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Research Article

Role of miRNA 199a-5p Expression in Iraqi Women with Breast Cancer

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Abstract

Background: Elevated levels of microRNAs have been linked to breast cancer and other types of cancer. It has been discovered that miRNA-199a-5p overexpression contributes to the epithelial-mesenchymal transition (EMT) in breast cancer. ***Objective***: To examine the expression of microRNA 199a-5p in tissues from both benign and malignant breast cancers. ***Methods***: We use 50 FFPE tissue samples divided into two groups based on their histology and cytological characteristics. The first group comprised 25 samples classified as benign, while the second group comprised 25 samples classified as malignant. To calculate the expression levels of miRNA-199a-5p, we use a real-time RT-PCR method based on SYBR green. ***Results***: It was found that the folding value of miRNA-199a-5p in malignant tumors was 2.908 compared to benign tumors (2.027). However, the statistical significance of these differences was not significant. ***Conclusion***: Depending on the specific context and molecular targets, miRNA-199a-5p plays a dual role in breast cancer development, acting as both an oncogene and a tumor suppressor.

Keywords: Breast cancer, Epithelial-mesenchymal transition, Gene expression, miRNA-199a-5p, N-cadherin, RT-PCR.

دور تعبير miRNA-a-5p199 في النساء العراقيات المصابات بسرطان الثدي

الخلاصة

الخلفية: النوع الأكثر شيوعًا من السرطان بين النساء في جميع أنحاء العالم هو سرطان الثدي. تم ربط مستويات مرتفعة من miRNA 199a-5p بسرطان الثدي وأنواع أخرى من السرطان وتسهم في التحول الظهاري الميزاني (EMT) في سرطان الثدي. **الهدف**: دراسة تعبير miRNA-199a-5p في الأنسجة الخاصة بسرطانات الثدي الحميدة والخبيثة للتحقق من ذلك بشكل أوسع. **الطريقة**: لحساب مستويات التعبير لـ miRNA 199a-5p ، استخدمت طريقة RT-PCR في الوقت الحقيقي استنادًا إلى اللون الأخضر SYBR . **النتائج**: في دراسة باستخدام RT-PCR لتحليل تعبير الجينات، تبين أن قيمة التعبير لـ miRNA 199a_5p كانت في الأورام الخبيثة 2.908 مقارنة بالأورام الحميدة (2.027). ومع ذلك، من المهم ملاحظة أن الأهمية الإحصائية لهذه الاختلافات لم تكن ذات قيمة معنوية. **الاستنتاج**: يعتمد دور miRNA-199a-5p كعامل سرطاني ومثبط للورم في تطور سرطان الثدي على السياق والأهداف الجزيئية المحددة.

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INTRODUCTION

Genetic predispositions and environmental triggers are just two of the many factors that affect the complex process of carcinogenesis. Tragically, the number of cancer-related deaths has been alarmingly increasing over time, cementing its position as a significant contributor to worldwide mortality rates [1]. Breast cancer is a complicated disease that has various molecular subtypes and classifications. According to the International Agency for Research on Cancer's (IARC) 2020 World Cancer Report, breast cancer is the most prevalent cancer among women worldwide. Unfortunately, it is also the leading cause of cancer-related deaths in females [2]. Most malignancies in the ducts and lobules of the

