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Role of miRNA-21-5p Gene Expression as a Tumor Marker and the Progression of Clinic-Pathological Characters in Breast Cancer Iraqi Female Patients

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Abstract: Breast cancer is a malignancy of the breast tissues that arise from uncontrolled proliferation of breast ductal and lobular epithelial cells. It's the most prevalent malignancy among the Iraqi women. The aims of study are analyzing the expression of serum (miR-21-5p) as a molecular tumor marker and main clinic pathological characters for patients and healthy control groups. Determination of microRNA gene expression by (qRT-PCR) for both BC patients' healthy control and statistical analysis of their demographic characteristics. The results show that high significant up regulation of serum miRNA-21 expression in BC patients (5.27) times than control group and the main clinic-pathological data show high percentage of (IDC) histopathology type, (T2) staging, the hormone receptors ER+, PR+, high metastasis LN+ and the molecular subtypes Luminal-A than other characters. In conclusions the high miRNA-21 expression levels make it act as noninvasive diagnostic tumor marker and show the aggressive phenotype progression BC diseases' characters.

Keywords: Breast cancer, microRNA-21, gene expression, RT-q PC.

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Introduction

Cancer is a genetic disease that produced as genetic defect in cell function, particularly cell growth and division, it's also recognized as a major source of death all around the world (1). Breast cancer (BC) is a heterogeneous diverse disease that divided into four main subtypes Luminal-A, Luminal-B, Triple Negative, HER2 each with its own clinical outcomes, such as patient survival and prognosis (2). All BC originate in the mammary glands' ducts and lobules, with ductal cancers that the majority of the malignancies (3). The identification of effective strategies for first diagnosis of BC is crucial to promote the survival rates of patients. The tumor biomarkers are molecules measured in the body's fluids and that can predict the risk of getting a cancer as prognostic biomarkers (4). MicroRNAs a class of endogenously non-coding RNAs having a length of ~22 bp found in different species, there have key regulators of biological processes by gene-silencing pathways, there deregulation of microRNAs in cancer cells, which is associated with tumor initiation, invasion and drug resistance. They are useful to detection of early cancer and significantly help to treatment response (5). MiRNA-21 is one of the first oncomiRs discovered, the up regulated it in breast cancer caused by decreased apoptosis and increased proliferation. As a result, miR-21 has been proposed as an acceptable prognostic, diagnostic biomarker and a therapeutic target for breast cancer (6, 7).

Materials and methods

Study plane and subjects

The study conducted from November 2021 to January 2022 in the laboratories of genetic engineering and biotechnology institute in university of Baghdad and the samples collected from patients with first diagnosed breast cancer patients consulted Al-Andalus Specialist Oncology Hospital in Baghdad and Al Anbar Specialized Center for Cancer treatment. The samples were taken from BC patients undergoing chemotherapy and grouped according to their BC histological subtype into five groups (IDC, ILC, DCIS, LCIS, MDLIS) and according to stage (I-IV) and grade (I-III).

Serum sample collection

Venous blood was taken from patients and healthy groups five milliliters (mL), all patients' diagnosis for primary BC by histology these samples were placed in gel tubes for 30 minutes at room temperature, and then centrifuged for 10 minutes in order to obtain serum.

Demographic and risk factors data collection

Their collected using a "short structured questionnaire", It is included the information of (name, age, weight, height, menopausal status, family history of breast cancer or other malignancies, that recorded on a data collection sheet, designed for the purpose of the study.

MicroRNA extraction

Total RNA included microRNA was isolated from sample according to the protocol of TRIzol Reagent, 0.2 mL of chloroform add for aqueous phase containing of RNA, 0.5 mL of isopropanol was added for RNA precipitated as white gel-like pellet, 0.5mL of 70% ethanol was added for RNA washing, finally Pellet was rehydrated in 50µl of Nuclease Free Water then incubated in a water bath at 55-60°C for 10-15 minutes.

Reverse transcription

Each 4 µl of RNA sample was mixed with 1 µl stem-loop RT primers of miR-21. The Primers for miR-21 were design in this study by using (The Sanger Center miR database Registry) to selected miR-21 sequence and using miR Primer Design Tool. The cDNAs were synthesized by reverse transcription of microRNA using script cDNA synthesis kit, with stem-loop primer "5GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAACA 3".

