1%) of the tota1 patients having a favorable family history, whereas the remaining 22 (44.9%) did not as shown in Table 4.

Moreover, many sex hormones including estrogen, progesterone, and others including were accused to take part in breast cancer development. Seventy-five percent (37/50)

Table 3: Demographic characteristics of patients with Gout and healthy control subjects

Characteristic	Patients *n* = 50	Healthy control *n* = 50	Total *P* value
Age (years)			
≤ 30, *n* (%)	2 (4%)	20 (41.6%)	0.001
31–40, *n* (%)	10 (20.4%)	13 (27%)	0.53
41–50, n (%)	16 (32.6%)	7 (10.4%)	0.016
51–60, n (%)	13 (26.5%)	9 (18.7%)	0.39
> 60, n (%)	9 (16.3%)	1 (2%)	0.02
P-value	0.021	0.001	

Table 4: Distribution of patients and control subjects according to family history

Family history	Patients *n* = 50		Control *n* = 50		*P*
	N	%	*n*	%	
Positive	28	55.1	4	6.3	0.001 ¥ HS
Negative	22	44.9	46	93.7	

n = number of cases, ¥ = chi-square test, HS = highly significant at $P \leq 0.01$

Table 5: Frequency distribution of some associated Hormone results in patients with breast cancer

Characteristic	Patients		P value
	N	%	
Estrogen			
Positive, n (%)	37	75.5 %	0.001 ¥ HS
Negative, n (%)	13	24.5 %	
Progesterone			
Positive, n (%)	35	71.4 %	0.001 ¥ HS
Negative, n (%)	15	28.6 %	
HER 2			
Positive, n (%)	22	42.8 %	0.31 ¥ NS
Negative, n (%)	28	57.2 %	

Table 6: Frequency distribution of patients with breast cancer according to BC type and Subtype

Characteristic	Patients		P value
	N	%	
BC type			
Invasive ductal carcinoma (IDC)	41	83.6 %	0.001 ¥ HS
Ductal Carcinoma in Situ (DCIS)	4	8.2 %	
Invasive Lobular Carcinoma (ILC)	4	8.2 %	

n: number of cases; ¥: Chi-square test; HS: Highly significant at $P \leq 0.01$

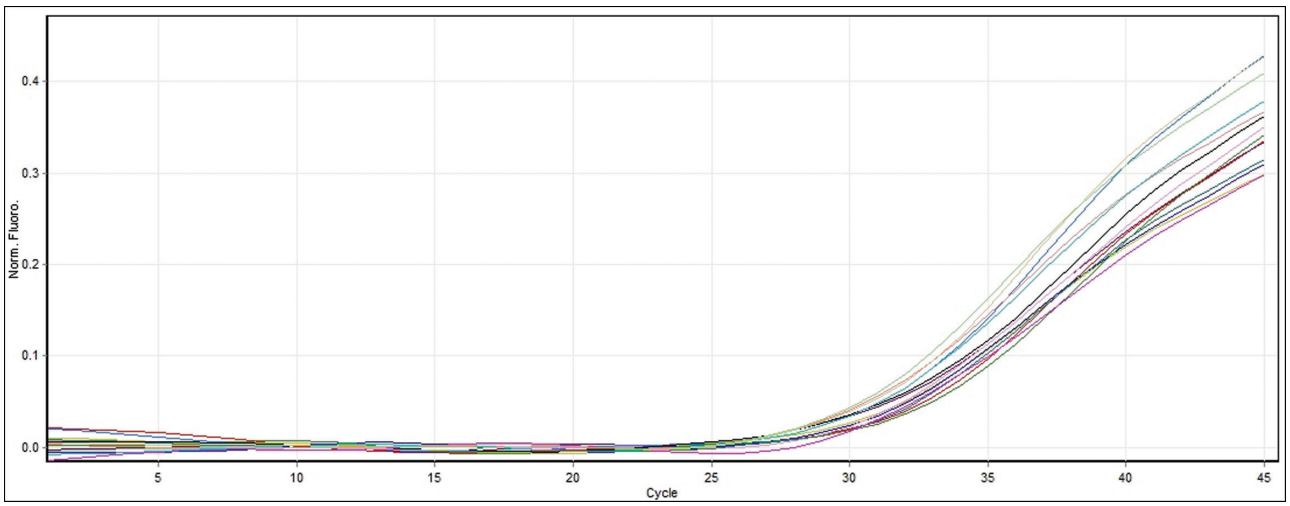

Figure 1: *miRlet-7* dissociation curves by q PCR Samples included all study groups

of the 50 BC patients presented had good results for ER receptors, whereas 71.4% (35/50) had positive results for PR receptors. It was statistically significant positive association with getting BC (P = 0.001) and (P = 0.001), respectively. On the contrary, HER-2 receptors were positive in 42.8% (21/50) of the cases. However statistically these relations of HER-2 did not reach the level of significance as in [Table 5].

The data in Table 6 showed a significance (P≤0.01) between invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), ductal 41 (83.6%) cases, and cases 4(8.2%) were lobular.

