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Correlation of Circulating microRNA-496 and vascular endothelial growth factor VEGF in triple negative breast cancer

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Abstract:
Background: *Any breast cancer that either lacks or exhibits low levels of the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) overexpression, and/or gene amplification is referred to as triple-negative breast cancer (TNBC) because the tumor tests negative on all three tests. MiRNAs are essential for governing many cellular and metabolic processes, most notably those that govern cell survival, differentiation, and proliferation.*
Objectives: *The study was aimed to investigate the correlation of miRNA-496 and VEGF in TNBC pateints compared with healthy indiveduals.*

Methods: *Using Real time PCR, micro RNA-496 gene expression was measured in plasma samples and ELISA used to measure VEGF for 40 Triple negative breast cancer patients diagnosed by clinical examination, mammography, ultrasound, and histology are included (Tru-cut)., 40 healthy controls with no history of cancer or inflammatory disorders*

Results: *The current study reveal a significant decrease in miRNA-496 gene expression with significant negative correlation to VEGF in TNBC group compared with healthy individuals.*

Conclusions : *Current research result of miRNA-496 in TNBC Suggest it as suppressor for VEGF and it may also serve as the novel therapeutic target for TNBC in the future.*

Keywords *: TNBC, miRNA-496, Real time PCR, Breast cancer, VEGF.*

Introduction

Breast cancer (BC) is the main cause of cancer-related deaths in women over the world [**Wu, D et al., 2021**]. Approximately one-third of patients die with BC as a result of regularly occurring metastases and recurrences, despite significant advancements in early detection and treatment.
[**LI**, **Baixue et al., 2020**]. TNBC is defined as one kind of tumor that is lack of expression of ER, PR, and HER-2 [**LI**, **Baixue et al., 2020**]. The main issue in treating TNBC is the metastasis and recurrence caused by the cancer cells' self-renewal. 15% to 20% of all cases of breast cancer are triple-negative.
(**Sporikova** et al., 2018), and affects more young women or women with a compared to other breast tumors, a mutation in the BRCA1 gene (Garrido et al 2019). The category of malignancies known as triple-negative breast cancers is extremely diverse. According to Cheang et al. (2018), TNBC is the most difficult kind of breast cancer to cure.
. TNBC is not responsive to hormone therapy used for other types of breast cancer (Cheang et al., 2018). Chemotherapy, radiation therapy, and surgery are usually used to treat cancer in its early stages. Chemotherapy and, in certain situations, additional targeted therapy are the only available treatments for advanced stages of the cancer when surgery is not an option or the disease has expanded beyond the original localized location (Cheang et al., 2018).
. A tiny, non-coding RNA molecule called microRNA (miRNA) is present in plants, animals, and some viruses. Its roles include post-transcriptional regulation of gene expression and RNA silencing (PJDexheimer et al., 2020). A member of the microRNA family, which is made up of short RNA strands involved in controlling gene expression, is microRNA 496 (SJ Lee et al., 2021). MicroRNAs do not code for proteins like messenger RNA does (SJ Lee et al., 2021). Rather, they serve to regulate the amount of a protein that is generated (SJ Lee et al., 2021). A signaling protein called VEGF encourages angiogenesis, the formation of new blood vessels, which aids in wound healing and the formation of new blood vessels during embryonic development.
. However, many disorders, including cancer, have unusually high levels of VEGF (ZL Liu et al., 2023). A key factor in angiogenesis, VEGF overexpression has been connected to the development of new blood vessels in a variety of tumor forms (Rao et al., 2024). It is well recognized that VEGF is a crucial signaling protein that influences angiogenesis and vasculogenesis (Jiang et al., 2020). Blood vessel production is essential for the development and metastasis of cancerous tumors (Kretschmer et al., 2021).
. By producing angiogenic growth factors like VEGF, solid tumors—which require oxygen and nutrients to grow larger than a few millimeters—can stimulate the formation of blood vessels (Kretschmer et al., 2021). Effectively blocking the VEGF signaling pathway has therefore emerged as a crucial area of study for medication development, potentially leading to the creation of novel cancer treatments (Kretschmer et al., 2021).

