RESEARCH ARTICLE

Expression of Inflammatory MicroRNA-146a in Iraqi Women with Breast Cancer

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ABSTRACT

Background: Breast cancer (BC) is the second foremost cause of cancer-related death in Iraqi women. MiR-146a had been latterly considered a remarkable modulator of differentiation and function of innate cells and adaptive immunity. This study aimed to elucidate a possible role of miR-146a in breast cancer progression.

Methods Sixty BC Iraqi women blood samples were collected from Oncology Teaching Hospital, Baghdad/Iraq. In addition to 25 healthy volunteers' women. Serum cancer antigen 15-3 was assessed using VIDAS. The total RNA was extracted and converted to cDNA using the stem loop technique and quantitative real-time PCR (RT-PCR) with specific primers to estimate fold change expression.

Results: The assayed serum CA 15-3 levels significantly increased in early and advanced stages compared to controls. Investigation of the miR-146a folding expression after normalization with U6 snRNA revealed lower folding expression ($2^{-\Delta\Delta Ct}$) of mir-146a in BC patients than controls.

Conclusions; Downregulation of mir-146a in peripheral blood of BC patients and the dramatically increased serum CA 15-3 suggests its dual role in cancer and possibly as therapeutic targets.

Keywords: Breast cancer, CA 15-3, miRNA, mir-146a.

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INTRODUCTION

Breast cancer (BC) is a most recurrent virulence illness in global women in which the cells in breast tissue are uncontrolled modified and continuously divide, about seventy to eighty percent of patients with initial-stage which is a non-metastatic stage. Progressive BC organ-related metastases is considered. Generality, the BC initiates from the lobules or ducts that join the lobules to the nipple. BC signs may contain a breast lump, breast shape change, skin dimpling, fluid flow from the nipple, and a newly-inverted nipple or a red or scaly skin patch.[1] Several factors behind developing BC e.g. being female, lack of physical exercise, obesity, menopause hormone replacement therapy, alcoholism, ionizing radiation, delayed or non-motherhood, early menstruation, older age, family history, and having a prior history of BC.[2]

Breast cancer is the most commonly occurring virulence in women and the 2nd most common cancer overall and its incidence altitude after being forty. At the same time, the highest occurrence (nearly 80% of aggressive cases) occurs when the women cross the age of fifty.[3] In Iraqi BC, patients have become a leading threat to a female health condition, where it is the major cause of death after cardiovascular illness between women, with a cancer-related mortality rate of 23%.[4] Micro-RNAs monitor proteins are binding short ssRNAs, directly or indirectly affecting all sides of cell biology.[5,6] They had been associated with illnesses such as viral infections, cancer, and auto-immune diseases. In recent years, they stand out as significant immune response regulators,[7,8] which act as regulators of inflammation and modulate signaling of onset and termination of inflammation. Depending upon the target miRNAs may either promote or suppress inflammation.[9] The miR-146a is a small non-translated mini-RNA; in humans, it is encoded by the miR146A gene[10] and functions an active part in the inflammatory process in several disorders, including diabetes.[11,12] In addition, miR-146a was recorded to exert anti-inflammatory impact in the pathogenesis of different diabetic complexities such as diabetic nephropathy, cardiovascular disorders, neuropathy, retinopathy, even tend to be a high potential biomarker of inflammatory status in these diseases.[13,14] It had been implicated to play a vital role in

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inflammation, innate immunity, and cancer, as well as shown to regulate mitochondrial functions involving inflammation-aging of all types of cancer.[15] The miR-146a had been linked to various critical physiological operations like inflammatory responses and innate immunity. It had been prior revealed that upregulation of miR-146a regulated downstream toll-like receptor 4 (TLR4), IL-1 receptor-associated kinase 1 (IRAK1) signaling, and TNF-receptor associated factor-6 (TRAF6) by a negative-feedback regulation loop.[16] Previous studies determined that mir-146a over various signaling pathways such as STAT1, IFN-γ, TNF-α, TGF-β, TRAF-6, and GPCR were talented at influencing gene expression and eventually having a binary function in cancer progression then metastasis.[17] MiR-146a is earning significance as a modulator of differentiation and function of innate immunity and adaptive immunity cells, given its significance in regulating vital cellular processes. It is also known as a tumor suppressor; thus, it is not surprising that miR-146a expression was found dysregulated in many tumor types, and it can be used as a potential biomarker for BC identification since it is deregulated in several pathways leading to the progression of BC. It behaves as a tumor suppressor gene. Thus, by correctly targeting miR146a, the proper functioning of its target genes can be restored.[18-20]

MATERIALS AND METHODS

Subjects

Sixty Iraqi women with breast cancer were sub-grouped into 26 stages I and II, and 34 with stages III and IV. The samples were collected from the Oncology Teaching Hospital, Baghdad, Iraq, their age was ranged from (35–70) years. Moreover, 25 age-matched healthy women and their age ranged was 27–68 years were enrolled in this study. Ten mL of venous blood was collected and distributed into 5 mL EDTA tubes for RNA and gene expression isolation, whereas the remaining about 5 mL was divided into gel-tube for the segregation of serum. Then serum was gathered by centrifugation (3000 rpm for 10 minutes). Then the serum was freezing at -20°C till estimation for cancer antigen experiments.

