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Evaluation of miR-206 as a Potential Biomarker in a Sample of Iraqi Breast Cancer Patient

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Abstract: Breast cancer (BC) is a heterogeneous malignant disease that begins in the tissues of the breast. It is characterized by the unregulated proliferation of cells, frequently leading to the development of tumors. The illness has a variety of molecular subtypes, including luminal A, luminal B, HER2-positive, and triple-negative, each characterized by unique genetic profiles and clinical outcomes. In this study the expression of miR-206 was examined in a sample of Iraqi female BC patients and its correlation with patients' demographic distribution and disease characteristics, potentially providing insights into BC diagnosis and progression.The study involved 60 female BC patients and 60 healthy controls. Demographic analysis showed most BC cases (70%) occurred in women aged 40-59 years, with 65% having a family history of BC. Invasive ductal carcinoma (IDC) was the most common type (85%), and luminal A the predominant molecular subtype (57%). Stage II and III showed the highest prevalence of BC, 40% and 36%, respectively. Using RT-qPCR with miR-16 as an internal reference, it was found that miR-206 was significantly downregulated (0.35-fold) in BC patients, with no significant differences among molecular subtypes but significantly downregulated in stages II, III, and IV compared to stage I. These findings suggested that miR-206 may play roles in BC development and progression.

Keywords: Breast Cancer, miR-206, RT-qPCR

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Introduction

Cancer is the leading cause of morbidity and mortality worldwide. Ferlay*et al*., (1) reported that almost 19.3 million people received cancer diagnosis globally in 2020, highlighting the substantial burden of cancer on a global scale. Cancer is an inherited condition marked by irregularities in cellular function, namely in the processes of cell growth and division. It is widely recognized as a major contributor to global mortality.

Breast cancer is the second leading cause of mortality worldwide and is the most detected tumor in females.(2). Annually, more than 1.5 million new instances of BC are detected, leading to around 460,000 fatalities due to resistance to chemotherapy and the spread of cancer to other parts of the body (3). The incidence of BC is experiencing a significant rise, with 2.3 million new cases reported in 2022. It is projected that there will be a 77% increase in BC patients by 2025 (4). The biological characteristics of BC, including histologic subtype, grade, lymph node status, hormone receptor status, and human epidermal growth factor receptor 2 (HER2) status, are commonly used to detect the disease

early, predict its outcome, and determine the appropriate treatment method (5).

MiRNAs are non-coding RNA molecules that are approximately 22 nucleotides in length. They play crucial roles in the regulation of gene expression following transcription (10). MiRNAs play a role in various biological processes, including cell proliferation, differentiation, apoptosis, and metabolism (11). MicroRNAs possess the capacity to exert either inhibitory or stimulatory control over signaling pathways, hence influencing the formation and advancement of tumors as well as other facets of cancer progression, specifically in the context of BC (9). Dysregulation of miRNA expression has been associated with a range of diseases, such as cancer, cardiovascular disorders, and neurological problems. Because of their capacity to maintain stability in various bodily fluids, they have demonstrated significant promise as biomarkers for the diagnosis and prediction of illnesses (12).

MiR-206 is a microRNA that is particular to muscles and has gained considerable attention in the field of cancer research. It is frequently reduced in many types of cancer, principally acting as a suppressor of tumor growth. MiR-206 specifically targets many oncogenes and pathways that are associated with cell proliferation, migration, and invasion(13). Studies have demonstrated that in cases of BC, it effectively inhibits the growth and spread of tumors by modulating the estrogen receptor-α (ERα) and the MAPK/ERK pathway(14). MiR-206 also contributes to the process of muscle differentiation and regeneration, particularly in malignancies that impact muscular tissues. The levels of its expression are associated with the prognosis of various cancers, indicating that it has the potential to be used as a biomarker. For this reason, the study aimed to evaluate the expression profile of miRNA-206 in serum sample from Iraqi female BC patients and investigating the correlation of miRNAs expression with some BC clinicopathological features.