Methods

The Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, Baghdad, Iraq, carried out a case control research on 80 women between April 2022 and December 2023. They ranged in age from (19-70) years, mean ± standard deviation of triple negative breast cancer and control group were (44.76±7.81 vs 46.08±7.47, respectively). Forty of them were triple negative breast cancer patients (18 IDC; 9 DCIS; 13 ILC). Eighteen patients stage I, eleven patients stage II, ten patients stage III, and one patient stage IV**.** Forty women in the control **group.**
Medical lab data were reviewed to get histopathological information from Teaching Oncology Hospital .
Institutional review board (IRB) of the College of Medicine at Al-Nahrain University in Baghdad, Iraq, provided approval for this study (IRB- 150/ in 28- April- 2022). Each subject provided written form of informed consent. This study has been performed in conformity with the Helsinki Declaration.
Blood samples (plasma and serum) collected then serum separation was obtained using the following criteria:
1- Collected at the time of diagnosis, prior to any surgery or therapy.
2- Blood, plasma, and serum samples from participants with no history of cancer or inflammatory disorders (e.g. Rheumatoid arthritis) were obtained. Samples with missing pathologic stage or histologic grade

information were discarded. Samples were collected from Teaching Oncology Hospital in Baghdad. At 8:00 a.m, seven milliliters of blood were drawn from each patient for serum separation then placed in a plain tube. They were taken to the Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University and centrifuged at (1500 x g) for 10 minutes. Following that, blood samples were pipetted into tubes then stored at -70ºC until time of assays of miR-496.and VEGF.

Inclusion and Exclusion Criteria

Inclusion criteria included Triple negative breast cancer patients diagnosed by clinical examination, mammography, ultrasound, and histology are included (Tru-cut). Control participants are chosen to be in the same age range as the patients (18-65 years) While Exclusion criteria included Every patient with any primary cancerous tumor or autoimmune desease.

RNA extraction

Kit contents of RNA extraction as shown in the Table

Kits contents of RNA extraction

Kits	Volume
DNase I² (2U/µl)	550 µl
DNase I Reaction Buffer	5 ml
Pre-Wash Buffer ¹	70 ml
RNase-free Water	15 ml
RB Columns	200
Wash Buffer ³	75 ml
2 ml Collection Tubes	400

RNA Extraction steps

A-Homogenization and Lysis of samples

1. A volume of 200 microliter of serum was added to a 1.5 ml microcentrifuge tube (RNase-free).
2. Six hundred microliter of GENEzolTM Reagent added to serum, and then thoroughly mixed by vortexing.
3. The sample was incubated for 5 minutes at room temperature.

B- Binding of RNA

1. Cell debris was eliminated by centrifuging the material for one minute at 15,000 x g. A fresh 1.5 ml RNase-free microcentrifuge tube was then filled with the clear supernatant.
2. A volume of Four hundred microliter of 100% ethanol has been transferred directly to the microcentrifuge tube containing the sample.
3. After ensuring that the sample mixture was well mixed using a vortex, an RB Column was loaded into a 2 ml collection tube.
4. A volume of Seven hundred microliter of the sample mixture has been transferred to the RB Column. Then centrifuged at 15,000 x g for 1 minute and the flow-through was discarded.
5. After transferring the remaining sample mixture to the RB Column, the RNA Binding Step was performed a second time. 6. After centrifuging the sample mixture for minute at 15,000 x g, the flow-through was discarded. The sample mixture had already been centrifuged. A new 2 ml Collecting Tube was placed into which the RB Column was positioned.

C- Wash of RNA

1. After adding 400 microliters of pre-wash buffer to the RB Column, the mixture was centrifuged at 15,000 times g for thirty seconds.
2. The flow-through has been discarded and then the RB Column was positioned inside of the 2 ml Collecting Tube.
3. A volume of 600 microliters of the wash buffer has been added to the RB Column.
4. After being centrifuged for thirty seconds at a speed of 15,000 x g, the flow-through was discarded. .then, the back of the RB Column was placed into the 2 ml Collecting Tube.

5. The RB Column was cleaned once again with 600 microliters of Wash Buffer.
8. To dry the column matrix, it was centrifuged at 15,000 x g for 3 minutes.

D- Elution of RNA

1. A clean 1.5 ml microcentrifuge tube was used to taken the dried RB Column (RNase-free).
2. At the center of the column matrix, 50 µl of RNase-free Water was added.
3. The column matrix was left standing for 3 minutes until the RNase-free Water was entirely absorbed.
4. Until the purified RNA has been eluted, the sample was centrifuged at a speed of 15,000 x g for one minute.

2.3.3.2 Preparation of Master Mix

A Kit of Master Mix component was prepared as presented in Table 2-5.

