Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.1093

Circulating microRNA-92a as biomarkers for primary woman breast cancer Iraq population

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Submitted: 17 November 2022; Accepted: 10 December 2022; Published: 12 January 2023

ABSTRACT

MiRNA genes have their own the beginning and end of RNA transcripts and may be intragenic or intergenic. When the host genes' intron sequences match exactly those of the splice-site-containing pre-miRNA, mirtons are created. Processing of Drosha is not necessary for the development of Merton, and they can be generated without it. This study included 75 patients suffering from breast cancer based on the oncologist confirmation and 50 healthy subjects. Using Friedwald's method, LDL cholesterol may be quantitatively determined from total cholesterol, triglycerides, and the concentration of HDL cholesterol. A The total RNA was extracted from blood samples using a ready-to-use reagent (TRI Reagent®; ZYMO RESEARCH; USA). Promega's QuantusTM Fluorometer (USA)and PromegaQuantiFluor® Dye Systems were used for the analysis. Total RNA, quantitative MIR29a gene expression was estimated by real time PCR in patient and control group. The mean level of BMI showed none-significant difference between the patients and control. High Density Lipid (HDL) levels showed a significant higher mean in patients than in control. The MIR29a gene expression levels of patients showed a significantly higher level than those of control. Levels of cancer marker Ca125 showed high levels in patients compared to control.

Keywords: *HDL, TG, TC, VLDL, miRNA, and pri-miRNA*

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INTRODUCTION

Short, endogenous, non-coding RNAs that are single-stranded and typically shorter than 19 to 25 nucleotides are referred to as microRNAs. They have undergone evolutionary conservation. 2018; O'Brien et al. The Caenorhabditis elegans worm's lin-4 locus generates the first miRNA, designated lin-4. It was found in 1993.. (Azlan et al. 2022). Database of microRNA Sequences (miRBase), (release 22.0, March 2018). As of right now, there are 2,654 complete sequences of miRNA in the human genome. (Birgaoanu, Griffiths-Jones, and Kozomara 2019). In many tissues and developmental phases, miRNAs are the main post-transcriptional regulators of gene expression. They do this by regulating gene expression by use of intricately interwoven regulatory networks and highly specific interaction types. (Cianflone et al. 2022).

MiRNA transcription in the nucleus is the first stage in the processes of miRNA creation or biogenesis, followed by development and refinement in the cytoplasm. MicroRNA genes may be located both within and outside of genes. Independent promoters, transcribed sequences, and transcription termination units characterize intergenic miRNA genes.(MacFarlane and Murphy 2010) However, intragenic genes share both intronic and exonic sections of host genes, sharing the same transcriptional units. (Zeidler et al. 2020) In contrast to intronic miRNAs, which are found in the intergenic regions between exons, exon-intron overlap is common for exonic miRNAs.. (Rodriguez et al. 2004) When the host genes' intron sequences match exactly those of the pre-miRNA, splice sites are added to both ends of the resulting mirtron to make the mature miRNA.. (Lin, Miller, and Ying 2006) Therefore, processing on the the pre-miRNA, splice sites are added to both ends of the resulting mirtron to make the mature miRNA.. (Loh et al. 2019) In the first stage of miRNA synthesis, drosha processing is the mechanism that produces The first miRNA (pri-miRNA) precursor).

Uncontrolled growth of cells or tissues lining the mammary glands and ducts is what causes breast cancer. Cancers of the breast, particularly adenocarcinomas, account for the vast majority of malignant lesions in the breast. A number of microRNAs have a robust link between their expression levels and details about breast cancer's morphology, immunohistochemistry, histology, diagnostic testing, pathology, medicine, prognosis, and treatment outcomes. (Lin and Gregory 2015) Additionally, investigations showed that in contrast to their non-cancerous relatives, breast cancer situations have abnormal miRNA expression patterns. (Fridrichova and Zmetakova 2019) MiRNA molecules, one of the most significant groups of gene regulators, have tremendous potential as novel biological treatment agents, targets, or biomarkers for individual breast cancer patients. therapies.