Methods

Study Subjects

In this research, Sixty Iraqi females with breast cancer who in attended Al-Andalus Specialist Oncology Hospital in Baghdad and Al Amal hospital, aged 25 to 67 years and sixty were healthy volunteers as a control all the whom had no family history of breast cancer between the first of December 2023 to beginning of March 2024.

Sample Collection

The blood was taken from the patients and healthy group using aseptic techniques ,taked 5 mL of venous blood extracted. The blood samples were collected in gel serum separation tubes and stored at a temperature of 18°C–22°C.Subsequently, the serum was isolated by centrifugation at 3000 rpm for 15 min. The serum, which had been separated, was promptly transferred to TRIzol for RNA extraction and held at a temperature of -20°C until it could be further analyzed

RNA Extraction

RNA was exteractedfrom serum samplesaccording to the protocol of TRIzol™ Reagent.The serum (400µl) was mixed with (600µl) of TRIzol™ Reagent in each tube, and the lysate was homogenized by pipetting upand down multiple times.For phase separation chloroform was used with a volume of 0.2 ml of and the aqueous was transferred into a new tube, then for the RNA precipitation 0.5 ml of isopropanol was added, the pellet was washed with 0.5 mL of 70%

ethanol, the supernatant was discarded and the pellet was kept only, 50 µl of Nuclease free water was added and the RNA kept in -70°C until RT-PCR reaction. Using Quantus Fluorometer (Promega, USA) the concentration of RNA in samples was quantified

The reaction setup and Thermal Cycling Protocol:

Two steps RT-qPCR to generate cDNA for the subsequent qPCR reaction.

A. First step Reverse Transcription for complimentary DNA synthesis with two reaction :

Every 4µl of the RNA sample has been mixed with 1µl stem-loop RT primer of the miR206 . The thermal cycle setting as table 1 below :

Table(1): Thermal Cycler Program for 1st reaction.

Step	ºC	Min:s	Cycles
Denaturation	70	5:00	1
Hold	4	10:00	
Cool in ice and spin			

With used OT-1Reagent Kits (Reverse Transcription kits), Nuclease Free Water (Synthol, Russia) in SimpliAmp Thermal Cycler(Applied Biosystem,USA) complementary cDNA was synthesized from miRNA reverse transcription. Based on manufacturer's instructions, which are displayed in Tables (2) and (3), the Reaction Volume was 7.5 µl from the 2nd Reaction Mix per tube and 5.0µl of the First Reaction Template.

Table (2): Components of cDNA synthesis reaction.

Second reaction	Volume for each 1 Sample(ml)
2.5 Reaction Mixture	10 µL
Enzyme MMLV-RT	1 µL
RNA-Primer Template	5µL
Nuclease Free Water	4µL
Total volume	20µL

Table (3): Thermal Cycler Program for 2nd reaction.

Step	ºC	Min: S	Cycles
Annealing	25	5:0	1
Extension	42	60:0	
Enzyme inactivation	70	15:20	
Hold	04	10:0	

The QuantusFluorometer was used to evaluate the concentration and quality of the extracted cDNA. Aliquot of 1 µL of cDNA was combined with 198 µL of diluted Quantifluor Dye. The solution was thereafter placed in a dark area and incubated for 5 minutes at room temperature. After the incubation period, the concentration of cDNA was measured using the QuantusFluorometer.

B.Second Step Quantitative Estimation of miR-206 and miRNA-16(housekeeping gene) by Gene expression (SYBR Green qPCR)

The miRNA expression levels were quantitatively evaluated using MicqPCR Cycle (Bio Molecular System,Australia) with the SYBR green master mix kit (Synthol/Russia) is listed in table 4**.**The RT-qPCR conditions are listed in Table (5).

Table (4): Gene expression reaction components.

Master mix components	Volume (µl) for each 1 sample
2.5 Reaction Mixture	10
$Mgcl_2$	1
Nuclease Free Water	4
Forward primer(10pmol)	1
Reverse primer(10pmol)	1
cDNA	3
Total volume	20

Table (5): The RT-qPCR Program.

