Study the Role Of Mirna -126 Gene Expression In A Sample Of Breast Cancer Iraqi Women

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KEYWORDS

Circulating micoRNA, miR-126, Breast Cancer, Gene Expression, RT-PCR

ABSTRACT

Background: MicroRNAs play a major function in gene expression and are associated with numerous cancer types. Breast cancer (BC) is aberrant breast cell proliferation induced by environmental and genetic factors with gene expression alterations. **Objective**: Study of clinical characteristics of women with breast cancer and estimation of circulating miR-126 expression in serum. **Methods**: This research involved the recruitment of a cohort consisting of seventy female BC patients from Oncology Unit of Al-Yarmouk Teaching Hospital located in Baghdad city, Iraq. Furthermore, thirty samples of healthy female volunteers were included as control subjects. The miR-126 expression levels were measured by means of a technique called real-time reverse transcription-polymerase chain reaction. **Results**: The age range between 45 to 54 included the higher number of patients with breast cancer comprised treated and untreated groups than other. Roughly 75% of the patients were discovered at an early stage (II), and all patients were in stages II and III. The majority of patients were found to be overweight or obese. This assignment also demonstrated that treated (60.0%) and untreated (68.6%) left breasts had more malignancies than right breasts. Active smokers were rare, whereas non-smokers and passive smokers dominated the group. This study found a substantial drop in *miR-126-5p* gene expression in BC patients' serum (treated and untreated) compared to healthy controls. **Conclusion**: The study found a decrease in circulating *miR-126-5p* gene expression in BC patients' serum.

1. Introduction

Breast cancer is the most prevalent malignancy among women and the second foremost cause of cancer-related mortality [1][2]. BC was influenced by environmental and hereditary factors and is a heterogeneous illness characterized by varied clinical and pathological, and molecular features [3][4][5]. The factors contributing to the increasing or decreasing trends in breast cancer incidence in Iraq, including advances in diagnosis, improved case reporting, availability of screening strategies in main hospitals, and lifestyle changes like overweight, obesity, physical inactivity, family history, delayed first birth, and not breastfeeding [6]. BC pathogenesis and progression involve gene expression alterations [7][8]. Noncoding single-stranded RNAs (ncRNAs), also known as microRNAs (miRNAs), are conserved RNAs that modulate gene expression via attaching to specific messenger RNA regions. This control affects physiological processes like cell proliferation, angiogenesis, invasion, differentiation, and embryonic development [9][10]. Some types of microRNAs have been associated with BC and several other malignancies. [11][12]. miRNAs serve as biomarkers for cancer diagnosis and early prognostic assessment, and have garnered acknowledgment as potential tools in the prognosis and identification of persons with breast cancer (BC) [10][13]. Circulating miRNAs in the bloodstream have significant candidates for use as biomarkers for diagnostic purposes and monitoring treatment efficacy [12][14]. MiR-126, a microRNA produced in endothelial cells, plays a crucial role in angiogenesis regulation. Researchers have shown a correlation between miRNAs and BC pathophysiology, with studies indicating its role in angiogenesiis, proliferation, migration,, invasion, and cell survival in some malignancies [15][16]. MiR-126, a tumor suppressor, has been found to downregulate expression in various malignancies, potentially facilitating or inhibiting tumor dissemination [17][18]. Clinical data suggests it serves as a biomarker for BC prediction and diagnosis due to altered gene expression in BC tissue [9].

2. Methodology

Study Design and Sample Collection

Seventy female breast cancer patients from Iraq's Al-Yarmouk Teaching Hospital in Baghdad city were included in this case-control research. Patients with BC were divided into two cohorts: those who had treatment and those who did not. The samples were included in this study who were women who had BC (invasive ductal carcinoma) for the first time, and it excluded men, pregnant women, patients treated with other types of cancer therapy like radiotherapy, those with any other cancer, and those who had a had a previous infection with BC. Also included as controls were 30 women who seemed to be in good health; none of them had a history of cancer. The ages of both the patients and the control group members were within the normal range, which was 25–75 years. We gathered these samples throughout the months of September 2022 and August 2023. Every person had venous blood obtained to get a peripheral blood sample. After blood samples were taken, they were spun at 3000 rpm for 10 minutes to separate the serum out of the gel. Prior to any further analysis, the serum that had been separated was swiftly moved to TRIzol reagent for use in RNA extraction. It was then kept at -20°C.