Table 2-5: Master Mix components

Components	20 µl Reaction	Final concentration
Luna Universal One-step Reaction Mix	10 µl	1X
Luna WarmStart®RT Enzyme Mix	1 µl	1X
Forward primer (10 µm)	0.8 µl	0.4 µm
Reverse primer (10 µm)	0.8 µl	0.4 µm
Template RNA	variable	< 1 µg (total RNA)
Nuclease-free Water	To 20 µl	

Procedure

1. Before being placed on ice, the Universal Luna One-Step Reaction mix and other reaction ingredients were allowed to defrost at room temperature. Following complete thawing, gentle vortexing was used to quickly homogenize each component.
2. The total volume has been determined for the required number of reactions plus 10% overage, and the assay mix of all components except the RNA template was produced appropriately. Thoroughly mixed, but slowly vortexed. A quick centrifugation pushed the Supernatant to the bottom of the tube..
3. Supernatants of the assay mix were loaded into RT-qPCR tubes. In order to get the best possible results, the pipetting quantities checked were precise and that bubbles were kept to a minimum..
4. The qPCR plate has been loaded with RNA template. Both the tubes and the plates have been sealed up using thin layer. The caps on the tubes are flat and optically clear. In order to avoid artefacts induced by evaporation, the plate's edges and corners were carefully sealed.
5. The tubes were centrifuged at a speed of 12,000 x g for one minute in order to remove any bubbles and liquid has been collected..
6.Indicated thermocycling was programmed into a real-time device. When the final extension is complete, a plate read is performed.

2.3.3.3 Primers

This study involves synthesis primers of miR-496in Macrogenand haouse keeping gene U6 as shown in the Table 2-6.

Table 2-6: Specific primers sequence used in the current study

Primers	Sequence	Tm (°C)
RT of mir 496	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCGGT-3'	
miR-496	f-5'- GGCCAACCCCGGACCA -3'	62.4

	R-5- AGTGCAGGGTCCGAGGTATT -3'	58.5
RT of U6	5'- CGCTTCACGAATTTGCGTGTCAT-3'	61
U6	f- 5'- GCTTCGGCAGCACATATACTAAAAT - 3	57
	R-5'-CGCTTCACGAATTTGCGTGTCAT -3'	61

2.3.3.4 *Quantitative Reverse Transcription PCR* (RT-qPCR)
The following were the conditions for the PCR reactions as shown in Table 2-8.

Table 2-8: PCR Reactions Conditions

Cycle step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 minutes	1
Initial Denaturation	95 °C	minute	1
Denaturation Extension	95 °C 60 °C	10 seconds 30 seconds	40-45
Melt Curve	60-95 °C	Various	1

Upon completion of the process, the melting and amplification graphs were analyzed. We first acquired Ct values for each microRNA sample and standardized them in order to identify relative expression levels. Results are shown as mean ± S.D., with GADPH as the benchmark for calculation (Livaket al, 2001).

2.3.3.4.1 *Quantitative Reverse Transcription PCR* Data Analysis and calculations

Relative expression and ΔCt were used to analysis RT-qPCR results for both housekeeping and target genes as shown below *(***Livak *et al*., 2001).**

$\Delta Ct\ (patients) = Ct\ (patients)_{mean} - Ct\ (reference)_{mean}$

$\Delta Ct\ (Controls) = Ct\ (controls)_{mean} - Ct\ (reference)_{mean}$

$\Delta\Delta\ Ct = \Delta Ct\ (patients) - Ct\ (controls)$

Using the up-described particular primer and the novel molecular technique Real time PCR (qRT-PCR), able to effectively identify the expression of gene. The value of Ct for the triplicate reactions was used to assess the precision of gene product amplification.

Ct values, determined from cycles, were utilized to identify data from real-time experiments; these values were approximated equally to the initial target copy number (logarithmic scale) employed in amplification (the point that the fluorescence signal increased above baseline is the Ct) which are proportional to the quantity of beginning template, therefore low Ct values represent high amounts of gene expression or amplification gene,

Fluorescent signal from samples may be plotted against the number of cycles in a RT-qPCR experiment, yielding amplification plots, which also show the buildup of product over time.
A popular relative quantification technique for analyzing quantitative real-time polymerase chain reaction (qPCR) data is the 2-ΔΔCT method. This method, which applies the 2-ΔΔCT method to data with automatic background fluorescence removal by the qPCR software, is a convenient way to determine relative gene

expression levels between different samples because it directly uses the threshold cycles (CTs) generated by the qPCR system for calculation. Results may be distorted if an incorrect background is subtracted since the background fluorescence is unknown. We offer an enhanced approach, the individual efficiency corrected computation, to deal with these issues (Rao et al., 2013).