Quantitative real time PCR

The PCR master mix preparation as shown in table 1 and Real Time PCR Program thermal cycling conditions for miR-21, in table 2.

Table (1): The PCR master mix preparation for miR-21.

Master mix components	Volume (µl)
SYBR Green Master Mix	5
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	3
MiRNA-21 cDNA template	1
Total volume	10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template

Table (2): Real Time PCR Program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	**1**
Denaturation	95	00:20	
Annealing	55	00:20 Acquiring on Green	**50**
Extension	72	00:20	

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) (8).

Results and discussion

1. **Demographic characteristics of BC patients and control groups**

This study involved seventy seven women were enrolled in the study, includes fifty women with BC at early state of diagnosis and had treatment with chemotherapy, along with twenty seven healthy women to serve as a control group.

A. All participants were females with age range between 21 to 74 years. It occur between 27 to 74 years for malignant group while 21 to 67 years for healthy women. Results shown that 70% of females with BC aged more than 50 years. The mean age of females with BC was high significantly increased (P≤0.01) as compared to control groups (53.64 ± 1.36 *versus* 39.55 ± 2.58, respectively), (table 3).

Table (3): Correlations between demographic data (Age) for patients and control.

Variables	BC patients N=50(%)	Control N=27(%)	P-value
Age / mean ± S.E.M	53.64 ±1.36	39.55 ±2.58	0.0001 **
Age (years): > 50	35 (70.00%)	10 (37.04%)	0.0026 **
Age (years): ≤ 50	15 (30.00%)	17 (62.96%)	

B. The results of BMI (Kg/m2) showed that 44% of BC were obese, overweight 42% compared to only 14.81% obesity and 33.33% overweight in the control group. The mean BMI of females with BC was high significantly increased (P≤0.01) as compared to control group (30.38 ± 0.78 *versus* 25.49 ± 0.65, respectively), (table 4).

Table (4): Correlations between demographic data (BMI) for patients and control.

Variables	BC patients N=50(%)	Control N=27(%)	P-value
BMI (Kg/m2) / Mean ± S.E.M	30.38 ±0.78	25.49 ±0.65	0.0001 **
(Normal) < 25	7 (14.00%)	14 (51.85%)	0.0001 **
(Overweight) 25-29.9	21 (42.00%)	9 (33.33%)	
(Obese) 30-40	22 (44.00%)	4 (14.81%)	

C. The same trends, menopausal period results indicate that higher percentages (54 %) of BC women were recorded in post-menopausal period, while the less percentage (18.52%) includes the healthy volunteer's women. The mean of post-menopausal status of females with BC was high significantly increased (P≤0.01) as compared to control groups (5 (18.52%) *versus* 27 (54.00%), (Table 5).

Table (5): Correlations of (Menopausal Status) for BC patients and control.

Variables	BC patients N=50(%)	Healthy Control N=27(%)	P-value
Menopausal Status			
Post-menopausal	27 (54.00%)	5 (18.52%)	0.0001 **
Pre-menopausal	23 (46.00%)	22 (81.48%)	

D. The Family History of BC result show only (18%) of BC patients gave positive family history of BC, in addition to no Significant results with only (4%) of positive history of other malignancies, (table 6).

Table (6): Correlations between demographic data (Family History) for patients and control.

Variables	BC patients N=50(%)	Control N=27(%)	P-value
Family History of BC			
Positive	9 (18.00%)	2 (7.41%)	0.0492 *
Negative	41 (82.00%)	25 (92.59%)	
Other malignancies			
Positive	2 (4.00%)	1 (3.70%)	0.607 N.S
Negative	48 (96.00%)	26 (96.30%)	

1. Clinic pathological characteristics of breast cancer patients' group

This study involved (50) patients with BC at early state of diagnosis who had treatment with chemotherapy. Their recorded data including (Stage of the tumor, lymph node invasive, Grade, ER-PR-Her2 receptors, molecular and histological subtype) have been studied and the results are presented in many tables.

A. According to the obtained results, the highest percentage type histologically is 37 patients as 74% were having invasive ductal carcinoma (IDC), the second common type was 14% as ductal carcinoma in situ (DCIS), just 4 patients with 8% having invasive lobular carcinoma (ILC) and only one patient with (2%) showed lobular carcinoma (LCIS) and Ductal- Lobular Carcinoma In Situ (MDLs), (table 7).

