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Demographic Study of Age, Family History, Stages, Grade and Expression of miRNA-195-5p in Sample of Iraqi Breast Cancer Patients

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Abstract: The most prevalent cancer in women and one of the leading causes of death is breast cancer. In Iraq, there were 35,864 new cases of cancer in total during 2019. Age is the most important known risk factor for breast cancer. The patterns of breast cancer in small groups of families appear to be consistent with the known patterns of genetic inheritance. New non-invasive prognostic biomarkers are required for the quick identification and differentiation of breast cancer (BC) stages for the improvement of treatment options. Small, non-coding RNAs called microRNAs (miRNA) are involved in many cellular processes, including metastasis, and regulate gene expression. Circulating miRNAs (found in the blood) have great potential as biomarkers to aid in diagnosis or monitor the effectiveness of treatments. Materials and Methods, total RNA was extracted from serum from (n=50) patients and (n=26) healthy control to measure the MicroRNA 195 expression by using SYBR green-based real-time RT-PCR technology. Result, breast cancer is more frequent in 50-59 age groups ($P \leq 0.01$) than others. The majority of BC patients in this study were 31 (62%) grade II out of the total than others. the expression levels of miR-195 in breast cancer patients' serum were greatly increased (up-regulated) when compared with those in the normal adjacent serum. Our results demonstrate that the pre-operative group patients have a higher expression of miR-195 than pre-operative group patients in their circulation.

Keywords: Breast Cancer, miR-195, pre-operative, post-operative, Iraq

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Introduction

Cancer considers the main cause of illness and death globally and it is responsible for 19.3 million patients has been diagnosed and 10 million deaths on earth. The number of people has cancer increases to a 70% rate in countries which have paid a low-medium salary to their employees and workers (1). Cancer can simply be defined as a class of diseases or disorders that is characterized by an uncontrolled division of cells and the ability of these abnormal cells to spread, either by direct growth into adjacent tissues through invasion or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system) (2). Breast cancer is the most common type of cancer in women worldwide (excluding skin and tissue cancers) and the second leading cause of mortality from cancer in women, is an illness in which normal breast cells begin to alter, expand

uncontrollably, and no longer die. Cancer that has not spread is referred to as in situ, which means "in place." Cancer that has spread is referred to as invasive or infiltrating cancer (3, 4). In Iraq, the total number of new cases of cancer during the year 2019 was 35,864 with an incidence of 91.66/100,000 P, While the rate that was recorded in 2010 was 18,482 with an incidence of 56.89/100,000 P. Approximately, 1.5 million new cases are annually diagnosed with breast cancer and almost 460,000 patients died each year due to BC chemoresistance and metastasis (5) Approximately, 7,109 new cases are diagnosed with breast cancer in 2019(6).

Cancer is the major reason for morbidity and mortality worldwide. In 2020, a global cancer burden approximated rate to 19.3 million patients diagnosed with cancer (1). In Iraq, the total number of new cases of cancer during the year 2019 was 35,864 with an incidence of 91.66/100,000 P, While the rate that was recorded in 2010 was 18,482 with an incidence of 56.89/100,000 P (6). Cancer can simply be defined as a class of diseases or disorders that is characterized by an uncontrolled division of cells and the ability of these abnormal cells to spread, either by direct growth into adjacent tissues through invasion or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system) (2). Breast cancer (BC) is a complex disease encompassing multiple tumor entities, each characterized by distinct morphology, behavior and clinical implications (7). Breast cancer is one of the most common malignant tumors and the second leading cause of cancer in women. Approximately, 1.5 million new cases are annually diagnosed with breast cancer and almost 460,000 patients died each year due to BC chemoresistance and metastasis (5). Approximately, 7,109 new cases are diagnosed with breast cancer in 2019 (6). Breast Cancer biological characteristics are routinely used for early detection, prognosis, and selection of the therapeutic strategy, including histologic subtype, grade, lymph node status, hormone receptor, and human epidermal growth factor receptor 2 (HER2) statuses (8). Some of the mentioned characteristics are related to patients' survival and posttreatment clinical outcomes (9). However, several breast cancer patients, who had identical characteristics, displayed various clinical results. Therefore, biological features have restrictions with regard to diagnosis, prognosis, and clinical outcomes' prediction (10). Thus, there is still require to develop a affordable and accurate screening method for this cancer and find new biomarkers to enhance diagnosis, prognosis and prediction (11), and novel diagnostic and prognostic approaches are urgently required for the identification of new personalized therapeutic approaches that enhance BC patients' quality of life.