The most common malignant kind worldwide is breast cancer. women, particularly those in the first grade, who have a Breast cancer in the family history. Bowel cancer accounts for 19% of all malignancies in women, making it a significant malignancy. The prevalence of breast cancer is growing worldwide, mostly in developing countries. If instances are disclosed and treated promptly, the global health organization (WHO) estimates that the cost of cancer therapy may be decreased by around one third. (Shihab and Al-Mualm 2021).

Although high Breast cancer in the family history. Bowel cancer cancer, Ovarian cancer biomarkers, including CA125, have been advocated for use in diagnostic blood tests.. (Fang et al. 2017) and in pancreatic cancer was connected with the metastasis-associated burden (Liu et al. 2016a) CA15-3 is often used for women who have breast cancer in monitoring treatment and predicting recurrences. (Chu and Ryu 2016) The therapy of It has been found to have a significant positive effect on breast cancer patients. elevated amounts of cancer-related proteins in the blood, such as CEA, CA125, and CA15-3, used as indications of prognosis and to track the progression of the illness. (Wang et al. 2014)

High-density lipoprotein (HDL) cholesterol and overall cholesterol indicators are inversely related to the risk of breast cancer. was shown in a recent comprehensive analysis of prospective studies; however, no significant relationships were found that is, cholesterol carried by low-density lipoprotein (LDL) particles.(Gupta et al. 2022)Based on geographic location, this analysis found there was a substantial amount of variation across the included studies for total cholesterol.

A second comprehensive study that also revealed a favorable correlation for LDL cholesterol mirrored HDL cholesterol's inverse correlation. (Weissglas-Volkov and Pajukanta 2010) Higher triglyceride levels were inversely linked with BC risk, Neither high-density lipoprotein nor low-density lipoprotein, according to a third meta-analysis.

METHODS

This study included 75 patients suffering from breast cancer based on the oncologist confirmation. anybody who had surgical excision for primary breast cancer between January 2021 and March 2022. 50 people in the control group, who were age-matched and free of breast cancer at the same period.

Calculation of By dividing the square of the height, we were able to calculate the body mass index (BMI).: BMI = weight (kg)/height (m2).

Total blood cholesterol was measured using a Biolabo laboratory kit; the method of measurement was based on the enzymatic hydrolysis. The amount of the produced red dye quinonimide is related to the level of cholesterol; quinonimine absorbance was measured using a spectrophotometer at 500 nm.

Glycerol and fatty acids were digested by enzymes to identify the triglycerides. The amount of red dye quinonimide produced is inversely related to the level of cholesterol. Using a spectrophotometer, the quinonimine absorbance was measured at 500 nm.

Using Friedwald's method, LDL cholesterol may be quantitatively determined from total cholesterol, triglycerides, and the concentration of HDL cholesterol: The formula for calculating LDL is LDL = Total Cholesterol - HDL Cholesterol - Triglyceride/5.

VLDL concentration is equal to one-fifth of serum TG.

Blood samples were utilized to isolate total RNA using TRI Reagent®, a ready-to-use reagent from ZYMO RESEARCH in the United States.. The The purpose of this reagent is to purify cellular total RNA. Due to the reagent's very efficient suppression of RNase activity, which occurs during sample homogenization while destroying cells and dissolving cell components, the RNA is kept in tact. (Kit leaflet). Up to 50 g (per prep) of high-quality RNA may be quickly and easily purified from samples in TRI Reagent with the Direct-zolTM RNA MiniPrep. Effective isolation of whole RNA, including short RNAs (17-200 nt)..