Table (7): The pathological characteristics (Histological type) of the BC patients.

Variable	BC N=50	BC %	P-value
Histological type			
invasive ductal carcinoma (NOS)(IDC)	37	(74.00%)	0.0001 **
Invasive Lobular carcinoma *(ILC*)	4	(8.00%)	
Ductal carcinoma *in situ* (DCIS)	7	(14.00%)	
Lobular carcinoma *in situ* (LCIS)	1	(2.00%)	
Ductal-Lobular carcinoma *in situ* (D-LCIS)	1	(2.00%)	

B. The molecular subtypes obtained result was the highest percentage of tumors were Luminal A in 29 patients (58%) while 10 (20%) were have Luminal B and only 3 (6%) were having Tripe negative and finally just 8 patients (16%) patients with HER^{2+}, (table 8).

Table (8): The pathological characteristics (Molecular subtype) of the BC patients

Variable	BC N=50	BC %	P-value
Molecular subtype			
Luminal A	29	(58.00%)	0.0001 **
Luminal B	10	(20.00%)	
Triple Negative (BCL)	3	(6.00%)	
HER2/neu	8	(16.00%)	

C. Based on TNM criteria result, it was found that 16 patients with the highest with an equal percentage for each stage (32%) having tumors in the stage II and III, while 10 patients (20%) were in stage I and only 8 patients (16%) were having tumor in stage IV, (table 9).

Table (9): The pathological characteristics (TNM staging) of the BC patients.

Variable	BC N=50	BC %	P-value
TNM Stage			
• **I**	10	(20.00%)	0.0274 *
• **II**	16	(32.00%)	
• **III**	16	(32.00%)	
• **IV**	8	(16.00%)	

D. Histological grade present result show the grade II which considers the highest percentage (46%) among the samples while only 11 (22%) were at grade I and the rest of the patients 16 (32%) were at grade III, (table 10).

Table (10): The pathological characteristics (Histologic Grade) of the BC patients.

Variable	BC N=50	BC %	P-value
Histologic Grade			
• **I**	11	(22.00%)	0.0084 **
• **II**	23	(46.00%)	
• **III**	16	(32.00%)	

E. According to the obtained results, Lymph node (LN) involvement were positive in 40 (80%) and negative in 10 (20%), table 11).

Table (11): The pathological characteristics (Lymph node) of the BC patients

Variable	BC N=50	BC %	P-value
Lymph node			
Positive	40	(80.00%)	0.0001 **
Negative	10	(20.00%)	

Finally, the hormone receptors studying showed (ER) and (PR) were both positive in 39 patients and negative in 11 with (78% and 22%, respectively). While the Her2/neu receptor was high percentage at negative state in 42 (84%) patients, (table 12).

Table (12): The pathological characteristics (Hormonal receptors) of the BC patients.

Variable	BC N=50	BC %	P-value
Estrogen receptor			
Positive	39	(78.00%)	0.0001 **
Negative	11	(22.00%)	
Progesterone receptor			
Positive	39	(78.00%)	0.0001 **
Negative	11	(22.00%)	
Her2/neu receptor			
Positive	8	(16.00%)	0.0001 **
Negative	42	(84.00%)	

2. Molecular analyses (quantitative expression of hsa-miR-21-5p)

A. The expression level of housekeeping gene amplification in study groups

The mean of Ct value of RNU43 in BC patients was 20.53 and in healthy control was 20.39, the Statistical analyses show no significant difference (0.902 N.S) between gene folding change of patients (0.9) and in control (1.0), (table 13).

Table (13): Comparison expression between study group of (RNU-43) as a reference gene depending on 2^-Ct Method.

Study Group of (RNU43)	No.	Ct-means RNU43	2^- Ct RNU43	Experimental / control	Folding of gene
BC Patients	50	20.53	6.60 E- 0.7	6.6047 E- 0.7 / 7.277 E- 0.7	0.90±0.17
Healthy Control	27	20.39	7.27 E- 0.7	7.2777 E- 0.7 / 7.277 E- 0.7	1.0±0.00
The Statistical analyses				0.902 N.S: Non-Significant.	