2.3.3.4.2*Quantitative Reverse Transcription PCR* Amplification Curve

The curves that resulted during this study as shown in Figures2-5 and 2-6.

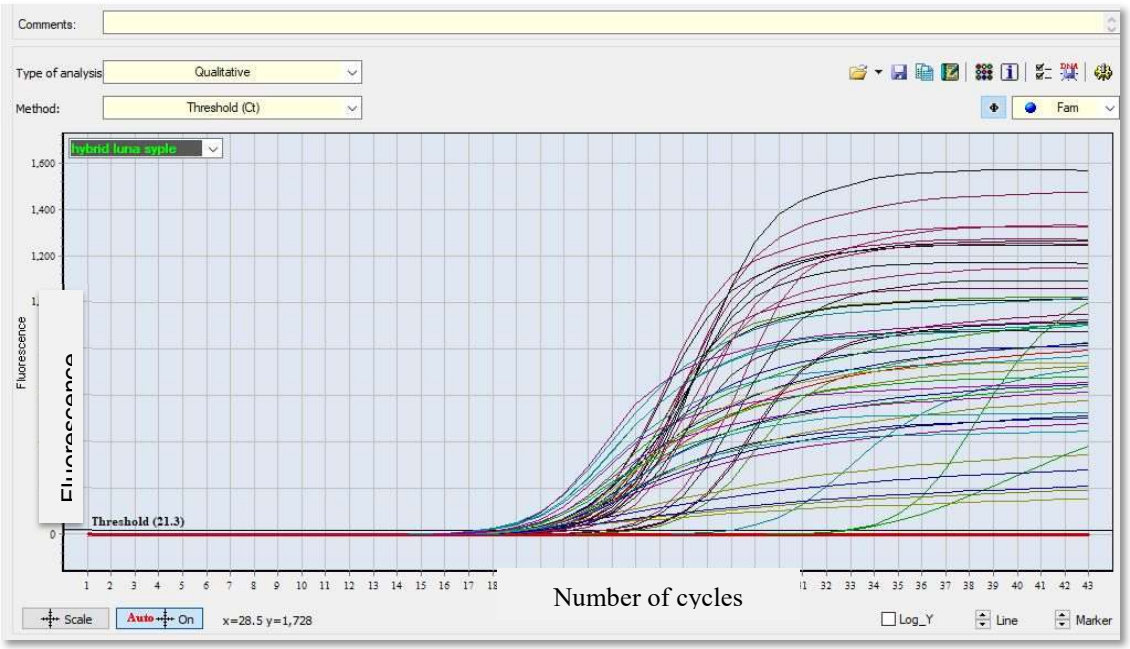
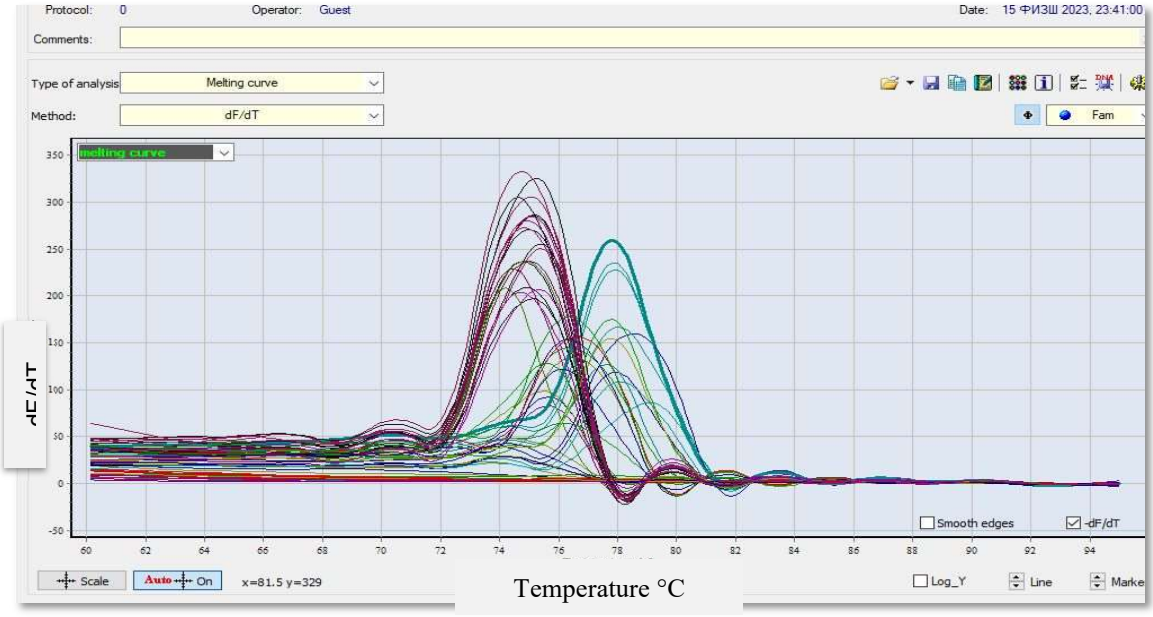