Molecular assay

RNA extraction

The TRIzol Reagent procedure was followed in order to isolate ribonucleic acid (RNA) from serum samples. 300 µL of serum were mixed by shaking with 500 µL TRIzol. Added 0.25 mL of chloroform to each tube then incubated for 2–3 minutes, and centrifuged for 10 min. The mixture was split into three different parts. The aqueous phase it was isolated that it containing the RNA and added 0.5mL of isopropanol then incubated for 10 minutes, and centrifuged for 10 min, it was precipitated total RNA as white gel. The washing process included adding 0.5 mL of 70% ethanol solution, briefly vortexing, then centrifuging for 5 minutes. The pellet was then air-dried after the ethanol was aspirated. 50 microliters of Nuclease Free Water was used to rehydrate the pellet. Prior to the RT-PCR experiment, the extracted RNA was kept at a temperature of -70°C. By using the Quantifluor dsDNA System (Promega/USA), the RNA concentration in the samples was determined.

Quantification of microRNA gene expression by real-time quantitative polymerase chain reaction

Two separate procedures make up the two-steps qRT-PCR method was depended for gene expression. The process begins with cDNA synthesis by reverse transcription of total RNA, and then moves on to PCR amplification. Starting with 4 µL of total RNA sample and 1 µL of the stem-loop RT primers specific to *miR-126-5p* and *miR-16-1* designed for this study as Table 1, the thermal cycler program denatured the RNA at 70 °C for 5 min and held it at 4°C for 10 min in a single cycle to extend microRNAs.

Table 1. Sequences of Primers used to detection miR-126-5p and miR-16-1

Primer		**Sequence (5′→3′ direction)**	**primer size bp**
miR-126-5	miR-126-5p RT-primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACCGCGTA -3̀	24
	miR-126-5p	F GGTTTTTTCATTATTACTTTTGGA -3̀	50
Universal Reverse		R GTGCAGGGTCCGAGGTAT -3̀	18
miR-16-1	miR-16-1 RT-primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACCGCCAAT-3̀	51
	miR-16-1	F GGTTTTTTTTAGCAGCACGTAAAT -3̀	24

Reverse transcription

The conversion of RNA to cDNA by reverse transcription of the miRNAs was achieved by using the EntiLinkTM Reverse Transcriptase Kit (ELK Biotechnology, Chine). The reaction was conducted in a volume of 20 µl following the manufacturer's guidelines. The thermal cycler step was carried out for the cDNA reverse transcription process in one cycle, which included annealing at 25°C for 5 min,

extension at 42°C for 60 min, enzyme inactivation at 70°C for 15 min, and finally hollding at 4°C for 10 min.

Quantitative RT- PCR

After converting RNA to cDNA, the expression levels of miRNAs (*miR-126-5p* and *miR-16-1*) were quantitatively assessed using real-time quantitative PCR (RT-qPCR) with the SYBR green master mix kit (ELK Biotechnology, China), and specific primers were designed for this study, as shown in Table 1. Throughout the procedure the reagents were quickly thawed and kept them on ice. The cycling conditions for the qPCR were set as follows: An initial denaturation phase at 95°C for 5 minutes (1 cycle), followed by 45 cycles including denaturation at 95°C for 20 seconds, annealing at 57.5°C for 20 seconds, and extension at 72°C for 20 seconds. The thermal cycling conditions varied, with the annealing temperature at 57.5°C for *miR-126-5p* and 59°C for *miR-16-1* (achieved separately). We used the expression of *miR-16-5p* as an internal control to normalize the data. Using the $2^{-\Delta\Delta Ct}$ approach, the relative quantification (fold change) of *miR-126-5p* expression was determined between the patient groups and the group of women presenting as healthy.