B. The expression level of miR-21-5p amplification in study groups

The RT-q PCR results for miR-21 analyzed by the relative quantification gene expression levels (folding changes) which were based on the (Ct) values.

Calculating gene expression depending on (2^-Δ Ct Method). By using Equation: (Fold change =2^- Δ Ct target gene / 2^- Δ Ct control gene). (Pfaffl formula).All 50 patients show high level of miR-21 level which was significantly high (**P>0.01) among BC patients (5.27 times increase) than healthy control (1.00), table (14).

Table (14): Comparison expression between study group of (MiRNA-21-5P) as a target gene depending on 2^-Δ Ct Method.

Descriptive Statistics	Breast cancer	Healthy control
C_t mature miR-21	31.13 ±4.05	33.39 ±3.63
C_t RNU- 43	20.53 ±2.91	20.39 ±3.08
Relative expression $\Delta C_t = (C_{t\,21} - C_{t\,RNU})$	10.60 ±1.07	13.00 ±1.59
Relative expression (2^-Δ Ct)	6.4429 E-0.4 ± 0.00083	1.2207 E-0.4 ± 0.000077

Experimental / control	6.4429 E-0.4 / 1.2207 E-0.4	1.2207 E-0.4 / 1.2207 E-0.4
Folding of gene	5.27803 ± 0.93	1.00 ± 0.00
The Statistical analyses	** (P≤0.01) : 0.0001**	

Results presented in this study related to information of different criteria obtained from the questionnaire forma answered by patients and control groups. This study involved 77 women, includes 50 women with BC at first state of diagnosis and had treatment with chemotherapy, along with 27 healthy women as a control group.

The age-related BC in table 3, the present study is similar to many Iraqi studies such as Abedalrahman *et al.* (9), Al-Naqqash and AL-Shewewed (10), their found that increased risk for BC associated with increased age especially ≥50 years. The age development gives the cells more time to turn faulty or mutate to grow into cancer or older age have been exposed to more sunlight, cigarette smoke, chemicals and other cancer-causing agents for a long time. While not similar to this result suggest the young women have BC related to abnormal hormonal status or increase pollution, remnants of war and weapons (11).

BMI result in table 4 was similar to Iraqi studies as Al-Naqqash *et al.* (12) and Al-Alwan *et al.* (13) They showed obesity related to high risk of BC. The fatty (adipose) tissue of breast is the largest source of estrogen come from conversion androgen to estrogen through aromatization make obesity relate to BC (14). Mutations linked to estrogen exposure or metabolic deregulation cause (Obesity-inflammation) creates a pro-tumor inflammatory environment increases cell proliferation develop to BC (15).

The present results in table 5 show the menopause state is similar to many studies shown that the menopause after 50 age is linked to risk of BC (16). According to 51 epidemiological recent studies shown that the hormone replacement treatment (HRT) after menopause increases the risk of BC (17). One hypothesis can relate of (HRT) positive with BC by derive estrogen from conversion of androgens by the enzyme aromatase. (18).

Table 6 show the result of family history effect, it has the same line with novel studies mention that the frequency of a family history only 11cases (7.33%) (19). Another study refers that 10.4% of patients had a family history of BC while 89.6% had no family history of BC (20) the development of breast cancer is influenced by a number of inherited factors, BRCA1 - BRCA2 dominant autosomal mutations (21).

A recent Iraqi study by Hussain and Lafta,, found the biological-chemical warfare in the following years (1980–88, 1990–91, 2003–2006 and 2014–2016) left significant quantities of uranium and radiation throughout the area, has unique effects, including " environmental contamination and a rise in the incidence of BC (22).

Breast carcinoma which arises from the inner lining epithelium of the ducts or lobules supply the ducts with milk, this classification primarily by its histological appearance. Our study in table 7 showed, that (IDC) is the majority form of invasive BC, it starts in a milk duct of breast and spreads into the breast fatty tissue through the lymphatic and circulation system. This result is similar to a number of Iraqi studies shown that IDC is the most predominant histological type (23),(10).

