Article

The expression miRNA-195 in a sample of Iraqi breast cancer patients.

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Abstract

Breast carcinoma is the most prevalent cancer-related cause of death in women, and metastasis is the main factor in morbidity. The total number of new cases of cancer in Iraq during the year 2019 was 35,864. New non-invasive prognostic biomarkers are needed for the rapid recognition and differentiation between breast cancer (BC) stages for treatment choice improvement. MicroRNA (miRNA) are small, noncoding RNAs regulating gene expression and involve many cellular processes, including metastasis. Circulating miRNAs (detected in the blood) show considerable potential as biomarkers for helping diagnosis or tracking treatment efficacy. Materials and Methods: total RNA was extracted from serum from (n=50) patients and (n=26) healthy control to measure the MicroRNA 195 expression using SYBR green-based real-time RT-PCR technology. As a result, the expression levels miR-195 in breast cancer patients' serum were significantly increased (up-regulated) compared to those in the normal adjacent serum. BC group showed a higher significant miR-195 expression (upregulation) when compared with those in the control group. While the highest expression of miR-195 was recorded in stage II.

Keywords: Breast Cancer, MicroRNA, miR-195, Stage, Iraq

Introduction

Cancer is the primary 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 cancer cases in 2019 was 35,864, with an incidence of 91.66/100,000 P, While the rate recorded in 2010 was 18,482, with an incidence of 56.89/100,000 P [2]. Cancer can 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) [3]. Breast cancer (BC) is one of the most common malignant tumors and the second leading cause of cancer in women. Approximately 1.5 million new cases are diagnosed with breast cancer annually, and almost 460,000 patients die each year due to BC chemoresistance and metastasis[4]. Approximately 7,109 new cases are diagnosed with breast cancer in 2019 [2]. Breast Cancer biological characteristics are routinely used for early detection, prognosis, and therapeutic strategy selection, including histologic subtype, grade, lymph node status, hormone receptor, and human epidermal growth factor receptor 2 (HER2) statuses [5]. Some

of the mentioned characteristics are related to patients' survival and posttreatment clinical outcomes [6]. However, several BC patients with similar characteristics showed different clinical outcomes. Therefore, biological features have limitations concerning diagnosis, prognosis, and clinical outcomes' prediction [7]. Thus, there is still a need to develop a cost-effective and accurate screening method for this cancer and discover new biomarkers to improve diagnosis, prognosis and prediction [8]. Novel diagnostic and prognostic approaches are urgently required to identify new personalized therapeutic methods that improve BC patients' quality of life.

High throughput studies highlighted the role of different sets of miRNAs in controlling gene expression during and after initiation stages of breast cancer as well as metastasizing stage [9]. However, a comprehensive overview and classification of the most essential miRNAs at different stages of this type of cancer remains to be elucidated [10].

MiRNAs (microRNAs) are small, endogenous, and noncoding RNA constructs of about 22 nucleotides. Cumulative evidence from biological experiments shows that miRNAs play a fundamental and essential role in various biological processes, such as regulation of gene expression by post-transcriptionally binding to 5'untranslated regions (UTR), coding sequences, or 3'UTR of target messenger RNAs (mRNAs) [11]. According to the latest release of an online miRNA database, miRBase (v22), there are 38,589 entries representing hairpin precursor miRNAs that express 48,860 mature miRNAs from 271 organisms such as humans, mice, rats, etc. As a sub-category of the organism classification, the human genome contains 1917 annotated hairpin precursors and 2654 mature sequences [12]. In mammals, approximately one-third of all protein-coding genes' activities are estimated to be controlled by miRNAs [13]. The discovery of the first miRNA started in Caenorhabditis elegans in 1993 by Ambros and Ruvkun's studies. They found that the lin-4 was a small noncoding RNA but not a protein-coding RNA [14-16].

MicroRNA and Breast Cancer: miRNAs participate in the regulation of essential biological processes and several diseases. There is a long history of regulatory relationships between miRNAs and the hallmark of cancer, especially breast cancer. As early as 2005, Lu et al. reported the differential expression of miRNAs in breast cancer [17]. Subsequently, many studies have suggested that miRNAs are closely associated with BC occurrence and development. miRNAs were reported to play two important roles as oncogenes (oncomiRNAs) and tumor suppressors.