Step	ºC	Min: Sec	Cycle
Initial Denaturation	95º	5:00	1
Denaturation	95º	0:20	45
Annealing	55º	0:20	
Extension	72º	0:20 Acquiring on Green	

The reagents were rapidly thawed and maintained on ice throughout the experiment.The cycle parameters for qPCR were configured as follows: The process begins with an initial denaturation phase at a temperature of 95°C for 5 min, which is performed only once. This is followed by a series of 45 cycles, each consisting of denaturation at 95°C for 20 sec, annealing at 55°C for 20 sec, and extension at 72°C for 20 sec. The miRNA expression levels were determined by calculating the fold change, which was normalized to the housekeeping gene miR-16-1, using the formula $2^{-\Delta\Delta CT}$.

Table (6): Primer sequence for miR-206 expression.

Primer Name	Sequencing	Annealing Temp	Refrences
miR-206 RT-primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACCCACAC	45ºC	Primer design
miR-16-1 RT-primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACCGCCAAT		Primer design
MiR-206-F	GGTTTTTTTGTGGAATGTAAGGAAGT	55 ºC	Primer design
miR-16-F	GGTTTTTTTTAGCAGCACGTAAAT		Primer design
Universal Reverse	GTGCAGGGTCCGAGGTATT		Primer design

Statistical Analysis

The statistical analysis was performed with GraphPad Prism version 9.2 (GraphPad Software Inc., LaJolla, CA). The student's *t*-test was applied to calculate the significance of the group variance. The Chi-square test was employed to comparethe significance between percentages. The data was expressed as the mean ± SD, and statistical significance was represented by * $p< 0.05$ and ** $p< 0.01$.

Results

Demographic Distribution

The distribution of BC patients according to family history showed thatthe number of BC patients with family history of BC was 39 (65%), which is significantly ($p = 0.0201$) different from the BC cases with no pervious family history (21, 35%).The

data collected of BC patients showed that the majority of BC patients were significantly (p<0.0001) invasive ductal carcinoma (IDC) which represent 85% (51 cases), while only 4 cases (6%) and 5 cases (9%) belonged to invasive lobular carcinoma (ILC) and other types, respectively. Based on molecular subtypes of BC, the highest percentage of BC patients was luminal A which represents 57% (34 cases). Luminal B and triple negative BC patients accounted for 17% (10 cases) and 20% (12 cases), respectively. Finaly only 4 cases (6%) were examined as HER2 subtype. The distribution of BC molecular subtypes showed significant (p<0.0001) differences among all types since luminal A contributed more than 50% of BC total. The distribution of BC patients depending on stage showed thatsignificant (p = 0.0117) differences were observed between the 4 cancer stages. Only one case (3%) was obtained from BC patient in stage I, stage II 24 cases (40%), stage III 22 cases (36%) and 13 cases (21%) for stage IV.

The BC patients were divided into 5 age groups ranging from 20 to >60. The distribution of BC patients showed significant (p<0.0001) differences among the different age levels with higher percentage of cases was detected 35% between 40-49 (21 cases) and 35% between 50-59 (21 cases). Only 3 cases (5%) were in the age range between 20-29. While 7 cases (11.67%) and 8 cases (13.33%) were distributed within the age range between 30-39 and ≥ 60, respectively.

Relative Expression of Circulating miR-206

The relative expression of miR-206 was measured depending on the relationship between the Ct (Cycle threshold) value of the target gene in patients and the control group, as well as the internal housekeeping referencemiR-16(15). Comparing to control group, the expression of miR-206 exhibited a significant (p< 0.0001) downregulation with 0.35±0.23-fold decrease (Table. 3).

Table (6): The gene expression results shown mean fold change inmiR-206 expression values of the control and patient.

Groups	Mean of Folding
Control	44.1856
Patient	11.6162

Correlation between miR-206 Expression and Demographic Parameters

The analysis of miR-206 expression based on BC molecular subtypes showed that there were no significant differences between the varied types of BC (Fig. 2). Luminal A, luminal B, triple negative and HER2 were exhibited miR-206 downregulation with relative expression of 0.36±0.2, 0.29±0.28, 0.4±0.19 and 0.15±0.032-fold decrease, respectively.