mammary glands are ductal cancers. Biomarkers are essential for providing insightful data for cancer diagnosis and prognosis [3,4]. Breast cancer is the major cause of cancer-related female mortality in Iraq and ranks among the population [5]. Eliminating breast cancer is a difficult task due to the dangerous side effects of conventional therapies like radiation and chemotherapy [6]. The CT scan is an essential step in detecting breast cancer. It involves using a specialized device to examine the breast for any tumors. A biopsy is then taken from any suspicious areas and reviewed to determine if it is benign or malignant. This process helps determine the severity of the condition [7]. In a recent study in Iraq, a total of 72,022 female cases of breast cancer were found [8]. Furthermore, according to the American Cancer Association, 268,600 women were given new breast cancer diagnoses in 2019 alone, and regrettably, 41,760 of them lost their battle with the illness [9]. The process by which cancer cells go through genetic reprogramming and transform from a non-motile, epithelial phenotype to a migratory, mesenchymal-like phenotype is known as epithelial-to-mesenchymal transition (EMT), which has been observed in many epithelial malignancies. The promotion of metastasis in many types of cancers depends heavily on this transition [10]. The downregulation of epithelial cadherin (E-cadherin) expression, together with the concomitant overexpression or de novo expression of neural cadherin (N-cadherin), was a common feature of the epithelial-to-mesenchymal transition (EMT). This process, also known as the "cadherin switch," has been connected to increased cell migration and invasion during EMT [11]. The notable effects of down-regulating E-cadherin were the loss of stable epithelial cell-cell adhesive junctions, apicobasal cell polarity, and overall epithelial tissue structure. This makes it easier for cancer cells to break free from the original tumor site, which increases their capacity for movement and metastasis [12]. E-cadherin, a Ca^{+2}-dependent transmembrane glycoprotein, mediates cell-to-cell adhesion and is encoded by the *CDH1* gene found on chromosome 16q22.1. E-cadherin is essential for controlling morphogenesis. Cancers with poor differentiation and advanced stages have been linked to decreased CDH1 expression [13]. Small non-coding RNAs called microRNAs (miRNAs) control post-transcriptional gene regulation [14]. Recent studies have shown that microRNAs are essential epithelial-to-mesenchymal transition (EMT) regulators, acting by carefully regulating several oncogenic signaling pathways. A growing body of research points to the possibility that microRNAs can influence the development of breast cancer by acting as both inducers and suppressors of transcription factors implicated in EMT. There are already 130 microRNAs known to impact the EMT process directly [15]. MiRNA-199a-5p expression was found to be significantly reduced in cancerous tissues compared to non-cancerous tissues [16]. The role of the miRNA-199 family has been extensively investigated in numerous cancer types. Recent research has focused on two specific members of this family: miRNA-199a and miRNA-199b [17]. Pre-miRNAs in hsa-miR-199a come in two varieties: pre-miR-199a-1 (MI000242) and pre-miRNA-199a-2 (MI0000281). These pre-miRNAs are derived from chromosomes 19 and 1, respectively, and were later renamed miRNA-199a-5p (MIMAT0000231) and miRNA-199a-3p (MIMAT0000232) [18]. Additionally, hsa-miRNA-199b (derived from chromosome 9) has two mature isoforms: miRNA-199b-5p (MIMAT0000263) and miRNA-199b-3p (MIMAT0004563). Different cancer cells use miRNA-199a for various purposes. Other research revealed that miR-199a-5p could decrease the levels of β1 integrin by targeting the 30-UTR of Ets-1 to alleviate the invasion of breast cancer via the FAK/Src/Akt/mTOR signaling pathway [19]. An earlier investigation revealed that miRNA-199a-5p prevents cancer cells from proliferating, migrating, and invading [20]. The microRNA miRNA-199a-5p promotes signaling pathways linked to EMT [21]. The expression levels of miRNA-199-a/b 5p, FAM83B, E-cadherin, N-cadherin, Snail, SMA, vimentin, and Twist were analyzed in various cancer cell lines [22].

METHODS

Online miRNA target prediction techniques described below were employed in this study to assess putative miRNA-199a-5p target genes: Mir-TV Database (https://mirtv.ibms.sinica.edu.tw/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), and CoMeta Database (https://cometa.tigem.it/).

Samples collection

The study included fifty participants with breast tumors of various ages who were recruited from the Teaching Oncology Hospital in Baghdad governorate between March and May 2022. Tissue samples were collected from different classifications and stages of breast tumors using microtome equipment to obtain thin sections. Specimens can be categorized according to their histopathology, which involves the examination of a biopsy or surgical sample by a pathologist. This examination occurs after the specimen has undergone processing and histological sections and has been mounted on glass slides for observation. Pathological grading systems are employed to assess the microscopic appearance of cells, including abnormalities and deviations in their growth rate. The primary objective is to predict developments at the tissue level based on these observations. In this study, the formalin-fixed paraffin-embedded tissue (FFPET) samples were divided into two groups based on their histology and cytological characteristics. The first group comprised 25 samples classified as benign, while the second group comprised 25 samples classified as malignant. Excess paraffin from the sample blocks (Formalin-Fixed Paraffin-Embedded) was carefully trimmed, and the resulting tissue sections were stored in

Eppendorf tubes. After that, 5–20 portions were taken out of each Eppendorf tube for further processing. Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections were deparaffinized using a solution that included different reagents such as heptane, xylene, limonene, or CitriSolv.

miRNA quantitation

The miRNAs were quantified using the Qubit 4.0 fluorimeter platform, which exhibits outstanding selectivity for miRNA in comparison to other RNA types. For assessing initial sample concentrations ranging from 10 pg/L to 100 ng/L, the assay provides high accuracy. The assay, when carried out at room temperature, may sustain steady signals for up to three hours. Furthermore, the performance of the Qubit 4.0 test can withstand typical impurities such as salts, free nucleotides, solvents, detergents, or proteins without being adversely affected.