Ethical approval

Following the guidelines laid forth by the Declaration of Helsinki, all methods used in this study were ethical. Once we obtained their verbal and analytical permission, we recruited patients for the trial. The institutional ethics committee approved the study (H.42765, 2022/8/15) after examining the research methodology, data from the subjects, and the consent form.

Statistical Analysis

The act of reviewing data by means of statistical analysis. To find out how different variables affected the study parameters, we used the IBM SPSS Statistics 29 program. A one-way analysis of variance (ANOVA) and t-test were used to compare the means and evaluate their significance in the statistical analysis. To compare percentages at 0.05 and 0.01 significance levels, a chi-square test was used. Would need an assess of the study's odds ratio and confidence interval.

3. Result and Discussion

The research included 100 women, with 70 breast cancer patients randomly assigned to 35 chemotherapy-treated and 35 untreated groups, as well as 30 healthy volunteers serving as a control group, selected according to certain criteria. The samples were subdivided into five age groups, spanning from 25 to 75 years old. The average age of women part of the control group was around 47.600±11.704 years old. Most of the women in the treated group were between the ages of 45 and 54 (31.4%), whereas the smallest age group was 25 to 34 (2.9%). The mean was 51.400±10.065. The average age of the untreated group was 53.342±9.511, with 54.3% of the participants falling within the 45–54 age bracket and 0% falling within the 25–34 age bracket, as detailed in the **Figure 1**.

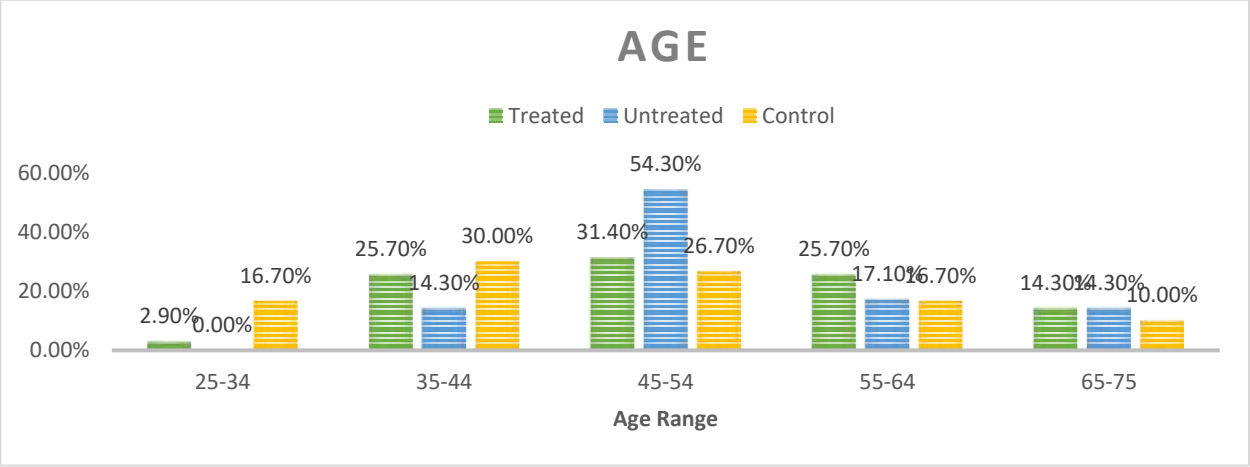

Figure 1: Distribution of patients (treated and untreated) and control according age subdivisions.

All of the diagnosed cases of BC in this study are only in stages II and III. In the treated group, 20 individuals (57%) are in stage II and 15 individuals (43%) are in stage III, while 33 individuals (94%) in the untreated group are in stage II and 2 individuals (6%) are in stage III. This indicates that the majority of cases are found in stage II as in **Figure 2**.