Figure 2-5: Relationship between cycle number and fluorescence as PCR curve **Figure 2-6**:RT-qPCR Melting curve



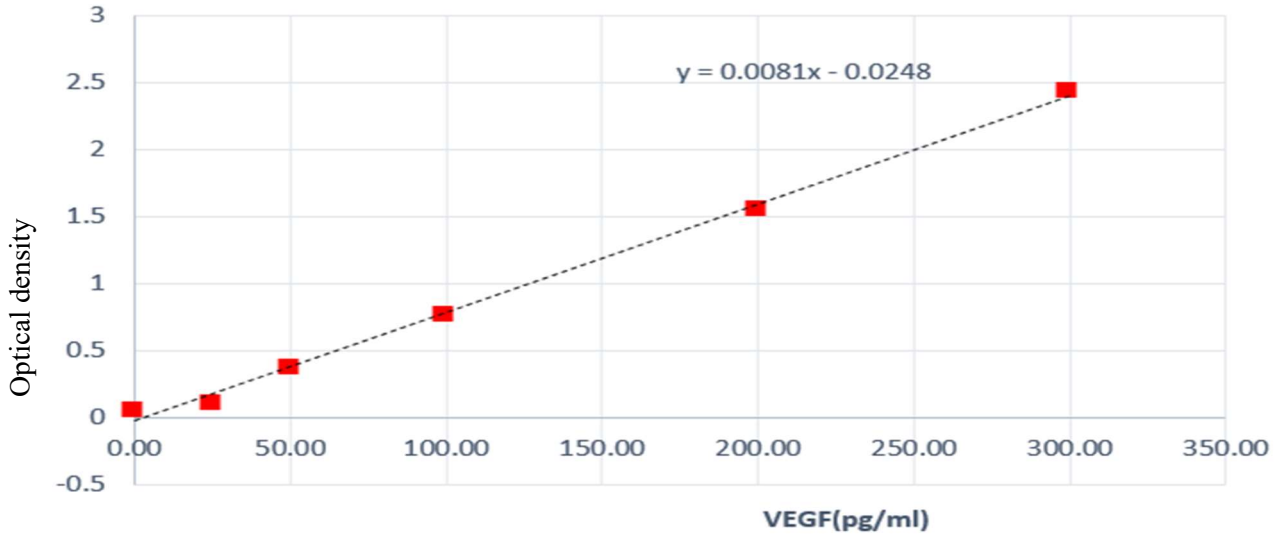
Measurement of Serum VEGF Level

Sample preparation

Concentrated Serum Preparation Whole blood clots when it is collected and then left at room temperature. The average time required for this is between 10 and 20 minutes. Then separated by centrifugation at 12,000 x g for twenty minutes to get rid of the clot.

Principle

In this ELISA kit, the Sandwich-ELISA method is used. This kit contain a Microelisa stripplate that has been pre-coated with an anti-VEGF antibody. Microelisa stripplate wells containing samples and standards are mixed with the suitable antibody. Next, in each Microelisa stripplate well, a HRP-conjugated antibody specific for VEGF is applied and incubated. Free particles were cleared. To each well, the TMB substrate solution was added. Only the wells possessing VEGF and HRP-conjugated VEGF antibody will initially show blue, turning to yellow once the stop solution is transferred. At a wavelength of 450 nm, O.D. is measured spectrophotometrically. The relationship between the OD value and VEGF concentration is linear. Finally, by comparing the OD of the samples to the standard curve, to measurement the concentration of VEGF in the samples.