The miR-195 with miR-497 is a highly conserved miRNA cluster located at Chromosome 17p13.1[18]. miR-195 was first predicted based on homology to a verified miRNA from the mouse and was later shown to exist in humans. Some studies have demonstrated that miR-195 expression is decreased relative to nonmalignant tissue in many solid tumors, including bladder, gastric, colorectal, and hepatocellular carcinoma. However, miR-195 expression has been reported to be increased in adrenocortical adenomas and breast cancer. Therefore, miR-195 may display either pro-proliferative or proapoptotic roles under specific physiological conditions and in different types of cancers [19]. In addition, forced expression of miR-195 had a similar effect to suppress breast cancer cell proliferation, blocked cell cycle G1 progression, and induced apoptosis. Such result suggested that miR-195 have similar effects to play a tumor suppressor role in breast cancer by the same cluster of gene regulation [20,21].

Materials and Methods
Fifty Iraqi Women patients with breast cancer who attended Al-Andalus Specialist Hospital and Oncology Teaching Hospital during the period extended from the first of December 2021 to the end of February 2022 with ages ranging

from 27 – to 72 years were enrolled in this study. The patients were compared with Twenty-six healthy volunteers aged 21 – 67 years. Peripheral blood was collected from the patients, and the healthy group served as controls, which was placed directly in Trizol preservation for RNA extraction and kept at -20 C° until used for study as described.

RNA extraction
RNA was isolated from serum samples according to the protocol of TRIzol™ Reagent. 0.2 mL of chloroform was added to each tube. All mixes were Incubated for 2–3 minutes, then centrifuged for 10 minutes. The mixture was separated into three phases. The aqueous phase containing the RNA was transferred to a new tube. 0.5mL of isopropanol was added to the aqueous phase, incubated for 10 minutes, then centrifuged for 10 minutes; Total RNA was precipitated. For each tube, 0.5mL of 70% ethanol was added, vortexed briefly, and then centrifuged for 5 minutes; Ethanol then aspirated and air-dried the pellet. The pellet was rehydrated in 50μl of Nuclease Free Water.

Detection of miRNA by RT-qPCR
Total RNA containing miRNA was the starting material in the RT-PCR reaction, performed in two steps. The miRNA gene miRNA195 and RNU expression were done using specific primers, as shown in (Table 1).

miR-195-5p-RT	5`-                            GTTGGCTCTGGTG-CAGGGTCCGAGGTATTCGCAC-CAGAGCCAACGCCAAT-3`	55
miR-195-5p-F2	5`-GGTTTTTTTGTAGCAGCACAGAAAT-3`	
RNU43_RT	5`-GTTGGCTCTGGTG-CAGGGTCCGAGGTATTCGCAC-CAGAGCCAACAATCAG-3`	
RNU43_F	5`-GTGAACTTATTGACGGGCG-3`	
Universal Reverse	5`-GTGCAGGGTCCGAGGT-3`	

Table 1. Primer Sequence for miRNA Gene Expression.

The first step was to test the expression of PCR target genes; the first method was reverse transcription using the GoScript Reverse Transcription System. The 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 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. In the first reaction, the total RNA and miRNA primers were added to PCR tube microfuge 0.2ml (4 - 1) μl, respectively, as in (Table 2).

	1 Sample
RNA	4
RT primer	1
Total volume	5

Table 2. The First reaction.

In the 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.

Results

MiR-195 expression in human breast cancer serum

Fifty breast cancer cases and 25 healthy controls were included in the analysis. Initially, we examined the expression levels miR-195 in breast cancer serum were studied using RT-qPCR. The expression levels of miR-195 in breast cancer serum were significantly increased compared to those in the normal adjacent serum (Figure 1). While some studies indicate that there was a decrease in miR-195 expression (downregulation) when compared to control group [24]. The expression levels miR-195 in stage II are significantly higher in this study than in other stages and healthy controls (Figure 2).