Circulating biomarkers play a critical role in clinical applications including prognosis, diagnosis, monitoring their effects and predicting recurrence in cancer patient. Although the roles of circulating miRNAs in disease therapy remain indistinct, recent studies have demonstrated that circulating miRNAs might be a novel strategy for breast cancer therapy due to their stability and predictive properties (12).

Small and non-coding RNAs called microRNAs (miRNA) constructs of about 22 nucleotides target messenger RNA (mRNA) to regulate gene expression by either suppressing translation or degrading RNA (13). The role of different sets of miRNAs in the regulation of gene expression during and after the initiation stages of breast cancer as well as the metastasizes stage was highlighted by high throughput studies (14). MiRNAs, of which more than 4000 have been described, are thought to control up to 30% of all human genes (15). MiRNAs can use as suppressors or promoters the tumor and their dysregulation is intricately linked to cellular processes involved in the metastatic cascade, such as sustained proliferation, angiogenesis and epithelial-mesenchymal transition (EMT) (16, 17).

The miR-195 belongs to the miR-15/107 family, which is triggered by stress and implicated in a number of disorders, including cancer, heart failure, and schizophrenia. The miR-15/107 family each member of this group has a similar sequence, AGCAGC, near the 5′ end of the mature miRNA, named AGCx2 miRNA (18). Intron 7 is the location where human miR-195 gene is found which is discovered on chromosome 17p13.1 and on the opposite strand of the mRNA gene AK098506, which codes for a protein with an unknown function (19). MiR-195 was initially predicted based on homology to a confirmed mouse miRNA, and it was later demonstrated that it also exists in humans (20). The "guide strand" miR-195-5p and the sister "passenger" strand miR-195-3p both originate from the miR-195 hairpin (figure 1) (21).

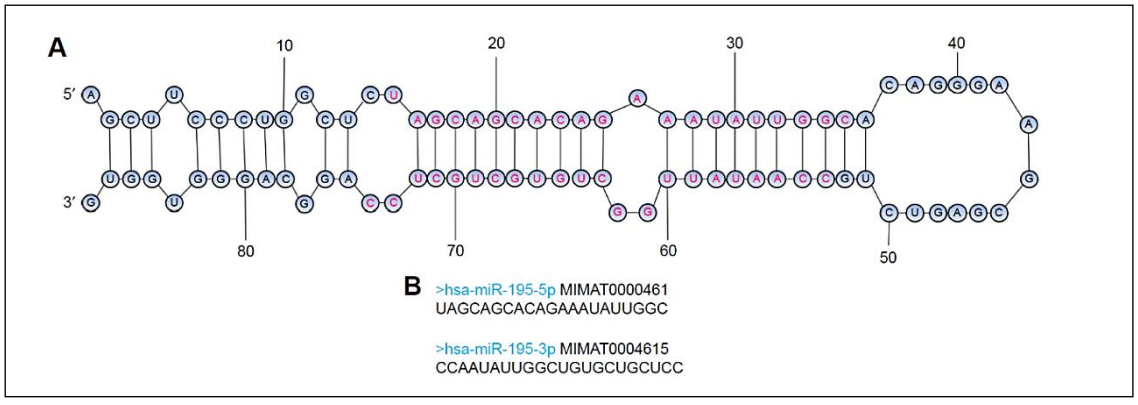

Figure (1): The structure and sequence of miR-195, (A) The miR-195 stem-loop structure. (B) The miR-195 mature sequence. MiR-195-5p and miR-195-3p are two different mature miR-195 sequences (21).

Dysregulation of miR-195 may impart to the etiology of multiple diseases Alzheimer's disease, lung cancer, Kidney cancer, Thyroid cancer and breast cancer. Additionally, it has been observed that miR-195 is upregulated such as Brain cancer and Skin cancer (21). For example, several studies found that miR-195 was significantly down regulated in breast cancer patients (22). The development of breast cancer has been shown to be inhibited by miR-195, according to numerous independent research teams (21).