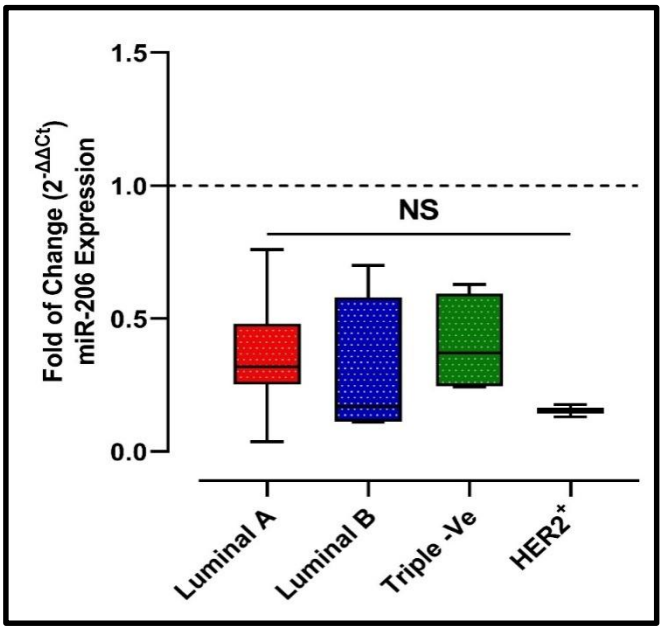

Figure (2): miR-206 expression in BC patients according to BC molecular subtypes. NS: Non-significant.

Based on stage the expression of miR-206 in stage I of BC patients1.2-fold of change. While the expression of miR-206 in stages II, III and IV were significantly different from stage I with fold decrease of 0.32±0.19 (p = 0.0052), 0.46±0.22 (p = 0.0213) and 0.3±0.22 (p = 0.0039), respectively (Fig. 3). No significant differences were observed between stages II, III and IV. No significant differences were recorded between IDC, ILC and other types of BC with respect to miR-206. The relative expression of miR-206 was 0.32±0.18, 0.36±0.17 and 0.31±0.25-fold change, respectively (Fig. 4).

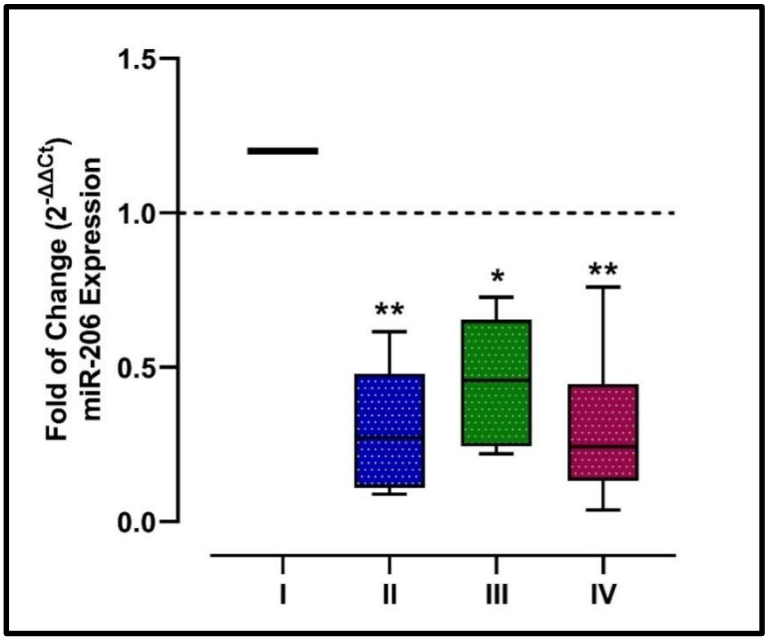

Figure (3): miR-206 expression in BC patients according to BC stage. *p< 0.05, **p< 0.01.