Concentration and purity of RNA were done for samples by using Quantus™ Fluorometer (Promega/ USA) and PromegaQuantiFluor® Dye Systems (Promega/ USA). The 20X Tris EDTA (TE) Buffer (pH 7.5) was diluted to The 1X TE buffer was made using nuclease-free water. Working solution of QuantiFluor® Dye was diluted to 1:400 in 1X TE buffer. In order to make the blank sample, we only added ing 200 l of a 0.5 ml PCR tube's previously produced QuantiFluor® Dye working solution (without RNA sample or standard). One liter of the QuantiFluor® ONE ssRNA System standard was mixed to 200 liters of the QuantiFluor® ONE ssRNA Dye. Pipetting gently was used to combine the mixture three times. To collect the liquid at the tube's bottom and eliminate any air bubbles, The centrifugation time for the tubes was 5-10 seconds at 2,000 g.Tubes light-protected, room-temperature incubation for five minutes. Then the prepared tubes of blank and standard placed in the Quantus apparatus to be read and calculate the standard to blank ratio. The samples then prepared by adding 1µl of the sample was added to 200µl, then placed in the calibrated Quantus apparatus to be read.

then, the eluted RNA converted to cDNA by adding 8µl of the RNA to a new sterile PCR tubes then the volume completed to 10µl by adding 2µl of the commercial kit provided by Takara prime script (Japan, Cat#RR014A) then the tubes contain reaction mixture was incubated under37°C, for 15 minutes, followed by Temperature: 85 degrees Celsius, 5 seconds (reverse transcription) (Heat-induced inactivation of reverse transcriptase) 4°C. The prepared cDNA then kept until the day of the PCR in the deep freezer.

PrimeScriptTM The reverse transcription process for real-time RT-PCR is improved by the RT reagent Kit. It leverages PrimeScriptTM RTase, a tool that has good extensibility and enables the synthesis of cDNA templates for Real Time PCR in a quick and effective manner..

For one reaction volume, the additions mentioned in the table (1) were added to a new sterile PCR tube and placed on ice. A slightly larger amount of master mix was prepared than is required to compensate for pipetting losses. The reaction included the use of stem loop- primer which sequence was as follow; GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCGCCTTC TTTCNNNNN.

TABLE 1: cDNA synthesis reaction components

Reagents	Volumes
PrimeScriptTM	2 µl
RNA	2 µl
RT-stem-loop primer	1 µl
RNase Free dH2O	up to 10 µ l
	Total 10 µ l

The reaction mixture was incubated under 37°C for 15 minutes, then 85°C for 5 seconds (Reverse transcription) (Inactivation of reverse transcriptase with heat treatment). The lyophilized primers were liquefied To provide a stock solution with a final concentration of 100 M in free DdH2O and maintain a stock at -20. Prepare a 10 M concentration as a suspended work primer by adding A 100-milliliter volume may be made by adding 10 milliliters of stock solution to 90 milliliters of DdH2O-free water.

Performing qRT-PCR

A reaction mix was prepared according to the appropriated volume of all reaction components listed in the table (2). After the addition of all the listed components in a new sterile PCR tubes then placed into a thermal cycler instrument which had programmed as followed; Enzyme activation at 95C for min; then immediately subsequent to 45 cycles of denaturation at 94° C, annealing at 60 °C, and extension at 72° C, each of those steps acquired 20 seconds, then followed the 40 cycles a melting curve analysis which took 15 min. the forward primer sequence that used to target the MIR92a is; CACTTGTCCCGGCCTGTAAA. And the reverse primer sequence is; GTGCAGGGTCCGAGGT. the reverse primer sequence that used to target the U6 is CTCGCTTCGGCAGCACA and the reverse primer sequence is AACGCTTCACGAATTTGCGT.

TABLE 2: qRT-PCR reaction components

Component	10µL (Final volume)	Final concentration
KAPA Master Mix (2X) Universal	5µL	2x
F primer	0.4 µL	0.2µM
Universal Reverse primer	0.4 µL	0.2µM
Nuclease-free water	Up to 10 µL	
Template DNA Sample Volume	-	1pg-100ng

Statistical Analysis

SPSS was used for the statistical analysis of the data. The data was summed up using typical statistical concepts including the mean, standard deviation, and standard error. Randomized samples We performed a T test to evaluate mean differences. It was decided that a probability was significant if it was equal to or less than 0.05. The following formulae were used to determine the fold change: CT that is equivalent to the discrepancy between the target gene's CT and the U gene's CT. CT is determined by subtracting the average control CT from each sample's CT. Change in fold equal to 2 to the power of - ΔΔCt.