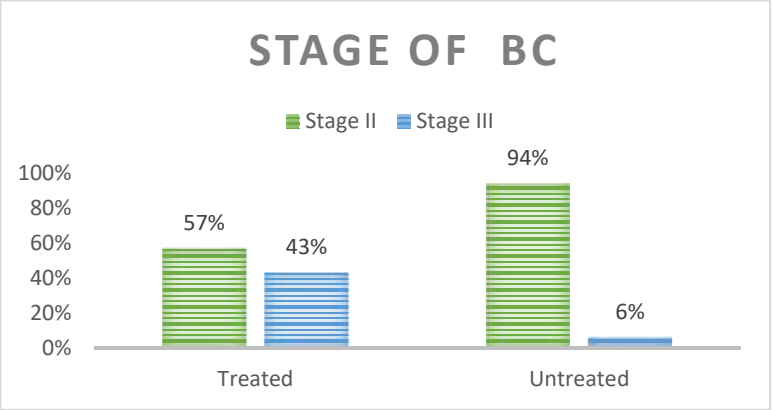

Figure 2: Sage of tumor of breast cancer cases according to treated and untreated groups.

Family history was linked to BC. In the **Figure 3** we identified a positive familial history of the disease in either the first or second degree of a relative in 18 cases (51.4%) among the treated group and 15 cases (42.9%) among the untreated group. Conversely, we discovered a negative familial history in 17 cases (48.6%) among the treated group and 20 cases (57.1%) among the untreated group. In contras, among of control group with no family history form 60% or 24 individuals and 6 (40%) with family history.

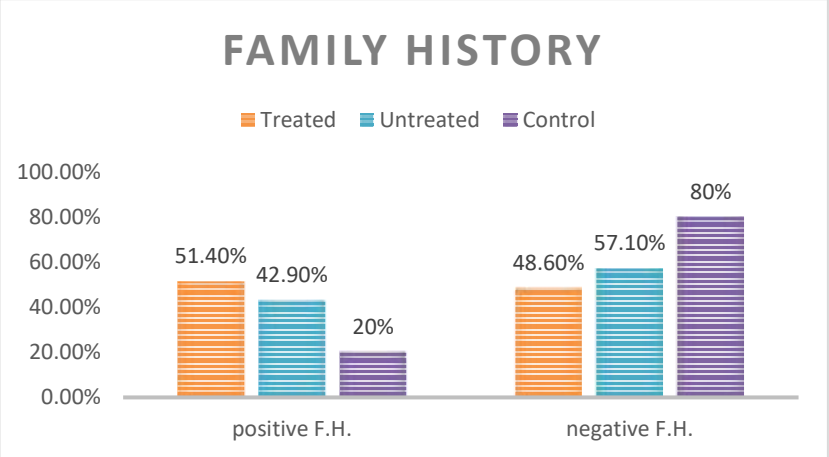

Figure 3: Family history of patient's groups

Among the three groups (control, treated, and untreated), three classifications were established based on their Body Mass Index (BMI kg/m2): normal-weight (18.5-24.9), over-weight (25-29.9), and obesity (>30). Within the treated group, 2 individuals (5.7%) had a normal-weight, 14 (24.0%) were over-weight, and 19 (54.3%) were obese. 3 individuals (8.6%) in the untreated group had a normal-weight, whereas 16 individuals (45.7%) were overweight and 16 individuals (45.7%) were obese. Within the control group, 3 individuals (10%) had a normal weight, 8 individuals (26.7%) were overweight, and 63.3% were obese explained in **Figure 4**.