Estimation of Serum Antigens Markers

Serum cancer antigen 15-3 was estimated using VIDAS® Mybiomerieux, France. Immuno-assay

Isolation of Total RNA

A ready-to-use reagent TRIzol® RNA isolation reagents and Directzol™ RNA-Mini-Prep, ZYMO-RESEARCH, USA was practical for total RNA isolation from peripheral blood samples. The RNA was eluted in 25 µL of nuclease-free water.

Quantitation of miRNA Concentration and Purity

MiRNA concentration and purity were done randomly for five samples only Calibrating the Quantus™ Fluorometer for use with the QuantiFluor® Dye System Follow this protocol to calibrate the Quantus™ Fluorometer before quantitation using the PromegaQuantiFluor® Dye Systems.

Conversion of RNA to cDNA

Prime Script TM RT-reagent kit is intended to perform the reverse-transcription adjusted for RT-qPCR The Stem-loop primer for has-miR-146a was 5′ GTCGTATCCAGTG-CAGGGTCCGAGGTATTCGCACTGGAT ACGACAACCCA 3′, and the forward primer 5′ CGCGCGTGAGTTCTGAAT3′. The U6 gene was used as a reference gene, the reverse transcription primer was 5′C GAAGCCGTCGTGTATAT-GATTTTA 3′, and amplification primer was 5′ GCTTCG-GCAGCACATATACTAAAAT 3′. The study used a universal reverse primer in reverse transcription, complementary to the stem loop primer 5′CGCTTCACGAAT TTGCGTGTCAT 3′. The primers were adopted using http://www.mirbase.org, 2 µL 20X prime script reaction buffer, 3 µL nuclease-free water, and 5 µL (100 ng/µL) of the total RNA up to obtain 10 µL as final reaction concentration. The reaction was running by using thermal-cycler (SaCycler-96, Sacase, Italy) at 42°C for 15 minutes, tracked by 1 minutes for heat deactivation at 85°C, then stored at 4°C for qPCR.

Performing RT-PCR

The KAPA-SYBR-FAST-qPCR master mix (2X) is intended for maximum real-time PCR performance. Twenty µL PCR reaction mixtures were prepared using KAPA SYBR® fast universal PCR master-mix (KAPA, USA) as suggested by the producer. Fleetingly, 10 µL of 2X SYBR green master-mix, 5 µL of cDNA, 0.5 µL of each reverse and forward primer mix were addition and 4 µL of nucleases free water was added to complete the reaction mix up to 20 µL. Then, as endogenous control, we used U6 gene as a reference gene. The quantitative RT-PCR was performed at 95°C for 7 minutes, tracked by 45 series cycles of 95°C for 10 seconds and 60°C for 1 minute. Lastly, melting-curve analysis was approved constructed on the separation features of ds DNA through the cycles with intensifying denaturing temperature.

Statistical Analysis

Statistics of obtained data were investigated using SPSS program version 26 and Graph-Pad prism 6, using modest statistical parameters (mean and standard error). The results were clarified. The differences between means were intended by independent varieties T-test. A possibility that equalizes or minimal than 0.05 was considered significant. The fold change was calculated using $2^{-\Delta\Delta CT}$ method after normalizing with u6 gene in which: ΔCT= CT of Target gene-CT of reference gene (U6). Then the ΔΔCT = ΔCT of each sample - average ΔCT for controls. The fold change = $2^{-\Delta\Delta Ct}$. The control value was advised as 1, the samples, minimum than 1 were suggested as down-regaled and maximum than 1 were proposed as upregulated.

RESULTS

Immunoassay of Serum CA15-3

The serum level CA15-3displayed a significant increase in both early (77.97 ± 2.44) and advanced stages (96.33 ± 3.66) than

Table 1: Folding expression of Mi-r-146a in breast cancer women

miR-146a	*mean of ΔCt ± SE controls*	*mean of ΔCt ± SE patients*	*ΔΔCt*	*P- value*	*Folding*
Early stages	1.19 ± 0.08	2.437 ± 0.07	1.244	0.001	0.422
Advanced stages	1.19 ± 0.08	2.889 ± 0.06	1.695	0.001	0.309