Expression of MiR-195	Mean	1.079	5.010	< 0.0056

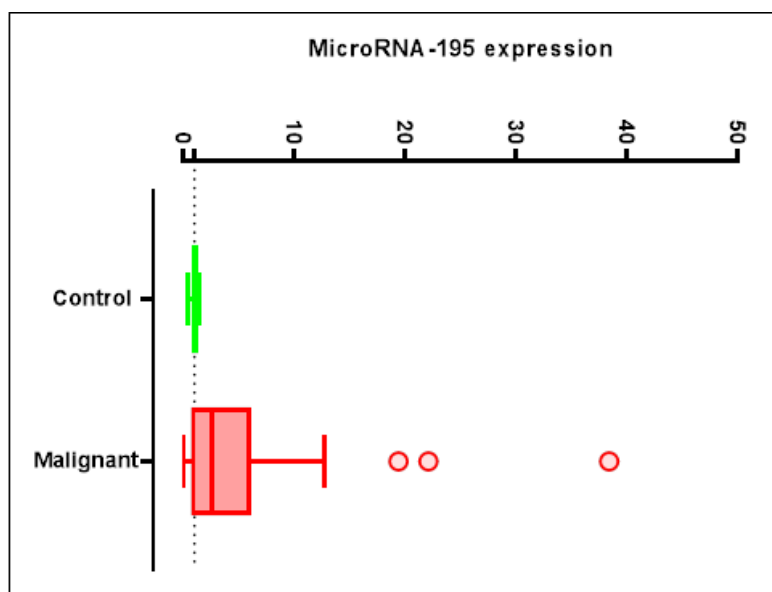**Table 3. Comparison between serum miR-195 expression between control and breast cancer groups.**

Figure-1. miR-195 expression between control and breast cancer patients.

Control	26	1.079 [a]
I	8	3.273 [ab]
II	17	7.236 [b]
III	12	3.943[ab]
IV	11	3.995 [ab]
P-Value	---	0.0196 [*]
This means that having the different letters in the same column differed significantly. * (P≤0.05).		

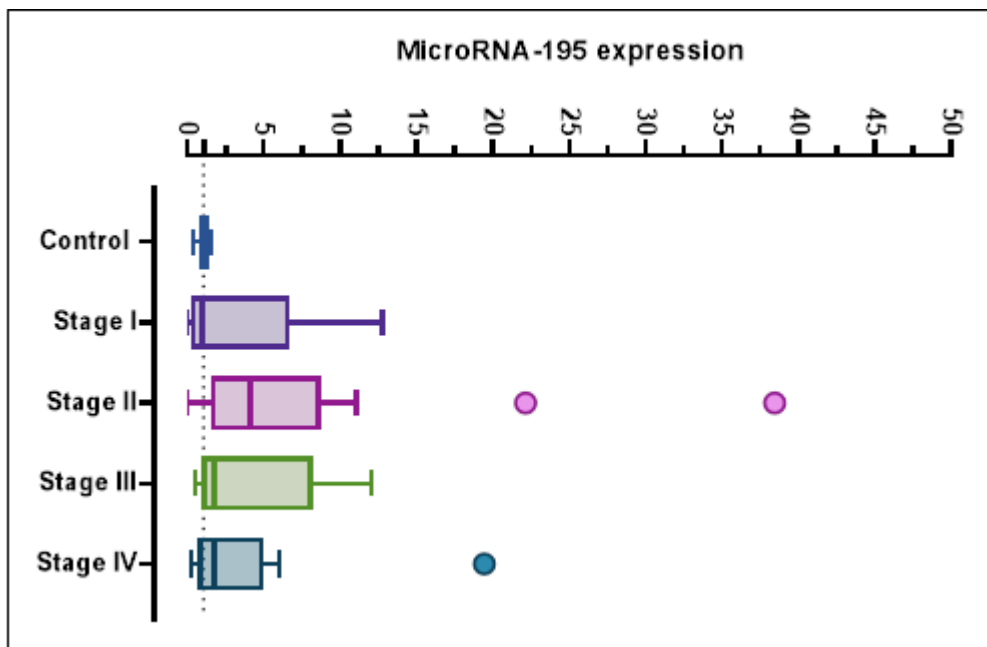**Table 4. Comparison between serum miR-195 expression between control and breast cancer stages.**

Figure-2. miR-195 expression between control and breast cancer stages.

Discussion

BC group showed significantly higher miR-195 expression (upregulation) when compared to the control group (p < 0.0056) (Table 3). In a recent study, the miR-195 expression was significantly higher than healthy controls that showed regular expression [22,23].