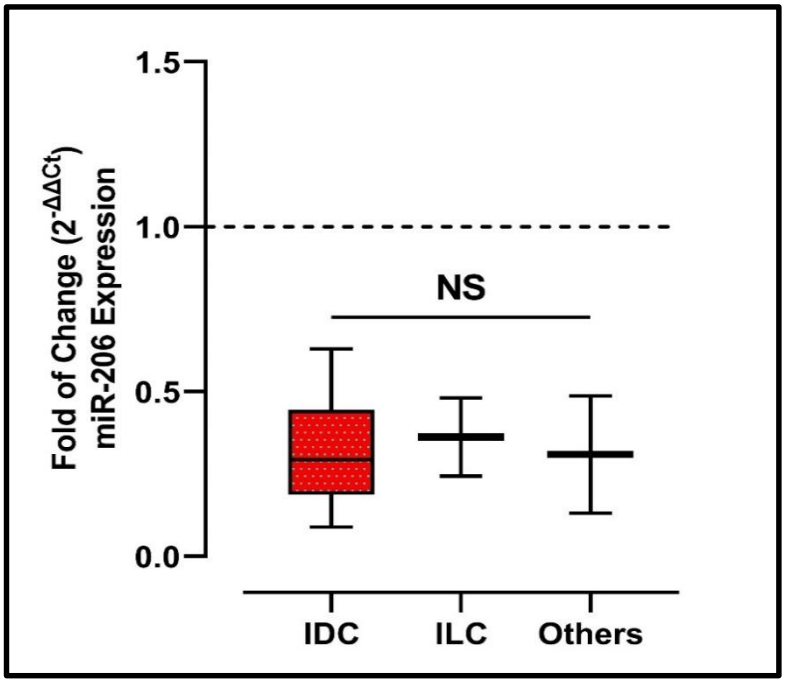

Figure (4): miR-206 expression in BC patients according to BC type. NS: Non-Significant. IDC: Invasive Ductal Carcinoma, ILC: Invasive Lobular Carcinoma.

Discussion

The statistical analysis performed in this study demonstrated a notable rise in the expression of miR-206.

Breast Cancer is a condition that can impact females of many age groups; however, it is typically identified in women who are 40 years old or older(16). Statistically, the likelihood of acquiring BC escalates notably after reaching the age of 40 and reaches its highest point between the ages of 50 and 60(17). A study involved 7,711 BC Thai women revealed that the higher incidence of BC was at age groups 40 and older(18). Another study involved 1172 Iraqi female with BC showed that peck frequency of age in Iraqi BC patients occurred between 35 – 49 years old with mean age of 51 (19). Iraqi women diagnosed with breast cancer have emphasized the very earlyage at which the affected patients were diagnosed(20). Among the greatest risk factors for BC is the family history of the illness. Women run a higher chance of developing breast cancer themselves if they have close blood relatives, a mother, sister, or daughter, who have been diagnosed(21). The correlation between genetic abnormalities and BC susceptibility, specifically in the *BRCA1* and *BRCA2* genes, is the primary reason for this association(22). IDC represent the major type of BC in this study, these findings align with the results reported in a local study conducted by Ibrahim and Nader, which indicated that 96% of the cases in the study were classified as IDC based on histological analysis(23). Same results were obtained by Li et al., (24)in which 72.8% of BC patients were classified as IDC, while 7.6% of the patients were classified as ILC. Analysis from China involved 796,335 BC patients showed that the majority type of BC was IDC (89.3%). ILC represents only 10.7% from all BC patients(25).

Based om molecular subtypes, the resultswere in agreement with different local and international studies that