RESULTS

Quantitative expression of Micro-RNA 29a was calculated using a real-time PCR-based method of reverse transcription (Reverse transcription qRT-PCR). The folding of gene expression were estimated using Ct value and folding (2-Ct) technique, and normalized into the realm of a housekeeping gene (U6 gene).A representative RT-qPCR plot is given in figure (1a) which represent the amplification of MIR29a and figure (1b) represent the amplification of U6 gene.

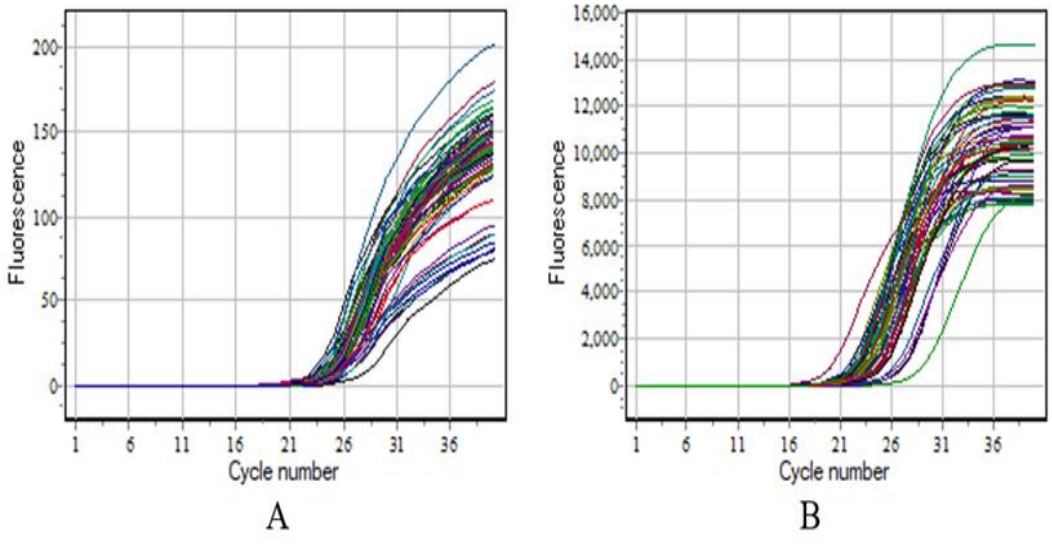

FIGURE 1 A: Rt-PCR result of MIR-29a amplification as given CT, B; Rt-PCR result of U6 gene amplification as given CT

The difference between the means of the studied parameters within controls and patients subjects are summarized in the table (3). The mean level of BMI showed none-significant between the patients and control (31.7 ± 0.79 and 29.8± 0.90, respectively). The age showed a none-significant difference between patients and controls subjects (50.70± 1.25 and 47.12± 1.08, respectively). The total cholesterol (TC) of patients and control showed none-significant difference (45.75± 3.99 and 171.60± 5.49, respectively). The values of triglyceride (TG) showed a significant lower levels in patients than control (47.47±3.25, 49.60±8.71, respectively). High Density Lipid (HDL) levels showed a significant higher mean in patients than in control (184.68±4.09, 154.46±1.51, respectively). Low Density Lipid (LDL) means of patient and control subjects showed a significant difference (201.82±8.49, 97.50±4.16, respectively). Very Low Density Lipid (VLDL) statistically significant distinction across the test subjects and controls studied subjects with a higher levels in patients group (43.60±1.01 and 32.65±2.32, respectively). Levels of cancer marker Ca125 showed a significant high levels within patients than in control (109.56± 4.43 and 8.54± 0.77, respectively). The cancer marker Ca15 also showed a higher level in patients than in control (41.99± 2.24 and 12.04± 1.07, respectively). the MIR29a gene expression levels of patients showed a significant higher level in patients than in control (9.50± 1.30 and 3.47± 0.70, respectively).