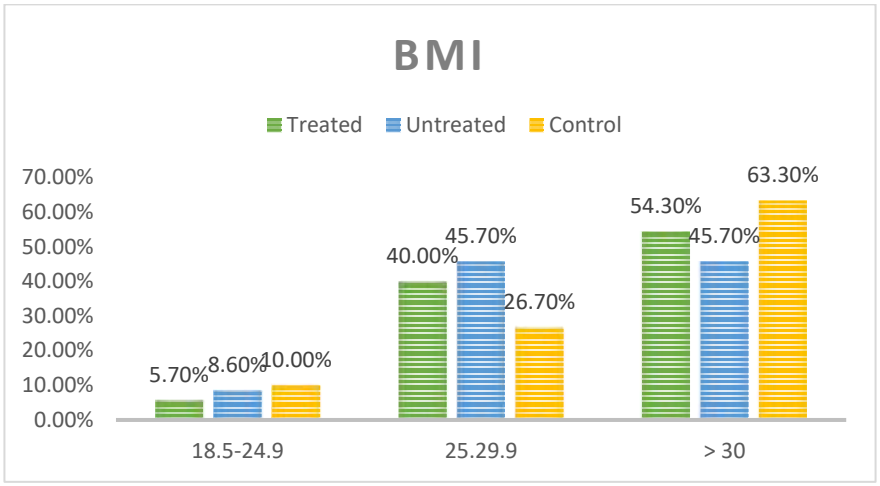

Figure 4: The body mass index (BMI) of three groups: treated, untreated and control.

Figure 5 show within the treated group, 21 patients (60.0%) had the tumor located unilaterally in the left breast, while 14 patients (40.0%) had it unilaterally in the right breast. In the untreated group, 24 patients (68.6%) had the tumor located unilaterally in the left breast, and 11 patients (31.4%) had it unilaterally in the right breast.

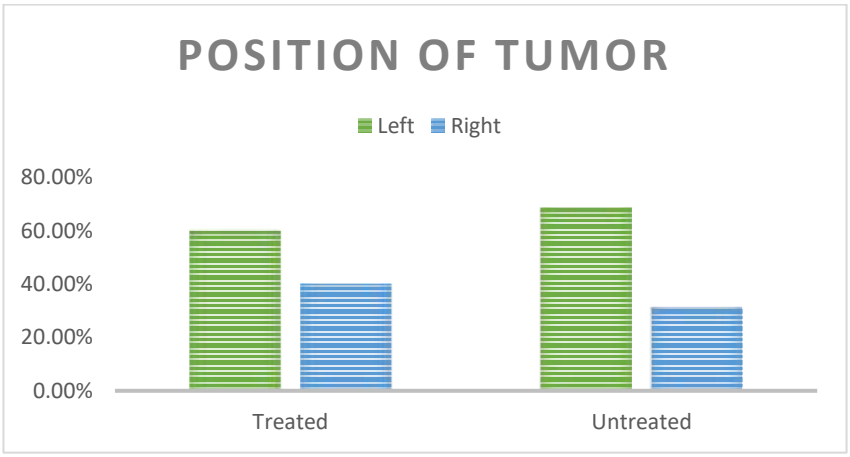

Figure 5: position of breast tumor of treated and untreated group.

The three groups (control, treated and untreated) were divided according to their smoking. In treated group 13(37.1%) were non-smoker, 3(8.6%) were active smoker and 19(54.3%) were passive smoker. The untreated group 21(60%) were non-smoker, 2(5.7%) were active smoker and 12(34.3%) were passive smokers. The control 18(60%) were non-smokers, 2(6.7%) were active smoker and 10(33.3%) were passive smoker, presented in **Figure 6**.