Materials and methods

In this research, 50 Iraqi women with breast cancer with ages ranging from 27 to 72 years who visited Al-Andalus Specialist Hospital and Oncology Teaching Hospital between the first of December 2021 and the end of February 2022 were included. Comparison of the patients with 26 volunteers, whose ages ranged from 21 to 67 who appeared to be good health. The blood was taken from the patients and healthy group. which was kept at -20 C^o until it was used for the study as described, and then placed directly in Trizol preservation for RNA extraction.

RNA purification

RNA was exteracted from serum samples according to the protocol of TRIzol™ Reagent. The serum (0.4ul) was mixed with 0.5 mL of TRIzol™ Reagent in each tube, and the lysate was homogenized by pipetting up and down multiple times. For each tube, 0.2 mL of chloroform was added to the lysate before securing the tube top. All mixtures were incubated for 2-3 minutes before being centrifuged at 10,000 rpm for 10 minutes to separate the mixture into a lower organic phase, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube. 0.5mL of 'isopropanol was added to the aqueous phase and incubated for 10 minutes then centrifuged for 10 minutes at 12,000 rpm'. Total RNA was precipitated and formed a white gel-like pellet at the bottom of the tube, Supernatant was then discarded. 0.5mL of 70% ethanol was added to each tube and vortexed briefly then centrifuged for 5 minutes at 10000 rpm. Ethanol then aspirated and air-dried the pellet. 'Pellet was rehydrated in 50µl of Nuclease Free Water then incubated in a water bath or heat block set at 55–60°C for 10–15 minutes'.

Primers design

Using the NCBI Gene Bank database and miRBASE, the sequences retrieved and used as a template for primer designs, stem-loop structure considered for efficient detection and quantification (23), Primer premier3 software considered for designing, RT-qPCR primers were synthesized by Macrogene (South Korea), primers set adopted in the current study listed in the (Table 1). For primer designing protocol, all data retrieved from miRBase https://www.mirbase.org/ (24) and confirmed with the published sequences in GenBank/NCBI (https://www.ncbi.nlm.nih.gov/genbank/), the primers designed via primer primers software (http://www.premierbiosoft.com/primerdesign/) (25).

Primer preparation and optimization

Macrogene Company provided these primers in lyophilized form. As a stock solution, 'lyophilized primers were dissolved in nuclease-free water to a final concentration of 100pmol/µL'. A working solution of these primers was generated by combining 10µl of primer stock solution (stored in the freezer at -20°C) with 90µl of nuclease-free water to yield a working primer solution of 10pmol/µL.

Detection of miRNA by RT-qPCR

Total RNA containing miRNA was the starting material in RT-PCR reaction which was performed in two steps. The miRNA gene miRNA195

and RNU expression was done by using specific primers.

Table (1): Primer sequence for miRNA gene expression

Primer Name	Seq.	Annealing Temp. (°C)	Reference
miR-195-5p-RT	5`GTTGGCTCTGGTGCAGGGTCCGAGGTA TTCGCACCAGAGCCAACGCCAAT-3`	55	Design in this study
miR-195-5p-F2	5`-GGTTTTTTTGTAGCAGCACAGAAAT-3`		
RNU43_RT	5`GTTGGCTCTGGTGCAGGGTCCGAGGTA TTCGCACCAGAGCCAACAATCAG-3`		
RNU43_F	5`-GTGAACTTATTGACGGGCG-3`		
Universal Reverse	5`-GTGCAGGGTCCGAGGT-3`		

First step to test the expression of PCR target genes, the first method was reverse transcription by using GoScript Reverse Transcription System Promega kit involves the conversion of RNA to cDNA. Total RNA containing miRNA was used as row material for reverse-transcription reaction. All RNA species were converted into cDNA, in RT-qPCR need primers such as mRNA using oligo-dT primers they were reverse transcribed into cDNA, and the miRNA should have specific primers for conversion of RNA to cDNA. First reaction, the total RNA and miRNA primers were added to PCR tube microfuge 0.2ml (4 - 1) µl respectively as shown in (Table 2). The mixture was incubated for 5 minutes at 70oC and then for 10 minutes at 4oC to break the bonds in the hairpin loop structures seen in (Table 3). The reverse transcription master mix was added to a new PCR tube and gently mixed; the reverse transcription master mix comprises all components required for cDNA first-strand synthesis except templet RNA (Table 4). Templet RNA was gently combined into each tube containing reverse transcription master mix, quickly centrifuged, and then incubated for 5 minutes at 25oC, 60 minutes at 42oC, and 15 minutes at 70oC using a thermal cycler to inactivate reverse transcriptase, as shown in (Table 5).