TABLE 3; mean levels of studied parameters differences between patients and control

group		Mean	Std. Error Mean	P- Value
BMI	patient	31.76	0.79	0.17
	control	29.80	0.90	
age	patient	50.70	1.25	0.250
	control	47.12	1.08	
TC	patient	171.60	3.99	0.461
	control	45.75	5.49	
TG	patient	154.46	3.25	0.001
	control	47.47	8.711	
HDL	patient	184.68	4.09	0.001
	control	49.60	1.51	
LDL	patient	201.82	8.49	0.001
	control	97.50	4.16	
VLDL	patient	43.60	1.01	0.001
	control	32.65	2.32	
Ca125	patient	109.56	4.43	0.001
	control	8.54	0.77	
Ca15	patient	41.99	2.24	0.001
	control	12.04	1.07	
MIR29a	patient	9.50	1.30	0.01
	control	3.47	0.70	

The table (4) summarizes the association between each of the analyzed parameters. Age of subjects correlated negatively with each of the following TC (r= -0.524, P= 0.001), TG (r= -0.461, P= 0.001), and positively with each of the following, HDL (r= 0.661, P=0.001), LDL (r=0.421, P=0.001), VLDL (r= 0.318, p= 0.001), Ca125 (r= 0.664, p= 0.001), Ca15 (r= 0.454, p=0.001), and MIR29a (r=0.250, p= 0.005). the total cholesterol levels correlate favorably only with TG (r= 0.736, p= 0.00), and adversely with the rest of parameters, HDL (r= -0.78, p= 0.001), LDL (r= -0.604, p=0.001), VLDL (r= -0.245, p= 0.009), Ca125 (r= -0.726, p= 0.001), Ca15 (r= -0.670, p= 0.001), MIR29a (r= -0.252, p= 0.007). HDL correlated positively with all of the measures tested, including LDL (r= 0.680, p=0.001), VLDL (r= 0.370, p=0.001), Ca125 (r= 0.924, p=0.001), Ca15 (r= 0.813, p=0.001), and MIR29a (r= 0.311, p=0.001). LDL exhibited a positive connection with all of the examined parameters, including Ca125 (r=0.578, p=0.001), Ca15 (r=0.813, p=0.001), and MIR29a (r=0.222, p=0.013). VLDL had a very significant positive relationship with Ca125 (r= 0.368, p0.001) and Ca15 (r= 0.183, p0.04). Ca125 may have a positive interaction with Ca15 and MIR29a. Ca15 had a favorable relationship with MIR29.

TABLE 4: the association between each of the analyzed biochemical markers and parameters

		TC	TG	HDL	LDL	VLDL	Ca125	Ca15	MIR
age	R	-0.524	-0.461	0.661	0.421	0.318	0.664	0.454	0.250
	P- Value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005
TC	R	1	0.736	-0.78	-0.604	-0.245	-0.726	-0.670	-0.252
	P- Value		0.00	0.00	0.00	0.009	0.000	0.000	0.007
TG	R		1	-0.740	-0.464	0.084	-0.694	-0.488	-0.182
	P- Value			0.000	0.000	0.376	0.000	0.000	0.052
HDL	R			1	0.680	0.370	0.924	0.692	0.311
	P- Value				0.000	0.000	0.000	0.000	0.000
LDL	R				1	0.123	0.578	0.813	0.222
	P- Value					0.172	0.000	0.000	0.013
VLDL	R					1	0.368	0.183	0.162
	P- Value						0.000	0.041	0.070
Ca125	R						1	0.547	0.280
	P- Value							0.000	0.002
Ca15	R							1	0.299
	P- Value								0.001
MIR	R								1
	P- Value								

