



miR-199a-5p Confers Tumor-Suppressive Role in MCF-7 Breast Cancer Cell Line

¹Arwa M. Salih, ²Ismail H. Aziz, ³Forat Y. Mohsin

¹ Forensic DNA Center for Research and Training, Al-Nahrain University

² Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

³ Oncology Teaching Hospital, The Medical City

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Abstract: Breast cancer is a leading cause of cancer death among women worldwide. Abnormal expression of microRNAs has been observed in various types of cancers, including breast cancer. This study aimed to evaluate and suggest the optimum quantities of lipoplex components to enhance the transfection efficiency of breast cancer cell line (MCF-7). Human breast cancer cell lines MCF-7 were grown through a transfection experiment. After RNA extraction and cDNA creation, real-time PCR was used to quantitatively measure miRNA expression levels. MCF-7 cells were transfected through a series of tests. The tests involved modifying the amount of Lipofectamine used. As a result, the optimal transgene expression time point and concentration were determined to be 1 μ l (with a folding value of 1.82675). This was found to achieve the highest transfection efficiency while causing minimal cytotoxicity. In conclusion, the results show that transfected MCF-7 cells exhibit increasing transgenic expression over time. 72 hours after transfection is a suitable period for further study or therapeutic application, which is more than 24 hours.

Keywords: breast cancer, miR-199a-5P, CDH2, Epithelial-Mesenchymal transition (EMT), N-cadherin, MCF-7 cell line, Stealth/siRNA Transfection Protocol Lipofectamine 2000 ThermoFisher, gene expression, RT-PCR.

Corresponding author: (Email: arwa.m.salih@nahrainuniv.edu.iq).

Introduction

Breast cancer is a complex disease that has many different molecular subtypes and classifications. International Agency for Research on Cancer (IARC) reported in their 2020 World Cancer Reports that its Breast cancer is the most commonly diagnosed cancer in women worldwide. Unfortunately, it also accounts for the majority of cancer deaths among women. (1). The breast cancer patterns in small family groups match known

genetic inheritance. Non-invasive biomarkers needed to distinguish Breast Cancer (BC) stages and improve therapy. New non-invasive prognostic biomarkers are needed for fast detection and distinction of breast cancer stages, improving therapy options (2). The genetic reprogramming and transformation of cancer cells from a non-motile, epithelial phenotype into a migratory, mesenchymal-like phenotype, known as epithelial-to-mesenchymal transition (EMT),

facilitates metastasis in many epithelial malignancies (3) During the Epithelial-to-Mesenchymal Transition (EMT), the expression of E-cadherin is often reduced, while N-cadherin expression is upregulated or newly emerges. This event, known as the "cadherin switch," there has been a numerical increase that is related to migratory and invasive tendencies (4). There are over 130 MicroRNAs that are known to regulate EMT, which leads to concerns over the impact of commonly used experimental techniques, such as miRNA overexpression. It is important to consider whether these relationships are biologically significant or simply a reflection of experimental manipulation. Certain miRNAs are known to play an important role in EMT. While it is important to study the effects of miRNA disruption, it appears that transcription factors are primarily responsible for mediating these effects on various targets. (5). Moreover, A significant portion of the population based on recent research, anticipated targets of miRNA remain unaffected at the endogenous levels of miRNA. In addition, a considerable number of miRNA target sites that have been discovered through computational methods might have been conserved due to other evolutionary influences (6). Currently, research is primarily centered about the biological process of miR-199a. Several studies have indicated that both mature forms of miR-199a regulate the behavior of healthy cells to participate in physiological or pathological processes. The breast, colon, and testis have shown high levels of miR-199a-5p expression, while low levels have been observed in the thymus, liver, and kidney (7).