Table (2): The First reaction

First reaction	Volume
	1 Sample
RNA	4 µl
RT primer	1 µl
Total volume	5 µl

Table (3): 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			

Table (4): Reaction components of cDNA synthesis for detection miRNA expression

Second reaction	Volume	
	1 Sample	29 Samples
Goscript 5x reaction Buffer	2	58
MgCl2	1	29
dNTPs	0.5	14.5
Rnasin	0.25	7.25
Goscript Reverse Transcriptase	0.25	14.5
Nuclease Free Water	3.5	101.5
Total volume	7.5	
Aliquot per single rxn	Add 7.5µl from Second reaction mix per tube and add 5 µl of Template	

Table (5): Thermal Cycler Program for Second reaction

Steps	°C	min: sec	Cycle
Primer Preparation	25	05:00	1
Incubation Enzyme	42	60:00	
Enzyme inactivation	70	15:00	
Hold	4	10:00	

Second step, the miRNA 195 was reversely transcribed to cDNA. following the conversion of miRNA into cDNA, SYBR Green reporter real time PCR reaction was used and the template this time is cDNA, mature miRNA detection using miRNA-specific primers and the SYBR Green qPCR Kit. For accurate results in miRNA quantification, a relative quantification method was used in this method normalization control was used in real-time PCR, where it is crucial to normalize the target miRNA amount using an appropriate endogenous reference RNA. In this experiment RNU43 was used as a reference gene

Table (6): Real Time PCR Program for miRNA gene expression

Steps	Co	min: sec	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:20	50
Annealing	55	00:20	
Extension	72	00:20	

Table (7): Reaction components for RT-qPCR for miRNA expression

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	5
Forward primer	10	µM	1	µM	0.5
Reverse primer	10	µM	1	µM	0.5
Nuclease Free Water					3
cDNA		ng/µl		ng/µl	1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

Results

Patients' distribution

According to age, Fifty female patients with breast cancer enrolled in this study, with a mean age of 53.92 ± 1.35 years ranging from 27 to 72 years old and a Body mass index of 28.93 ±0.65, Including; 1(2%) case between 20-29 years old, 2(4%) cases were between 30-39 years old, 11(22%) cases were between 40-49 years old, 23(46%) cases were between 50-59 years old, 13(26%) cases were between 60-75 years old. The age group at (50-59), which indicated that breast cancer is more frequent than other age groups P≤0.01 (Table 8).

Table (8): Distribution of sample study according to age groups

Age	Patients		Control		P-value
	No.	%	No.	%	
20-29	1	2	6	23.08	0.0092 **
30-39	2	4	8	30.77	0.0063 **
40-49	11	22	3	11.54	0.074 NS
50-59	23	46	8	30.77	0.0382 *
60-75	13	26	1	3.85	0.0081 NS
Total	50	100	26	100	---
P-value	--	0.0001 **	--	0.0001 **	---
*** (P≤0.05). ** (P≤0.01).**					

According to Majid et al., (26) research, women under the age of 60 had a considerably higher breast cancer rate between 2006 and 2012 (P< 0.001). The BC Iraqi studies such as Al-Naqqash and AL-Shewered, (2019) (27), which divided the Patients into two age groups (<50 and >50), Age-related to BC, found that risk for BC associated with increased age, especially ≥50 years, the age developed give 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.

In this study, 26 healthy females were used as a control, the mean age of this group was 39.88 ± 2.42 years old and a BMI of 24.93 ±0.57. The highest percentage of breast cancer cases was found in the fourth age group which reached 46% of total patients, followed by 26% for the fifth age group and 22% for the third age group, while the lowest percentage was observed in the youngest age group which was 4% with a highly significant difference. These results revealed that breast cancer risk exists at any age but it was increase in the middle age of women's life, and that also confirms the need for screening and early detection of breast cancer.