exhibited the predominant of luminal A over the other types of BC. An Iraqi study conducted 920 BC female patients who attended Al-Hussein Cancer Center in Karbala province of Iraq, showed that luminal A exhibited the highest percentage 53.69%(26). Another study enrolled 686 Iraqi BC patients revealed that the main identified phenotype of BC was luminal A (45%), followed by triple negative (15.6%), luminal B (14%) and HER2 (10.3%)(27).Thepercentages of BC subtype were almost similar to the percentages obtained in this study. In a recent study conducted by Abbas, in Iraq, the findings regarding BC molecular subtypes revealed that the majority of tumors (58%) belonged to the luminal A subtype. Additionally, 20% of patients had luminal B subtype, 6% had triple negative subtype, and only 16% had the HER2 molecular subtype(28). Mutar et al., (29)were also obtain close proportions for luminal A (42.2%), luminal B (14.6%), triple negative (15.6%) and HER2 (11.2%). Breast Cancer staging is crucial for treatment selection and prognosis. Most diagnoses occur at stage II, followed by stages III and IV, with few at stage I, highlighting the need for earlier detection(30). Diagnosis timing varies globally: Japanese women were often diagnosed at stage I (56.1%), compared to black American (37%) and white non-Hispanic women (50.8%)(31). In Iraq, stage II was most common (47.5%), followed by stage III (31.9%), stage I (12%), and stage IV (8.6%)(19). Iraqi women tend to be diagnosed at later stages, emphasizing the importance of improved early screening and detection efforts in the country(32).

Micro-RNA206 is a muscle-specific microRNA that has gained significant attention for its emerging roles in breast cancer pathogenesis. While initially recognized for its functions in skeletal and cardiac muscle development, recent studies have uncovered its dysregulation and potential tumor-suppressive effects in various cancers, including BC(33). The expression of miR-206 is often reduced in BC relative to normal breast tissue, indicating its possible function as a tumor suppressor. Decreased levels of miR-206 have been linked to more aggressive characteristics of BC, including heightened cell proliferation, migration, invasion, and metastasis. Functionally, miR-206 inhibits the growth of tumors by specifically binding to and suppressing the activity of multiple cancer-causing genes and cellular communication pathways that contribute to the advancement of BC(34).Micro-RNA206, which is well recognized for its involvement in the formation of skeletal muscle, has been discovered to be significantly decreased in BC samples compared to nearby healthy tissue. Ge *et al.*,(35)revealed the downregulation of miR-206 and upregulation of 6-Phosphofructo-2-kinase (PFKFB3) in BC patients. Overexpressing miR-206 hindered the formation of fructose-2,6-bisphosphate (F2,6BP), decreased lactate generation, and resulted in lower cell proliferation and migration in breast cancer cells.

By using a comprehensive data analysis, the consistent miR-206 downregulation was detected in all main molecular subtypes of BC, highlighting the probable vital role of miR-206 in the formation of BC. Luminal A and B are two subtypes that bear the presence of ER positive and miR-206 is lower significantly in both Luminal A and B(36). This downregulation is particularly important as miR-206 directly targets ESR1, the gene that encodes ERα. Thus, reduced levels of miR-206 could permissively allow for

higher ERα expression, fueling the development of estrogen-dependent tumors(37). The expression pattern of miR-206 in BC has a unique profile that varies depending on the stage of the disease. It was worth mentioning that miR-206 was noticeably reduced in later stages (II, III, and IV) of BC. However, its expression in stage I BC remains rather stable when compared to healthy breast tissue(38). The reason for stabilizing expression of miR-206 in stage I could be attributed to the small size of tumor (≤2 cm) that hasn't spread beyond the breast, miR-206 levels are comparable to normal tissue. This suggested that in early, localized breast cancer, the regulatory mechanisms governing miR-206 expression are still intact(39). However, as the disease advances to stages II and III, and/or IV characterized by larger tumors and lymph node involvement, miR-206 levels drop markedly. Finally, the decrease in expression of miR-206 is a characteristic feature observed in all main forms of BC, regardless of their histological source. In cases of IDC, which is the most prevalent kind, there is a correlation between a decrease in miR-206 and an increase in tumor size and grade. Similarly, low levels of miR-206 are linked to increased invasiveness in ILC. Even in less common kinds of carcinomas such as medullary and mucinous carcinomas, the expression of miR-206 is reduced(40).

Conclusions

Based on the study results, miR-206 shows significant downregulation in BC patients compared to healthy controls. While no significant differences were observed among molecular subtypes or histological types, miR-206 expression varied significantly across cancer stages. Stage I showed upregulation, while stages II-IV exhibited downregulation compared to stage I. These findings suggest miR-206 could be a potential biomarker for breast cancer detection and staging.

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