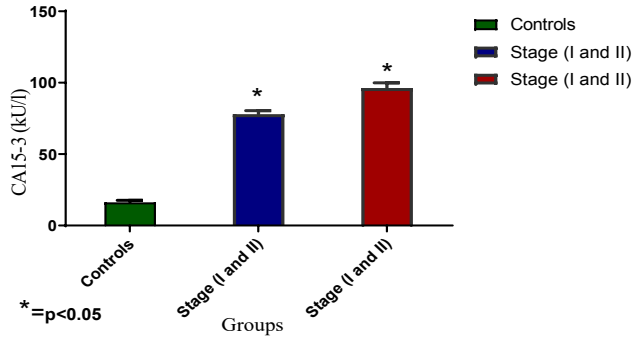

Figure 1: Serum level of CA15-3 in healthy controls and BC patients expression of miR-146a

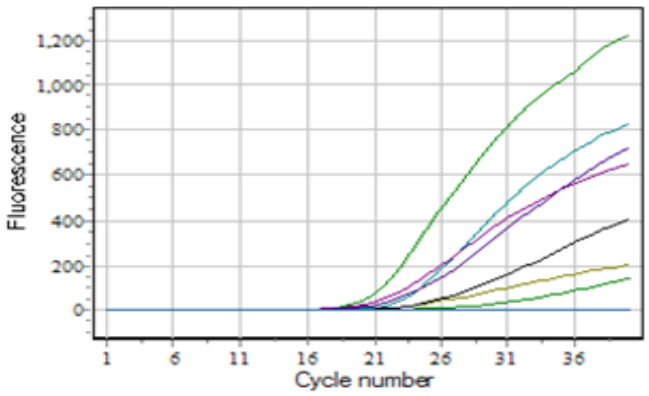

Figure 2: Real time PCR gene expression results of associated miRNA as given threshold (CT).

the control group (16.3 ± 1.35) kU/L, respectively. There were no significant differences between cancer stages ($p < 0.05$); Figure (1 and 2).

The expression of target miRNA (miR-146a) gene was examined by using real-time PCR after reverse transcription of RNA to cDNA via specific stem-loop primer that extends the target miRNA and the resulted curves represented in CT threshold of target and reference gene as shown in (Figure 2) then by folding analysis using Livak method (Livak and Schmittgen, 2001) (Table 1).

Micro RNA-146a

The results showed that miR-146a was significantly down-regulated in BC patients in early (I-II) and advanced stages (III-IV) p=0.001. The fold change in early-stage (stage I and II) was 0.422 compared with 1, and in the advanced stages (III-IV) was 0.309 compared with 1.

DISCUSSION

A growing gathering of scientific evidence had been reported miR-146 as the unity of the high probable epigenetic controllers, disturbing cellular and metabolic pathways resulting in fundamental inflammatory stimulations in different cell types.[21] The expression of mir-146a was significantly down-regulated in BC women that play a suppressor part in cancer cells and decreased its expression level by stimulating the nuclear factor kapa-B (NF-κB) signaling pathway income a part in the development and cancer metastasis. Though, in numerous metastatic cancers comparable to metastatic brain cancer, colorectal cancer, and lymphoma, the expression level of microRNAs is raised, denoting its double function in cancer cells.[22] Investigates carried out on hepato-cellular metastatic carcinoma exposed that the mir146a gene expression in this cell has failed compared to normal cells due to mir146a promoters being highly methylated, and its rises will decrease the level of invasion. This promoter decreases VEGF expression by raising the expression level of APC and reducing the level of NF-κB p65. These researches presented that the mir146a performance as a tumor suppressor in hepatocellular cancer invasiveness and talented reduces cancer cells relocation, metastasis, and invasion.[23] Subsequently, additional studies exposed that mir146a takes a portion in cancer cells' metastasis and invasion by disturbing the cmet. In the colorectal cancer cells, the expression of mir146a deteriorates, and the expression level of cmet is elevated, similar to the targets involved in the colorectal cancer cells invasion and metastasis.[24]

A study on hepatocellular carcinoma cells confirmed that the expression of miR146a is low. The miR146a can inhibit the cell cycle and elevation by apoptosis in the peripheral cells. Consequently, raising in the expression levels of miR146a in the cancer cell lines could be a magnificent strategy in the therapy of this sympathetic cancer.[25,26] Studies verified the variation of miRNAs expression profile in human BC and resolute that there were 29 types of miRNAs with an expression condition, in which the expression of miRNA-21 and miRNA155 was significantly upregulated. In contrast, the expression of miRNA-10b, miRNA-125b, and miRNA-145a were significantly reduced. In previous work, it was determined that the expression of miR146a5p in BC tissue was significantly higher compared with paraneoplastic tissue.[27] In contract another study found that the miR-146a-5p expression in breast cancer cell line MCF7 was also significantly higher, compared to control cells, and BRCA1 was moreover identified as a miR-146a-5p target gene by bioinformatics analysis and the reporter of fluorescent gene detection and may regulate the MCF-7 proliferation by BRCA1.[28]

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