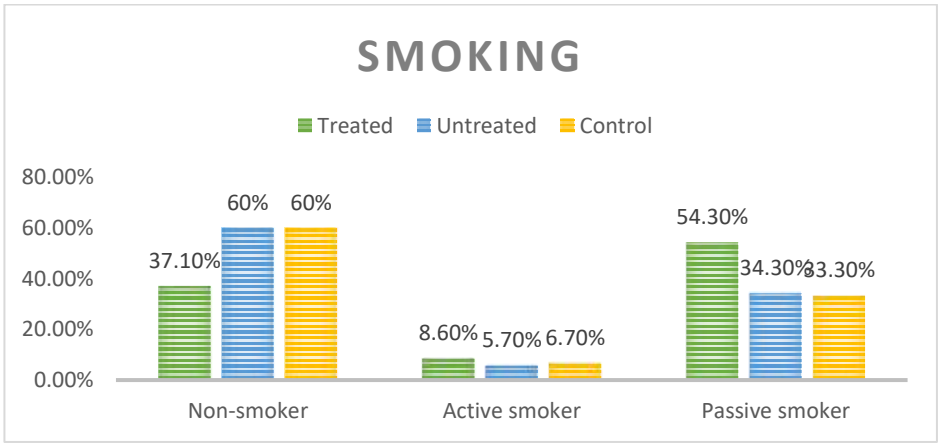

Figure 6: Smoking of treated, untreated and control groups.

Within the treated group, 20 people (57.1%) had no hypertension, while 15 individuals (42.9%) had hypertension. In contrast, the untreated group consisted of 29 individuals (82.9%) who did not have hypertension, and 6 persons (17.1%) who had hypertension. The control group consisted of 22 individuals (73.3%) without hypertension and 8 individuals (26.7%) with hypertension, as shown in **Figure 7**.

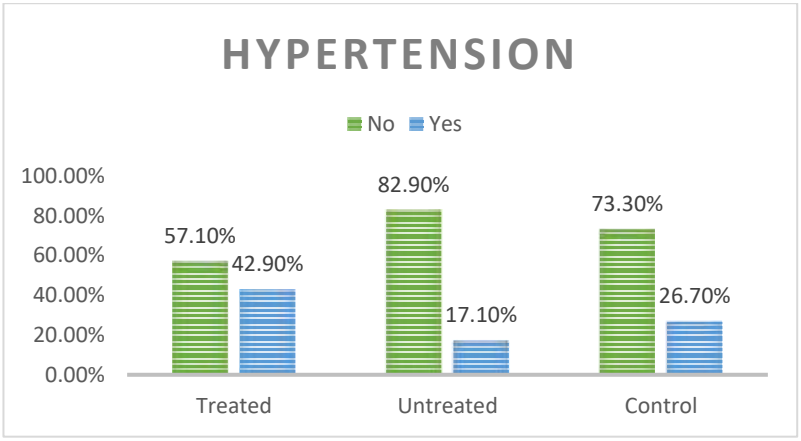

Figure 7: hypertension of groups (treated, untreated and control).

In the treated group, there were 28 people (or 80.0% of the total) without diabetes and 7 (20.0%) with diabetes, 29 people in the untreated group (or 82.9% of the total) without the disease and 6(17.1%) have diabetes. In the control group, 26 people (or 86.7% of the total) did not have diabetes, whereas 4 people (13.3%) did, as depicted in **Figure 8**.

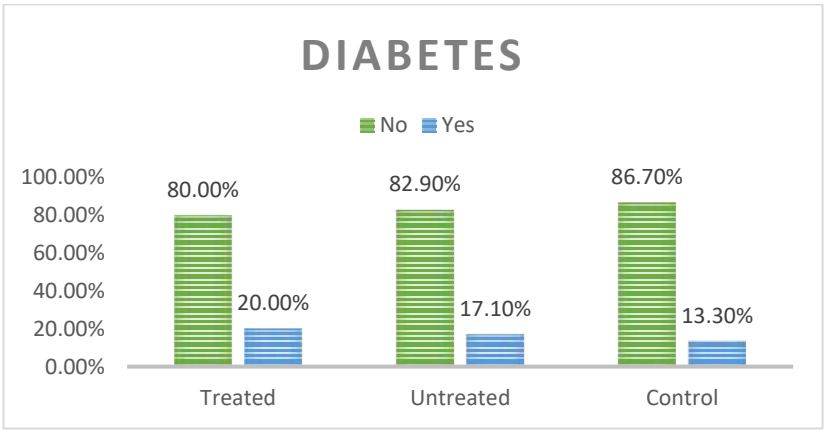

Figure 8: Diabetes of patients (treated and untreated) and control.