Reference range *between 31 and 86 pg/mL*

Figure 2-3: Standard curve of VEGF (pg/ mL)

Procedure

1. The volume of 50 ul from each small tube containing diluted standards was pipetted into each of the 10 wells of the microplate.
2. Microelisa stripplates have a blank control well that may be left unfilled. Ten microliters of sample and forty microliters of sample dilution buffer were injected into each well (dilution factor is 5). Samples were placed on the bottom of the well so they don't touch the sides, and then shake the well shacked gently to mixing the contents.Samples were Incubated for 30 minutes with the closure plate membrane in place at 37 °*C*.
3. Distilled water was used to dilute the concentrated washing buffer (30 times).
4. The membrane on the closure plate was cleaned off, aspirated, and filled with the wash solution. After 30 seconds of rest, the wash solution was discarded. The washing process was carried out five times.

5. Fifty hundreds microliters µl of HRP-conjugate reagent has been added to each well except the blank control well.
6. Samples were incubated for 30 minutes at 37°C after being closed with a Closure plate membrane.
7. The membrane on the closure plate was cleaned off, aspirated, and filled with the wash solution. After 30 seconds of rest, the wash solution was discarded. The washing process was carried out five times.
8. To each well, 50 µl Chromogen Solution A and 50 µl Chromogen Solution B were added, then gently shaken and incubated at 37°C for 15 minutes to protect light during coloring. Fifty hundreds microliters stop solution was transferred to each well to terminate the reaction. The color in the well changed from blue to yellow.
9. Optical density has been measured at 450 nm using a microtiter plate reader. In the blank control well, adjusted the OD to 0. Within 15 minutes after stop solution (H2SO4) has been added, the assay was completed.

Calculation the results

The standard concentrations of human VEGF (x-axis) and the corresponding optical density (y-axis) are shown in comparison to each other. We can determine the amount of human VEGF present in the sample by plotting O.D. on the Y-axis. The original concentration is obtained by multiplying the dilution factor (5) by the starting concentration.

Statistical analysis

All statistical comparisons were conducted using the independent t-test and the analysis of variance (ANOVA) test, and the findings were displayed as mean ± standard deviation (SD).In statistical testing, a difference was considered significant if its P level was less than 0.005. Along with the areas under the curve (AUC) of receiver operating characteristics (ROC) curves, specificity and sensitivity were also assessed.

Results

Comparison of MicroRNA-496 between control group and TNBC group:

Comparison of MictoRNA-496 between study groups was performed in the present study. the values of relative fold gene expression level that was calculated by the formula ($2^{-\Delta\Delta Ct}$) had been tested by the normality test and revealed there wasnot normal distributions, so that, Mann-Whitney U test was used to compare.p. value was calculated at 95% of confidence intervals.the date was expressed as median and (min-max) table below

Table (3.4): comparison of MicroRNA-496between study groups

Variables		Control Group *n*= 40	TNBC Group *n*=40	U. test	p value
MicroRNA-496	Median(min-max)	2.09(0.006-8.42)	0.45(0.03-3.19)	439.00	0.001*

n: number of samples, *significant deference

significant increase of MicroRNA-496fold gene expression level was shown in control group in comparison with TNBC Groupand p. value 0.001 figure 3.4

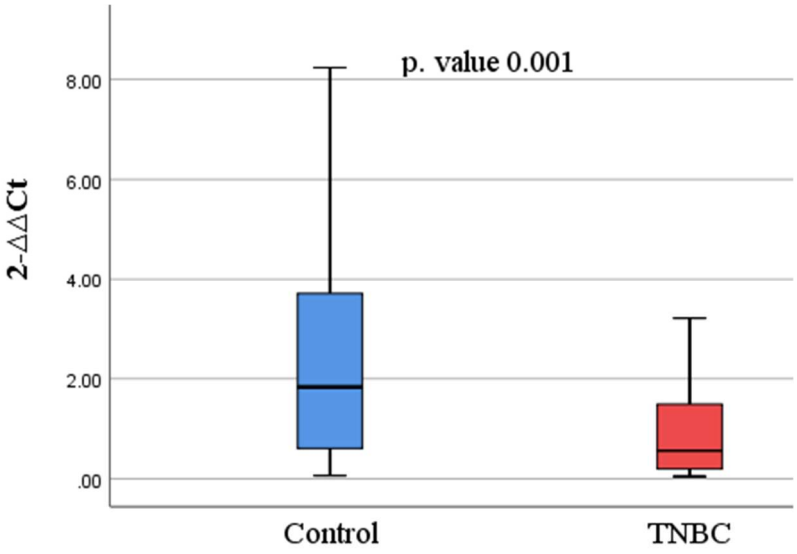


Figure 3.4

Comparison of serum levels VEGF between study groups:

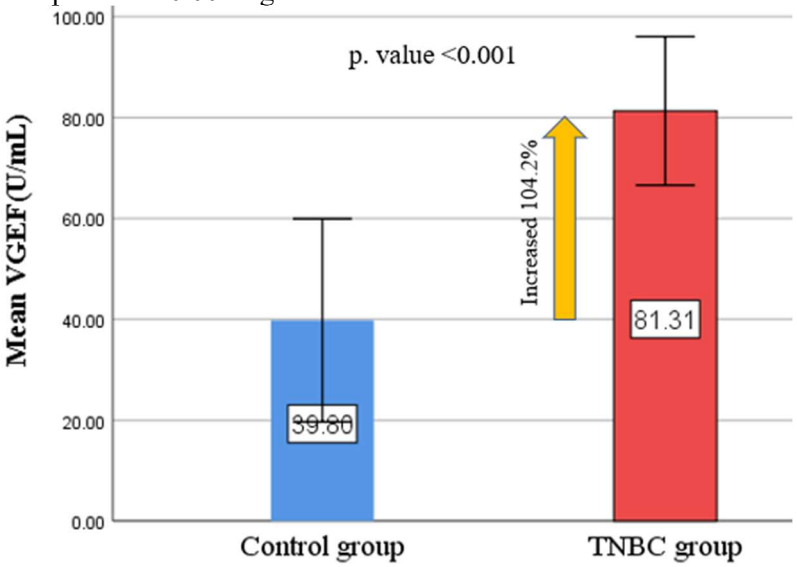
In the present study , VEGF was measured in the sera of all patients and control group and then compared between these groups. t test was used to Compare between mean of concentrations between study groups, the Values of results expressed as mean ± standard deviation and shown in table below.

Table (3.3): comparison of VEGF between study groups

Variables		Control Group *n*= 40	TNBC Group *n*=40	t. test	p value
VEGF(U/mL)	mean	39.80±20.12	81.31±14.74	10.52	<0.001*

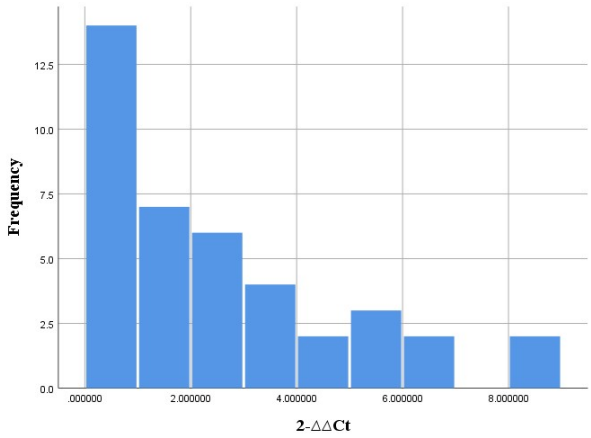
n: number of samples, *significant deference

significant increase of concentration level VEGF was shown inTNBC Group in comparison with control group with p. value <0.001 figure below

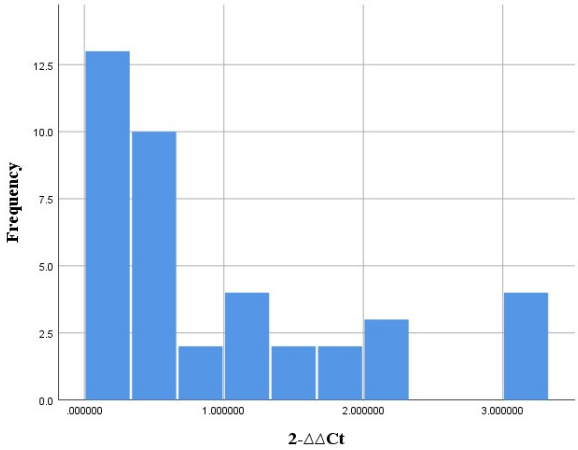


Correlation between MicoRNA-496 and VEGF in the TNBC group

In the present study, correlation study between MicoRNA-496 and VEGF in the TNBC group was performed. because of the formula ($2^{-\Delta\Delta Ct}$) that was used to calculated the values of relative fold gene expression level is exponential function, so the distribution of data was exponential distribution figure below