Conclusion

Our observations from this study found that miR-195 levels increase in female serum with breast cancer. The results show that patients with breast cancer have a higher expression of miR-195 than the control group. However, this study identified and validated circulating miR-195 with differential expression able to separate stage II from other breast cancer stages. Our results demonstrate that patients with stage II breast cancer have a higher expression of miR-195 than at other stages in their circulation.

Reference

1. SHKUR AZEEZ, S. KNOWLEDGE, ATTITUDE, AND PRACTICE TOWARDS BREAST CANCER, RISK FACTORS, AND SCREENING AMONG IRAQI WOMEN, **2021**.
2. Iraqi, A. R. in the Republic of Iraq Ministry of Health and Environment Iraqi Cancer Board 39 - 70 **2019**.
3. Mohammed Ghanim, M. A Molecular Study of Loss of Heterozygosity in Tissue Samples Isolated from Breast Cancer Patients in Relation with Their Sex Hormones Status, College of Medicine, Babylon University, **2009**.
4. Bray, F. et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians 68, 394-424 **2018**.
5. Yang, Z. & Liu, Z. The emerging role of microRNAs in breast cancer. *Journal of Oncology* **2020**.
6. Singletary, S. E. et al. Revision of the American Joint Committee on Cancer staging system for breast cancer. Journal of Clinical Oncology 20, 3628-3636 **2002**.
7. Schnitt, S. J. Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. Modern pathology 23, S60-S64 **2010**.
8. Bartels, C. L. & Tsongalis, G. J. MicroRNAs: novel biomarkers for human cancer. Clinical chemistry 55, 623-631 **2009**.
9. Lafta, H. Y., Allami, R. H. & Hussein, S. M. Evaluation the Role of Micro-RNAs During Stages of Breast Cancer: A Review. Medico-legal Update 21 **2021**.
10. Sohel, M. M. H. Circulating microRNAs as biomarkers in cancer diagnosis. Life sciences 248, 117473 **2020**.
11. Tasdelen, A. & Sen, B. A hybrid CNN-LSTM model for pre-miRNA classification. Scientific reports 11, 1-9 **2021**.
12. Kozomara, A., Birgaoanu, M. & Griffiths-Jones, S. miRBase: from microRNA sequences to function. Nucleic acids research 47, D155-D162 **2019**.
13. Saliminejad, K., Khorram Khorshid, H. R., Soleymani Fard, S. & Ghaffari, S. H. An overview of microRNAs: biology, functions, therapeutics, and analysis methods. Journal of cellular physiology 234, 5451-5465 **2019**.
14. Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. cell 75, 843-854 **1993**.
15. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75, 855-862 **1993**.
16. Lee, R., Feinbaum, R. & Ambros, V. A short history of a short RNA. Cell 116, S89-92 **2004**.
17. Lu, J. et al. MicroRNA expression profiles classify human cancers. nature 435, 834-838 **2005**.
18. Poel, D. et al. Proteomic analysis of miR-195 and miR-497 replacement reveals potential candidates that increase sensitivity to oxaliplatin in MSI/P53wt colorectal cancer cells. Cells 8, 1111 **2019**.
19. Luo, Q. et al. MicroRNA-195-5p is a potential diagnostic and therapeutic target for breast cancer. Oncology reports 31, 1096-1102 **2014**.
20. Gollob, J. A., Wilhelm, S., Carter, C. & Kelley, S. L. in Seminars in oncology. 392-406 (Elsevier).
21. Chen, Q. et al. CircWHSC1 promotes breast cancer progression by regulating the FASN/AMPK/mTOR axis through sponging miR-195-5p. Frontiers in Oncology 11 **2021**.
22. Peña-Cano, M. I. et al. Deregulated microRNAs and adiponectin in postmenopausal women with breast cancer. Gynecologic and Obstetric Investigation 84, 369-377 **2019**.
23. Jawdat Noori Gaaib, A.-F., Abdul Hussein M. Molecular analysis of CK19 , MGB1 ,MUC-1, microRNA-195 and microRNA-let 7a expression in breast cancer PHD thesis, University of Baghdad, **2014**.
24. El-Shafae, M., Behiry, E. G., Abd El-latif, M. E., Ahmed, E. S. & El-Fallah, A. A. Clinical value of serum microRNA-195 expression in invasive ductal carcinoma of the breast. Gene Reports 19, 100635 **2020**.