Sensitivity (or TPR) and specificity (1 - FPR) illustrate the trade-off between the two. Figure (2) and summarized in table (5). The parameter that produce curves that are more disproportionally concentrated in the upper left region of the graph. The cancer marker Ca125 gave the best AUC equal to (1.00m P-value=0.001) in response to sensitivity, followed by HDL (AUC=1.00, p-value= 0.001) also to sensitivity. Then Ca15 (AUC= 0.94, P-value= 0.001) also to sensitivity. Followed by LDL (0.917, p-value= 0.001). Then MIR29a (AUC=0.803, p-value= 0.001).

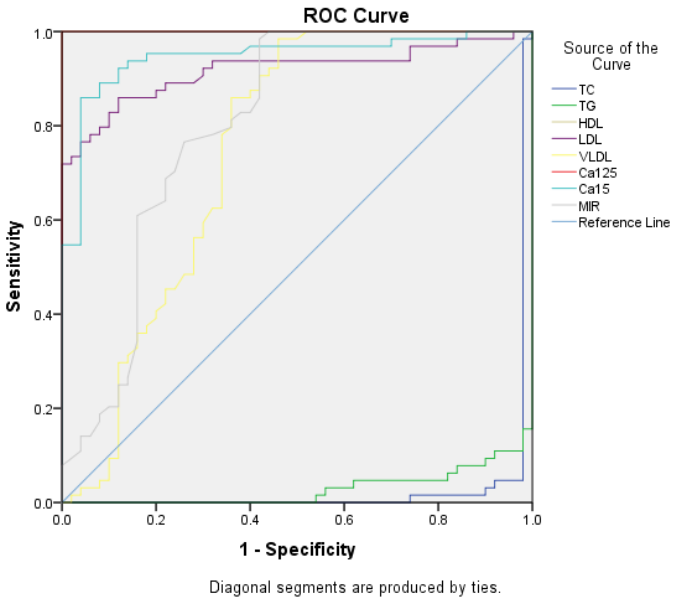

FIGURE 2: Sensitivity (or TPR) and specificity (1 - FPR) illustrate the trade-off between the two.

TABLE 5: the parameter that produce curves that are more disproportionally concentrated in the upper left region of the graph.

Test Result Variable(s)	Area	Std. Errora	Asymptotic Sig.b	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
TC	0.026	0.020	0.000	0.000	0.065
TG	0.029	0.013	0.000	0.003	0.055
HDL	1.000	0.000	0.000	1.000	1.000
LDL	0.917	0.028	0.000	0.863	0.971
VLDL	0.747	0.051	0.000	0.647	0.848
Ca125	1.000	0.000	0.000	1.000	1.000
Ca15	.946	0.022	0.000	0.903	0.989
MIR	0.803	0.045	0.000	0.714	0.891

DISCUSSION

Milk duct lining cells and lobule tissue from a breast are used to create a milk sample. contribute to incidence of breast cancer rising (Denton 2017). While breast cancer's precise cause is unknown, uncertain, estrogen, a female sex hormone, may encourage cellular proliferation that is carcinogenic in breast tissues and reproductive systems. Additionally, environmental and lifestyle elements, a lack of nursing or breastfeeding for a short amount of time, poor parity, irregular menstruation, a DNA that are changed at birth and run in families may increase the risk of developing breast cancer. are also factors. (de Sousa-e-Silva et al. 2014).

About 2.1 million people worldwide have cancer, accord with the 2018 GBCR. instances of breast cancer worldwide, accounting for 11.6% of all cancer-related deaths. (Bray et al. 2018). According to estimates, the lifetime risk of breast cancer for women who live to be 85 years old is 1 in 9. Due to inadequate care, a lack of knowledge about the disease, and a delay in diagnosis and treatment, A rising number of women in South and Central America, Africa, and Asia are being diagnosed with breast cancer, making it a serious public health issue.. (Rahimzadeh et al. 2014). By 2025, it's predicted that over 19.3 million women, the most of whom are from the Sahel region of Africa, one in four will acquire breast cancer in their lifetime. cancer diagnoses and one in five cancer deaths among women. (Ferlay et al. 2010).