According to a study by (8), breast tissue has higher levels of miR-199a-5p expression which prevents cell proliferation and development of the cell cycle, reducing the risk of breast cancer. Several human gastric cancer cell lines such as AGS, MKN-45, MKN-28, SGC-7901, NCI-N87, MCF-7, and BGC-823 also show significant miR-199a-3p expression. Further investigations have revealed that miR-199a-3p obstructs the production of zinc fingers and homeobox 1 (ZHX1) by binding to the 3' untranslated region of ZHX1 mRNA (9). In order to create cationic liposomes, cationic lipids are used. These lipids have two aliphatic long chains that are hydrophobic and positively charged functional groups in their head regions. They are often combined with neutral lipids such as cholesterol (Chol) or dioleoylphosphatidylethanolamine (DOPE) for gene transfer applications. The combination of cationic and neutral lipids allows cationic liposomes and negatively charged small interfering RNA molecules to interact electrostatically, resulting in the formation of "lipoplexes." Lipoplexes are lipid-siRNA complexes that have a net positive charge and have been proven to effectively deliver siRNA molecules (10). Cationic lipid-based therapy using siRNA has greatly improved treatment for multiple diseases in recent years (11). Many studies have been conducted on using cationic lipid-based gene delivery to treat illnesses (12). There are different types of delivery vectors, but nonviral ones are considered more effective due to the immunogenic effects of viral vectors (13). Numerous studies have investigated the effectiveness of

cationic lipid-based RNAi technology in halting tumor growth and restoring oncogene expression (14).

Materials and methods

Bioinformatics method

Online miRNA target prediction techniques described below were employed in this study to assess putative miR-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/>).

MCF7 cell line

Michigan Cancer Foundation-7 (MCF-7) cells are a type of epithelial cells that were first developed in 1973 at the Michigan Cancer Foundation. These cells were obtained from Biotechnology Research Center/Al-Nahrain University. MCF-7 cells are classified as luminal breast cancer cells and express receptors for glucocorticoid, progesterone, and estrogen (15). In RPMI 1640 media supplemented with 10% FBS and 1% penicillin-streptomycin, MCF-7 cells were cultured and incubated at 37°C in 5% CO₂.

Transfection efficiency

The miRNA mimics, inhibitors, and a negative control (NC) were custom-synthesized and then labelled using the Stealth/siRNA Transfection Protocol from ThermoFisher® (USA). Subsequently, for transfection, when the cells attained 80-90% confluence, a mixture consisting of 25 µl of miRNA, 25 µl of transfection reagents, and 100 µl of OPTIMEMI media was introduced into the cells. To preliminarily assess the transfection efficiency, an inverted

phase contrast microscope was employed. Subsequent to this verification, total RNA was extracted for RT-PCR analysis to further ascertain any alterations in miRNA levels. The relative mRNA expression level of the miR-199a-5p family, normalized to the reference gene U6, was determined using the $\Delta\Delta$ CT method.

Transfection efficiency protocol

Day 1: Seeding Cells

Seedwells of 96-well plate, in each well seed 1*10⁴ cells/well.

Day 2: Oligomer A preparation and adding.A. (Preparation of Oligomers)

Addition 18 µl from each ready-made stock miRNA to 282 µl of opti-MEM media. (300 µl of MiRNA-optimum will be the end product. "For the four tubes, use the same dilution." Note: Mix gently.

B. (Lipofectamine - complex preparation):

Gently mix the lipofectamine before use, then put 2 µl, 3 µl, and 4 µl of lipofectamine into three tubes with 96 µl, 97 µl, 98 µl of opti-MEM. (This complex contains lipofectamine. Gently combine, then incubate for five minutes at room temperature. Note: proceed to next step within 25 minutes. Each of the "four tubes" should have 100 µl of lipofectamine complex added before being gently mixed and left to incubate for 20 minutes at room temperature. To each well containing cells and 100 µl of media, add 100 µl 1 of the oligomer-lipofectamine complex. By gently shaking the plate, you can combine.

Day 3 or 4 or 5: To further determine any changes in miRNA levels, total

RNA was collected for Realtime-PCR examination.