Data distribution in control group



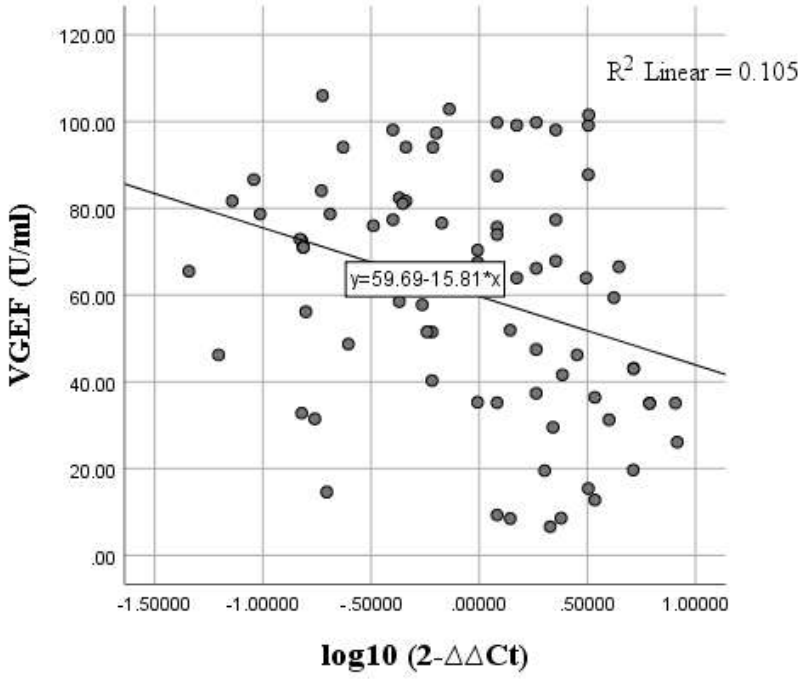
Data distribution in TNBC group

Figure

Accordingly, in order to adjust these data to agree withassumption of Pearson correlation coefficient (Daniel, W.W. and Cross C.L., 2018. Biostatistics), results were represented as$\log_{10}(2^{-\Delta\Delta Ct})$. Pearson correlation coefficient was used to measure this correlation, and the p. value was calculated at 95% confidence intervals. table below

Correlation test		VEGF (U/ml)
$\log_{10}(2^{-\Delta\Delta Ct})$	r	-0.32
	p. value	0.003*

Negative Significant correlation between $\log_{10}(2^{-\Delta\Delta Ct})$ and VGEF was shown in the patients of TNBC group with p. value 0.012 figure below



Discussion

Negative Significant correlation between $\log_{10}$ ($2^{-\Delta\Delta Ct}$) and VGEF was shown in the TNBC group patients, with a p-value of 0.012.Since VEGF is a crucial regulator of angiogenesis and encourages the formation of new blood vessels to support tumor progression, this could have a number of ramifications. However, in TNBC, miR-496 has been shown to function as a tumor suppressor and may prevent angiogenesis. Since VEGF and miR-496 have a negative correlation, higher levels of miR-496 may be linked to less VEGF-mediated angiogenesis in TNBC tumors.

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Through the inhibition of VEGF production and downstream angiogenic signaling pathways, miR-496 may decrease the growth of TNBC tumors. Decreased tumor vascularization and reduced tumor progression may come from lower VEGF levels brought on by increased miR-496 expression. In TNBC, miR-496 appears to function as a tumor suppressor, preventing the growth, migration, invasion, and metastasis of tumor cells. Higher levels of miR-496 may be linked to a lower tumor burden and a decreased release of VEGF into the bloodstream, as indicated by a negative correlation with VEGF.

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Conclusion :

Although the molecular mechanisms of the miRNAs in oncogenesis are remained need to be fully explained, evaluating the miRNA expression in circulation provides a tool to understand and explain varios cancers.

We have demonstrated an aberrant expression VEGF in TNBC regulated by miRNA-496. Our results indicate that VEGF expression may play a specific role in TNBC which may be regulated by one or more miRNAs. And this result outcome suggest miRNA-496 as a novel therapeutic target to be applied to improve TNBC outcome .

Ethical approval

All participating women have completed a written consent form indicating their agreement to participate in the study, which was authorized by the Al-Nahrain Medical College Institutional Review Board under number /.

Declaration of conflicting interests

The author(s) detected no potential conflicts of interests with respects to the research, authorship, and/or

publication of this research.

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