An experimental molecular assay was conducted to identify the target's amplification plot circulating *miR-126-5p* and the house keeping gene *miR-16-1*, expressed as fold changes, using the Ct values. The research reported a significant reduction in *miR-126-5p* gene expression in a serum sample obtained from individuals afflicted with BC (both treated and untreated groups). As shown in **Figure 9**, the fold change in *miR-126-5p* expression in treated patients was (0.86) and untreated patients (0.12) was much lower than in the controls (1.01).

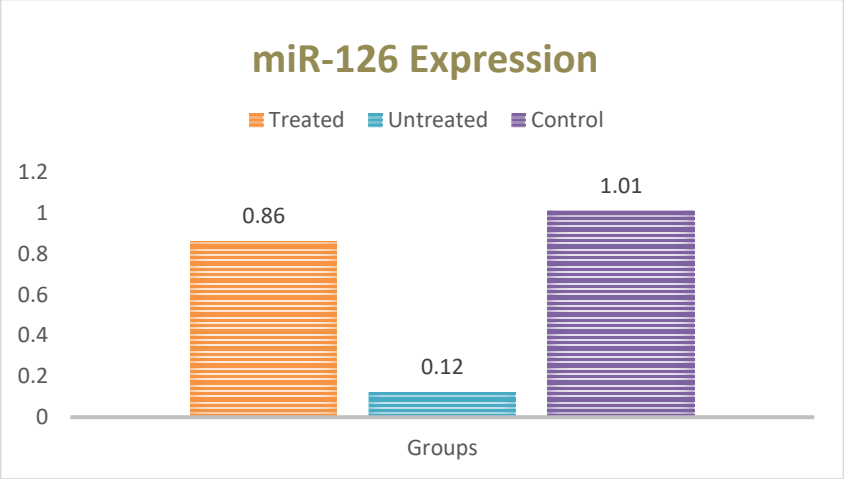

Figure 9: The difference of fold change of miR-126 between treated and untreated BC patients and control groups.

Discission:

Breast cancer (BC) is a hereditary disorder affecting the ducts and lobules of the mammary glands; ductal tumors account for the majority of these cancers. In addition to genetic predispositions, environmental variables also play a major role in initiating the multifactorial process of carcinogenesis. There are a great deal of risk factors for BC, and some of them are changeable while others are not [19][20]. Biomarkers may be used to predict and evaluate cancer diagnosis [21]. The results of this study divided people into three groups: treated patients, untreated patients, and a healthy group as a control.

The age range 45-54 years has the highest infection rates with BC; these findings align with other Iraqi studies conducted by AL-Saqabi, *et al*. (2022) [14] and Mohsin and Mohamad (2024) [22], as well as comparable studies in other nations, like research conducted in Palestine with 170 patients revealed a mean age of 51.71±11.11 years, with 39.4% of participants aged between 50 and 59 years [23]. Carcinogenesis progresses with time due to the accumulation of several cellular alterations and exposure to possible carcinogens. A complex interplay of hereditary, immunological, hormonal, and environmental variables contributes to the aging-related rise in breast cancer incidence [19].

All patients were in stages II and III, with roughly 75% identified at the early stage (II), consistent with the research conducted by Nasser & Behadili (2022) [24], and AL-Saqabi, *et al*. (2022) [14] who found the most of BC patients in this research were classified as grade II, including 31 individuals, which represents 62% of the overall cohort, this is due to the increasing awareness and need for breast cancer detection [25].

Patients included in this study had a slightly higher positive family history than the control group. The frequency of occurrences of BC is elevated in individuals with a familial history, irrespective of age, attributable to epigenetic modifications and environmental variables serving as potential catalysts [26]. The danger increases with the quantity of afflicted relatives, particularly those below the age of 50 [27].

In our results, we discovered that the majority of patients were overweight or obese. Previous Iraqi research emphasizes the significant correlation between increased BMI and important breast cancer characteristics, underscoring the therapeutic relevance of BMI in influencing breast cancer profiles [28]. The mechanism by which obesity induces cancer remains partially elucidated, including adipokines, inflammation, altered fatty acid metabolism, a modified extracellular matrix, and the release of estrogen and insulin-like growth factors [29].