Quantitative PCR (qPCR)

High-capacity cDNA Kit (ProtoScript® II First Strand cDNA Synthesis Kit, NEW ENGLAND BIOLABS) was used to reverse transcribe the RNA.

RNA reverse transcription

We used the reverse transcription technique with the ProtoScript® II First Strand cDNA Synthesis Kit to evaluate the expression of PCR target genes. Using oligo-dT primers, the ubiquitous tag sequence on the 5' end of these oligo-dT primers allowed for the subsequent amplification of mature miRNA during the real-time PCR step. With the exception of the template RNA. It was then carefully combined and added to PCR tubes. The template RNA was put into each tube, and then, following quick centrifugation, the lines were put into a thermal cycler. The reverse transcriptase enzyme was inactivated by incubating for 5 minutes at 95°C after 60 minutes at 37°C.

Primer preparation

The amplification of miRNA genes was carried out using specialized primers, as listed in Table 1. MacroGen® provided these primers in a lyophilized form. To create a stock RNA solution, the lyophilized primers were dissolved in distilled water, resulting in a final concentration of 100 pmol. Subsequently, a working solution of the primers at 10 pmol/µl was prepared by combining 10 µl of the primer stock solution with 90 µl of deionized distilled water.

Table 1: PCR primers

Primer name		Primer Sequence ('5----3')	Reference
miRNA-199a-5p	RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAACAGGT	[23]
	Forward Primer	ACACTCCAGCTGGGCCCAGTGTTCAGACTAC	
	Reverse primer	CTCAACTGGTGTCGTGGAGTCGGCAA	
U6 Housekeeping gene (Reference gene)	Forward Primer	CTCGCTTCGGCAGCACA	[23]
	reverse primer	AACGCTTCACGAATTTGCGT	

Detection of miRNA by RT-qPCR

SYBR-Green reagents were used for the RT-qPCR measurement of mRNA levels. Following 40 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds, the amplification conditions were established as follows: The second step of the methodology involved choosing and processing cDNA samples from malignant cells simultaneously. Every sample was run through two different PCR tubes, one for miRNA-199a-5P and the other for U6 snRNA, the study's housekeeping gene. Specific ingredients were carefully combined to create the reaction mix components and their respective quantities, as outlined in Table 2.

Table 2: Reaction components of miRNA expression

Component	20 ul Reaction
Luna Universal qPCR Master Mix	10 ul
Forward primer (10 µM)	1ul
Reverse primer (10 µM)	1ul
Template cDNA	5ul
Nuclease-free Water	3ul

The PCR tubes were subjected to a rapid centrifugation step lasting 1 minute at 2000g to eliminate bubbles and gather the liquid. The real-time PCR program was configured according to the specified thermocycling protocol, as shown in Table 3.

Table 3: Real-time-PCR program for amplification of miRNA 199A-5P and U6 snRNA

Cycle Step	Temp (°C)	Time (Sec)	Cycles
Initial Denaturation	95	60	1
Denaturation	95	15	40-45
Extension	60	30 (+plate read)	
Melt Curve	60-95	2400	1

Statistical analysis

A Statistical Analysis System [24] program was used to detect the effect of groups (Benign and Malignant) on Gene expression of miRNA-199a-5p fold change. Student's *t*-test was used to compare between means. A *p*-value less than 0.05 was considered for statistical significance.

RESULTS

Multiple web servers were used to browse, search, and query miRNA and target gene data. This made it possible to use expression data from hundreds of tissues and cell conditions to figure out miRNA targets and gene networks that are controlled by miRNA. These networks were then used for dynamic visualization of clinical data and possible predictions of miRNA-binding sites within the complete sequence of all known genes in three genomes. After RNA extraction, a wide range of RNA concentrations, from low concentrations (38.17–42.5 ng/l) to high concentrations, were obtained. Notably, the total RNA concentrations of the tumor samples did not differ significantly from one another. Furthermore, the RNA purity was evaluated, and it was shown that there were no appreciable variations within the same groups. The miRNA 199a-5p and the reference gene U6 were amplified in the molecular experiment to determine each threshold cycle (Ct) value (Figures 1 and 2). The melting temperature curve is calculated using the Ct values, which are also used to quantify real-time RT-PCR data that are inversely related to the amount of the beginning template.

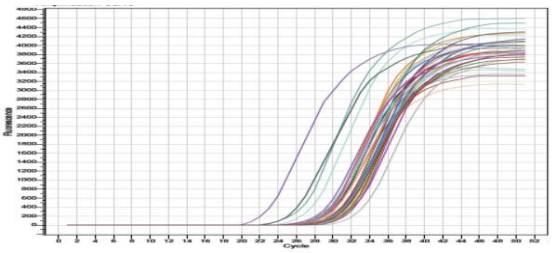

Figure 1: Amplification plots for miR199a-5p expression obtained by Real time PCR.