The data show a higher significance (P≤0.01) in Ductal than lobular, Ductal 39(82%) cases and 8(17%) cases were lobular. According to (Table 9), for BC types which arise from the inner lining epithelium of the ducts or lobules supply the ducts with milk, this classification by its histological appearance, the (Ductal) is the majority form of BC, it starts in a milk duct of the breast and may spread into the breast fatty tissue through the lymphatic and circulation system. This result is similar to many Iraqi studies showing that Ductal is the most predominant histological type (27, 28, 29).

Table (9): Distribution of breast cancer types according to age groups

	Ductal	lobular	P-value
	No. (%)	No. (%)	
Patients	39 (82%)	8 (17%)	0.0001 **
Total	47 (100%)		
**** (P≤0.01).**			

According to the result, it was found that 19 (38 %) of total patients had a positive family history, while the remaining 31 (62 %) did not (Figure 4-1). One of the main risk factors for breast cancer was family history; women who had a family member diagnosed with breast cancer before the age of 50 had a higher chance of developing than women who had family members diagnosed older age (30).

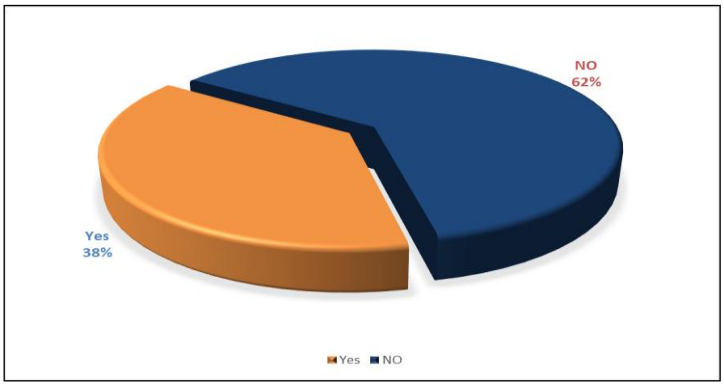

Figure (2): Distribution of breast cancer patients according to family history.

Suleiman, (31) revealed that positive family history was found in 41.67 % of cases in a recent study of 60 Iraqi patients. According to Hatif (2020), 11 out of 48 patients (or 24%) had a positive family history, while 37(or 76 %) did not.

Although 38% of patients have a positive family history of breast cancer was a small percentage, the current findings revealed the impact of family history on the incidence of breast cancer in Iraqi women.

The most prevalent stage among patients with breast cancer in this study was Stage II; 18 out of 50 (or 36%) had stage II breast cancer (Chi-square value = 2.434, p 0.4873). Additionally, only 13 (26%) patients had stage III breast cancer, while stage IV and stage I were present in 11 (22%) and 8 (16%) patients, respectively, as shown in (figure 3). The majority of breast cancer patients enrolled in this study were 31 (62%) grade II out of the total; the rest of the number was 10 (20%) grade I and 9 (18%) grade III.

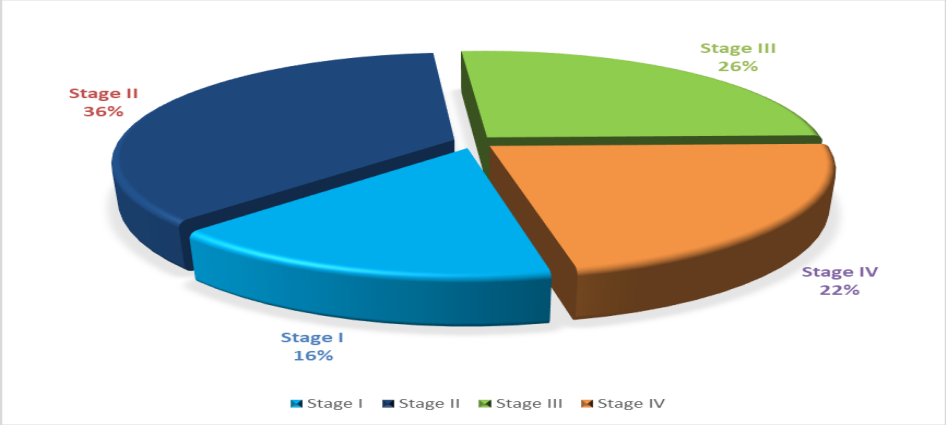

Figure (3): Distribution of breast cancer patients according to disease stage.