This assignment also showed that, in comparison to the right breast, the left breast had a greater incidence of malignancies in both treated individuals (60.0%) and untreated patients (68.6%). Abdou

et al. [30] state that prior studies have shown that BC a lot affects the left breast more frequently, with a left-to-right ratio that may range from 1.05 to 1.26. According to their research, 50.8% of the left and 49.2% of the right breasts, respectively, had BC. Additionally, they noted that the biology and pathology of the left breast exhibit more aggression than those of the right, although there were no alterations or variations in the clinicopathology of either side [30].

All participants in this research (patients and controls) were categorized into three categories according to smoking status: active smoker, passive smoker, and non-smoker. The sample data indicated that active smokers constituted a minimal fraction, while non-smokers and passive smokers were the majority. The relationship between smoking and this link may be affected by variables such as the duration of smoking, the total lifetime exposure to smoking, or the patient's age [31][32]. Exposure to secondhand smoke significantly augments the chance of getting breast cancer, but to a lesser extent than direct smoking [32][33].

Hypertension raises the risk of BC, as shown by a comprehensive evaluation of 30 research [34]. G protein coupled receptor kinase 4 (GRK4) has been linked to both diseases, according to research [35].

Diabetes is a significant chronic ailment globally, whereas BC is the most prevalent malignancy among women worldwide. Diabetes has been linked to breast cancer through three mechanisms: activation of the insulin signaling pathway, insulin-like growth factor signaling pathway, and modulation of endogenous sex hormones [36]. Chronic hyperglycemia, known as the Warburg effect, can increase the risk of breast cancer. Hyperglycemia increases concentrations of IGF-1 and inflammatory cytokines, affecting cancer cell metastasis, apoptosis, and proliferation [37][38]. Insulin resistance, which decreases estrogen levels, increases the likelihood of cancer development in tissues with elevated estrogen receptors, such as the breast, endometrial, and ovaries [39].

MicroRNA-126 (miR-126) appears to be reduced in several cancer types and is proposed to act as a potential tumor suppressor [40]. The two mature strands of pre-miR-126 are miR-126-3p and miR-126-5p, both exhibiting unique cell-type and strand-specific roles in angiogenesis [41]. The statistical analysis performed in this research showed a significant reduction in gene expression of miR-126-5p in the serum of BC patients (treated and untreated) compared to samples from healthy controls, these results are consistent with the previous studies, as compared to normal neighboring tissues, breast cancer tissues were shown to have downregulated miR-126 [42][43]. According to what is known about circulating miR-126, It could turn out to be a useful biomarker for predicting the outcome of different types of cancer [44]. miR-126 participated in the advancement by focusing on many important genes, such as PAK4, CADM1, and SOX2, of angiogenesis, migration, proliferation, invasion, and cell survival [45]. The overexpression of miR-126 in BC cells reduced the expression of CD97, a G-protein coupled receptor that promotes angiogenesis and cell invasion via integrin signaling [46]. MiR-126 significantly influences targeting vascular endothelial growth factor-A to promote tumor vascularization and development (VEGF-A) [47]. MiR-126 reduces proliferation and mammosphere development in the MCF-7, ER+ BC cell line, indicating that this microRNA may function as a tumor suppressor in ER+ BC by possibly targeting SLC7A5 (LAT1), a sodium-independent transporter that is overexpressed in neoplasia [48].

4. Conclusion and future scope

The study found that breast cancer patients aged 45-54 had a higher number of treated and untreated groups, with 75% discovered at early stages. The majority were overweight or obese, with both treated and untreated left breasts exhibiting a higher incidence of cancers. Active smokers were rare, while non-smokers and passive smokers dominated. The study also found a reduce in miR-126-5p gene expression in BC patients' serum suggesting that miR-126 is excreted by cancer cells, possibly it was affecting other target gene expression.

Acknowledgments

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