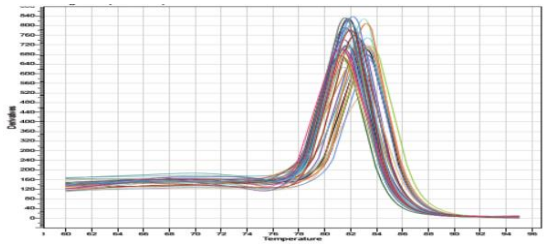

Figure 2: The miR199a-5p expression melting curve.

The expression fold of miRNA-199a-5p is displayed in Table 4. The results of a gene expression analysis using RT-PCR for miRNA-199a-5p in vitro showed interesting findings. Specifically, the folding value of miRNA-199a-5p in malignant tumors (2.908±0.52) compared to benign tumors (2.027±0.51) However, it is important to note that the statistical significance of these differences was not significant (*p*-value = 0.237).

Table 4: Fold of MiRNA 199a-5p expression

Group	miRNA-199a-5P	U6	ΔCT	ΔΔCT	Fold change
Benign	29.49	29.84	-0.344	0.000035	2.03±0.51
Malignant	28.66	29.67	-1.007	-0.662	2.91±0.52
p-value	--	--	--	--	0.237

Values were expressed as mean±SEM.

DISCUSSION

The activation of CDH2 by miRNA-199a-5p appears to promote cell invasion and migration and points to this gene as an oncogene in breast cancer. In 2003, miRNA-199 was first found and characterized [25]. In the human genome, chromosome 19 and chromosome 1 include the two potential hairpin precursors [26]. MiRNA-199a-5p has been detected in various tumor types, such as testicular, hepatocellular, breast, and ovarian cancer. Through emerging evidence, this microRNA has been linked to cancer migration, invasion, and cell growth in these different types of cancer [27]. Recent research has demonstrated that miRNA-199a-5p has opposing roles in developing and spreading specific cancer types, acting as a tumor suppressor gene for some and an oncogene for others [28]. According to Chen *et al.*, an imbalance of miRNA-199a-5p in triple-negative breast cancer affects the regulation of the epithelial-mesenchymal transition and cell stemness. MiRNA-199a-5p is a mature splice variant of miRNA-199a that functions as an epigenetic regulator in several diseases. It also showed that overexpression of miRNA-199a-5p altered the expression levels of EMT-related markers, including *CDH1* (E-cadherin), ZEB1, and TWIST [29]. According to Wang *et al.*, miRNA-199a-5p inhibits the overproliferation of hemangioma cells by reducing the activity of the HIF1A gene [30]. Breast cancer progression may be inhibited by miR199a-5p, suggesting that malignant metastasis was caused by the down-regulation of miR-199a-5p [31]. Additionally, miRNA-199a-5p may prevent the development and progression of cancerous tumors by interfering with the MLK3/NF-B pathway [32]. MiRNA-199a-5p has an impact on various physiological processes, including cell division, proliferation, and apoptosis. In breast cancer, it may act as a tumor suppressor by targeting genes that promote cancer growth. It is important to note that the outcomes of miRNA-199a-5p targeting specific genes may vary depending on individual circumstances. The deregulation of miRNA-199a-5p and its downstream target gene (*CDH2*) can lead to various cellular processes that contribute to the development and progression of breast cancer as part of the miRNA-199a-5p pathway. Moreover, breast cancer cell lines show common methylation of miRNA-199a/b and reduced invasive potential [33]. Research has shown that miRNA-199a/b overexpression in cancer cells can reduce migration, multidrug resistance, and proliferation [34]. Specifically, miRNA-199a-3p attaches to a certain plate number and stops the expression of zinc fingers and homeoboxes 1 (ZHX1), which promotes the growth and division of cancer cells [35]. MiRNA-199a-5p inhibits breast cancer invasion by targeting the 3'-UTR of Ets-1, reducing the level of integrin via FAK/Src/Akt/mTOR signaling [36]. In BC tissues, urine, and blood samples, there are frequently abnormally expressed miRNAs associated with pathologic traits and the chemical sensitivity of BC [37].

Conclusion

The miRNA-199a-5p pathway is complex in its role in breast cancer. It can act as both an oncogene and tumor suppressor gene, and its function depends on the context.

Conflict of interests

No conflict of interest was declared by the authors

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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