In addition, the molecular experiment of *miRlet-7* expression was performed to detect the amplification plots of *miRlet-7* and *miR-16* (reference gene) to find the threshold cycle (Ct) value for each. The curves of *miRlet-7* are shown in Figure 1.

The average cycle threshold (Ct) value of patients and control for target miRNAlet-7 and housekeeping gene miRNA16 were calculated based on the Ct values using the Livak method.

ΔCT = CT miRNAlet-7 gene – CT house keeping gene miRNA16

ΔΔCT =ΔCT treated or control – average ΔCT control

The ΔΔCt was used for the determination of fold difference in the miRNA expression for both miRNAlet-7 and miRNA34a-5p

Table 7: miRlet-7 level in patients with breast cancer and healthy control

MiRlet-7	Fold change
Patients N: 50	1.29
Controls N:50	1

n = number of cases

The fold-change for genes was calculated as follows [Table 7]:

folding = $2^{-\Delta\Delta CT}$

Discussion

The results of this study found that breast cancer can develop at any age; it is most common among women in middle age. These results agree with the previous Iraqi studies such as,[14] which divided the patients into two categories of age (<50 and >50). The recent findings confirmed the need of screening and early detection of breast cancer, which is a disease that can affect women at any age but tends to occur in the middle years of their lives. A recent Iraqi study about breast cancer done by [15] agrees with the results of the current study. Results showed that those between the ages of 35 and 49 and 50 to 64 made up 42.4% and 42.2% of the population, respectively. Moreover, breast cancer rates increased between the ages of 51 and 60 (38.19%), according to a few studies.[16] Cells have more time to mutate with age,

increasing the risk of cancer, cigarette smoke, chemicals and other cancer-causing.[14]

This study showed that the patients with a positive family history of breast cancer 27 (55.1%) cases compared to 3 (6.3%) healthy control women have a positive family history, and the differences were highly significant. This finding supports the genetic basis of the disease. Research showed that breast cancer rates among Iraqi women are affected by family history. This is not unexpected given the prevalence of consanguineous marriages in Iraq. Breast cancer risk factors largely included a family history of the disease. The present result is higher than the results of,[17] which found that 19 (38 %) patients had a confirmed case of breast cancer in their family.[18] It was discovered that among 204 Iraqi patients, 43.7% having a positive family history. Other studies found that 9 (18.00%) and (10.4%) of breast cancer patients have a positive family history.[19] The percentage of patients who have a family history of breast cancer may vary depending on the size of the sample and the overall population, as suggested by these data. Women with this condition have a significantly increased cancer risk compared to the general population, so it is advised that they undergo extensive screening as quickly as possible.[20]

Estrogen hormone is one of the steroid hormones with a vital role in the etiology of breast cancer.[21] Estrogen and progesterone receptors are hormone receptors found on breast cells that pick-up hormone signals resulting in cell growth. In breast cancer status the determination of these receptors is useful for therapeutic options and provide prognostic information.[15]

The present results are consistent with [20] who showed (ER) and (PR) were both positive in 39 patients and negative in 11 with (78% and 22%,) respectively, while [22] found a low level of estrogen which have antioxidant characteristic causes reduction in the levels of antioxidant parameters and then increase breast cancer risk factor.

For a median of 8.3 years, 18 586 women in a large-scale American study were diagnosed with invasive breast cancer; of them, 14 969 were ER-positive (80.5%) and 3617 were ER-negative (19.5%).[21] In AL-Bahrain,[23] tumors were ER-positive in 65.7% of cases, PR-positive in 57.8%, and HER2 amplified in 31.1% of patients. The present results showed 21 (42.8 %) cases of patients with breast cancer have positive for HER 2 results. these results were in agreement with [24] which showed that 30 % of patients were HER2 positive.

Several studies in Iraq have come to the same conclusion: ductal is the most common type of histology.[25,20] Showed most predominant histological type of breast cancer (74.0%) was invasive ductal carcinoma (IDC).[26] Showed of cases 72 (90.0 %) with breast cancer having IDC. But the present results higher than the results of,[27] which found that Invasive ductal carcinoma accounts for 48% of all cases of breast cancer, followed by ductal carcinoma in situ (34%), and finally invasive lobular carcinoma (18%).

Abnormal regulation and expression of MiRlet-7, also known as the "keeper of differentiation," has been linked to carcinogenesis.[22] Early cancer identification by less invasive methods, such as detecting differential let-7 levels in physiological fluids, could reduce the hazards associated with biopsies. The results current came in agreement with,[28] who found increased plasma 1et-7 levels are seen in patients with breast in compared to healthy control. Also the present findings consistence with,[10] who found increased plasma let-7 levels are seen in patients with breast and ovarian cancer. But the current results disagree with results of,[29,30] who reported decreased serum let-7 levels in ovarian and breast cancers. It is important to consider 1et-7 would be the mostibeneficia1 to patient surviva1 before developing it into therapy.[31,32]

Conclusion

Based on the results of this investigation, we find that miRlet-7 expression is significantly higher in breast cancer patients than in healthy controls, therefore, it is correlated with breast cancer. Also, this study found that among Iraqi women with breast cancer, the occurrence of the Ductal subtype is more significant than that of the Lobular subtype.

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Conflicts of interest

There are no conflicts of interest.

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