The ER and PR are hormone receptors that pick up hormone signals and cause cell development in BC, detection of these receptors routinely

assessed by immunohistochemistry (IHC) helpful in detection prognostic and therapy options of BC cases. The molecular subtypes result in table 8 was agree with studies shown that Luminal-A accounts higher percentage and more than 50% of invasive breast cancer (24) ,(25).

The TNM system result in table 9 seem to be in the study for chemotherapy treated patients shown higher (83.6%) at BC II and III stage (25). There consistent with Iraqi study reported higher percentage stages were II and III (23). Also consisted to the study found that II and III stages have high percentage (20).

Cancer's grade describes abnormal cancer cells look under a microscope compared to healthy cells (low grade is well differentiate and vice versa). Table 10 show the same results of many Iraqi study who reported the low degree of differentiation with high percentage histological grade (26).

The LN present result in table 11 was consistent with Iraqi study that found the lymph nodes state is the most important prognostic factor in BC, the increases (70%) of LN+ in BC patients (26). The present study similar to result suggest accurate lymph node staging study is essential for both prognosis (of early-stage disease) and treatment (for regional control of disease) in patients with breast cancer (27).

Immunohistochemical estimation of ER, PR, HER2 by (Anti-ER, PR, and HER2 antibodies) is significantly higher marker and providing prognostic and therapy options for better outcome in BC cases. The (HR) result was similar to recent Iraqi studies shown that HR positive higher than negative, while the high Her2/neu were negative (10), (28).

Many recent studies show the same result of higher (HR+) positive in (19).

The results of the present study show the microRNA was successfully extracted from serum samples of patient's groups and control group. The fold changes in miR-21-5p expression were measured by using quantitative real-time PCR (qTR-PCR).

According to the Ct value of RNU43 in BC patients and healthy control group statically no significant difference observed between them (N.S), no difference in expression level between patients' group and control groups. While Statistical analysis revealed a significant increase of relative expression of serum miRNA-21 in BC patients was higher than that in the normal breast samples.

This result that similar to many Iraqi studies mention that high expression level of miRNA-21 in BC Iraqi patients (10 times) compared with those of the control group (29) The (3.39 times) increase of miRNA-21 median fold in BC Iraqi patients (30).

Recent studies have shown that microRNA-21 has highly expression correlation with increase initiation, proliferation, inhibited apoptosis, high invasion of human cancer cells (31).

The present study result contact with recent studies found MiR-21 expression increase in BC regardless of exposure to chemotherapy associated with tumor has resist to cell death and drug resistant. (25), (5). It has same result in study shown no difference between high miR-21 expression before and after chemotherapy (32).

In cancers, epigenetic alterations such as DNA methylation are involved in the earliest phases of tumorigenesis, the most common molecular abnormalities in BC and is considered a candidate biomarker of diagnosis (33). The miRNAs expression regulated by epigenetic machinery, hypomethylation is a general characteristic of breast

tumors, hypomethylated of CpG island in promoter region of miRNA-21 sequence in BC causing up-regulation of its expression (34).

Oncogenic miRNA-21 is able to promote tumor growth, invasion, angiogenesis and metastasis by target and suppress tumor suppressor genes in post-transcriptional such as PDCD4, PTEN (35, 36).

The ct-miRNAs are resistant to RNase degradation and expression levels are stable during freezing / thaw period (37). MiR-21 overexpression is related to the development of Multi Drug Resistance (MDR) (38).

Conclusion

MiRNA-21-5P represent as noninvasive blood-based tumor marker, fluid biopsy help to minimize the unnecessary breast tissue biopsies, whereas miRNA-21 implicated in carcinogenesis by their high expression in biological serum fluid of BC patients than that HC. The role of miRNA-21 as an oncomiR, metastatic biomarkers and prognostic factors related to metastatic breast cancer (MBC), whereas up-regulation of it in 80% of +Lymph node metastasis among Iraqi female BC patients regardless exposure to chemotherapy treatment. Indicator of aggressive phenotype development and progression BC diseases' characters whereas provide additional division of patients to (invasive breast carcinomas than non-invasive, luminal compared to non-luminal subtypes and differentiate of early from advance stage and early from advance grade). The family history (genetic cause) is not the main etiological cause of BC at Baghdad and Al-Anbar in Iraq, especially as this region have been subjected to frequent wars and hazard weapons, that cause of increasing the rate of BC.

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