MiR-195 expression in human breast cancer serum

In this study. The analysis included 25 healthy controls and 50 cases of breast cancer. The RT-qPCR was applied to initially examine the expression levels of miR-195-5p in breast cancer serum. The expression levels of miR-195-5p in BC serum were considerably increased when compared with those in the normal adjacent serum. BC group demonstrated extremely higher miR-195 expression (upregulation) when compared to control group ($p < 0.0056$) (Table 10). In a contemporary study, the miR-195-5p expression was significantly higher in comparison with healthy controls that showed normal expression (12, 32). While some investigations show that there was a drop in miR-195 expression (downregulation) when compared to control group (22). The expression levels of miR-195 in stage II is significantly higher in this study in comparison with other stages and healthy controls.

Table (10): Comparison between serum miR-195 expression between control and breast cancer groups.

		Control N=26	BC N = 50	*P*
Expression of MiR-195	**Mean**	1.079	5.010	< 0.0056

MiR-195 expression in pre and post-operative

In this study, the expression of miR-195 was examined on 34 pre-operative and 14 post-operative breast cancer patients. Our findings show that the expression levels of miR-195 in pre-operative were dramatically heightened when compared with those in post-operative (Figure 4). When compared to the post-operative group, the pre-operative group had significantly higher miR-195 expression (upregulation) ($p<0.0001$).

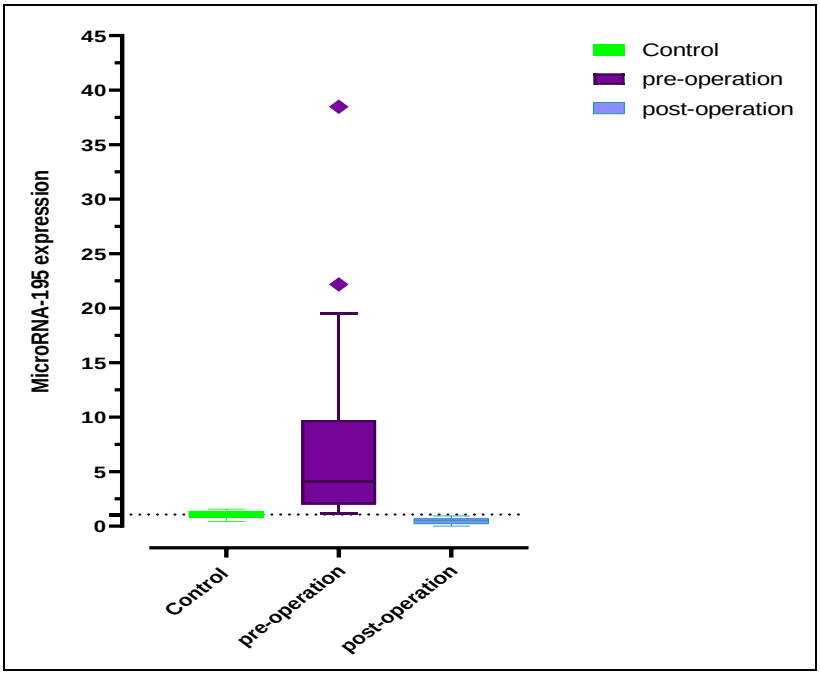

Figure (4): miR-195 expression between post-operative and pre-operative.

A Turkish study done by Cecene et al., (33) indicated that miR-195 expression has down-regulation in post-operative samples as compared to the pre-operative BC patients. the present study demonstrated that pre- operative had high levels of tumor suppressor hsa-miR-195 while post- operative had low levels suggesting that hsa-miR-195 is secreted out of cancer cells, possibly to facilitate the increase of other target gene expression.

Conclusion

Our observations from this study found that the breast cancer is more frequent in 50-59 age groups (P≤0.01) than others. The majority of BC patients in this study were 31 (62%) grade II out of the total than others. the expression levels of miR-195 in breast cancer patients' serum were greatly increased (up-regulated) when compared with those in the normal adjacent serum. Our results demonstrate that the pre-operative group patients have a higher expression of miR-195 than post-operative group patients in their circulation, suggesting that hsa-miR-195 is secreted out of cancer cells, possibly to facilitate the increase of other target gene expression.

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