Changes in blood lipid concentrations will impede the breakdown of very low-density lipoprotein cholesterol (VLDL-c), which is controlled by endogenous hormones, body weight, pregnancy, dietary fat intake, alcohol use, smoking, and physical inactivity. Tumor necrosis factor alpha (TNF-alpha) production will increase as a result of these changes, but adipose lipoprotein lipase activity will be inhibited. (2021, Sethi and Hotamisligil). Furthermore, developing cells, such as cancer cells, need more cholesterol and stimulate lipid production and metabolism, raising blood cholesterol levels in breast cancer patients. Furthermore, since cholesterol is carried in the blood by two kinds of lipoproteins, high levels of both low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) are protective against breast cancer. Breast tumors' levels are regarded to be prognostic markers. (Khader and colleagues, 2010). Increased levels of sex hormone-binding globulin, total cholesterol (TC), and triglycerides (TG) may increase the risk of breast cancer through increasing cell proliferation and fibrosarcoma. 2020 (Kumie, Melak, and Baynes).

In this investigation, the serum lipid profile of the patients was shown to be greater than that of the controls. This research's findings were consistent with those of the prior study. that mentioned Lipid and lipoprotein abnormalities are alarmingly more common when TG, low-density lipoprotein, and cholesterol levels are greater (LDL-c)but in another hand disagreed with the same study with the results of which showed in the previous study lower level HDL-c in women with breast cancer (Mariam, Technol, and 2016 2016). the creation of quantifiable changes in blood tumor markers as non-invasive methods for assessing the efficacy of cancer treatment in people (Liu et al. 2016b).

The most frequently used blood tumor markers in clinical practice The usefulness of cancer antigens 125, 15-3, and carcinoembryonic antigen (CA15-3) as breast cancer indicators is still up for debate. (2013) Falzarano et al. The first tumor antigen to be examined was CEA, a glycoprotein that is involved in cell adhesion. 2014; Wu et al. When endodermal tissues, such as the pancreas, breasts, and digestive system, are afflicted by inflammation or cancer, blood CEA levels may rise. Mukkamalla and Kankanala, 2022. Ovarian cancer biomarkers, including CA125, have been promoted for use in diagnostic blood tests and connected to the suffering caused by pancreatic cancer metastases, despite high levels being present in as many as 84% of people with breast cancer that has spread to other regions of the body. (2016) (Liu et al.). The breast cancer patient's treatment trajectory and likelihood of recurrence are often predicted using CA15-3. (2018) Mudduwa et al. Blood levels of CEA, CA125, and CA15-3, which may also be used as indicators of prognosis and to follow the evolution of the disease, have been shown to offer significant benefits for breast cancer patients. 2016; Zhao et al.

Using a microRNA microarray, we discovered that miR-29a was one of the most highly up-regulated miRNAs in BCSCs. The results of this study showed that MIR29a gene expression is elevated in breast cancer patients and also positively correlated with the other cancer markers ca125 and ca15-3 as well as the lipid profile of subjects. 2018; Yang et al. Additionally, it was in agreement with a prior research that found that the expression of MiR-29a in breast cancer cells was substantially higher than that of non-cancerous tissues. The same held true for MDA-MB-231 cells when compared to MCF-7 cells. Their findings suggested that miR-29a may support the tumorigenesis of breast cancer. 2019 (Wu et al.).

CONCLUSION

Our results demonstrated the higher levels of lipid profile, cancer markers and MIR29a accompanied with the breast cancer occurrence. In addition, MIR29a can be used as prognostic marker for breast cancer.

ACKNOWLEDGMENT

Despite the fact that they may not agree with all of the interpretations and findings in this study, I am grateful to my colleagues for their knowledge and insight, which substantially aided the research.

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