RNA extraction

A total of genomic RNA was extracted from MCF-7 cell lines by using the RNeasy® Mini Kit (QIAGEN).

Quantitative PCR (qPCR)

High-capacity cDNA Kit (ProtoScript® II First Strand cDNA Synthesis Kit, NEW ENGLAND BIOLABS performed reverse transcription of the RNA after extracting total RNA samples.

RNA reverse transcription

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, all RNA species were converted into cDNA during this procedure, which involved the conversion of RNA to cDNA. The oligo-dT primers have a common tag sequence at their 5' end, which helps in amplifying mature miRNA during the real-time PCR step. The total RNA, including miRNA, serves as the starting

material for the reverse transcription process, except for the template RNA., the reverse transcription master mix contained all the elements required for cDNA first-strand synthesis. 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 and optimization

The amplification of miRNA genes was carried out using specialized primers, as listed in Table (1). These primers were provided by the company MacroGen® in a lyophilized form with a concentration of picomols. 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.

No.	Primer name	Primer Sequence (5'---3')	Reference
1.	miRNA-199a-5p	RT primer	(16)
		Forward Primer	
		Reverse primer	
2.	U6 Housekeeping gene (Reference gene)	Forward Primer	(16)
		reverse primer	

Detection of miRNA by RT-qPCR

SYBR-Green Reagents were used for the RT-qPCR measurement of mRNA levels. The amplification conditions were established after 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 second section of the methodology involved choosing and

processing cDNA samples from malignant and benign 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. The fluorescent signal that SYBR-Green

emits was used to estimate the amount of the amplified products. Specific ingredients were carefully combined to create the reaction mix. components and their respective quantities, as outlined in Table (2) below:

Table (2): Reaction components of miRNA expression

No.	Component	20 ul Reaction
1.	Luna Universal qPCR Master Mix	10 ul
2.	Forward primer (10 μ M)	1ul
3.	Reverse primer (10 μ M)	1ul
4.	Template cDNA	5ul
5.	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): Realtime-PCR program for amplification of miRNA-199a-5P and U6 snRNA

No.	Cycle Step	Temperature	Time	Cycles
1.	Initial Denaturation	95°C	60 seconds	1
2.	Denaturation	95°C	15 seconds	40
	Extension	60°C	30 seconds (+plate read)	
3.	Melt Curve	60-95°C	40 minute	1

Statistical analysis

Microsoft Excel 2019 was used for statistical analysis. Error bars display descriptive analysis of investigated parameters.

Results and discussion

In silico study

By utilizing various web servers, data on miRNA and target genes were researched, analyzed, and investigated. This approach enabled the prediction of miRNA targets and gene networks regulated by miRNA through the combination of expression data from numerous tissues and cell types. Subsequently, the complete sequences of all known genes from the human, mouse, and rat genomes were integrated

into these networks to showcase clinical data dynamically and provide potential predictions of miRNA-binding sites.

In vitro cell culture studies

MCF-7 cells were initially seeded in 24-well culture plates. Subsequently, transfection was carried out in OptiMEM media, employing miRNA mimic, miRNA inhibitor, and a control (obtained from ThermoFisher®, USA), with LIPOFECTAMINERNAIMAX (ThermoFisher®, USA) as the transfection agent, following the manufacturer's instructions. After a 72-hour incubation period, the culture medium was removed, and the cells were rinsed with PBS. Next, Triazol was applied to facilitate mRNA

extraction, ensuring an appropriate concentration of lipofectamine. To further validate any potential alterations in miRNA levels, total RNA was isolated for subsequent qPCR analysis. The relative mRNA expression level of the miR-199a-5p family, normalized to the reference gene U6, was determined using the CT technique.

RNA extraction

After RNA extraction, a wide range of RNA concentrations, from low concentrations (42.5-38.17 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.

miRNA 199a-5p expression in MCF-7 cell line

In order to determine the Ct value of miRNA 199a-5p and the reference gene U6, they were amplified in MCF-7 cell (Figures 1, 2).

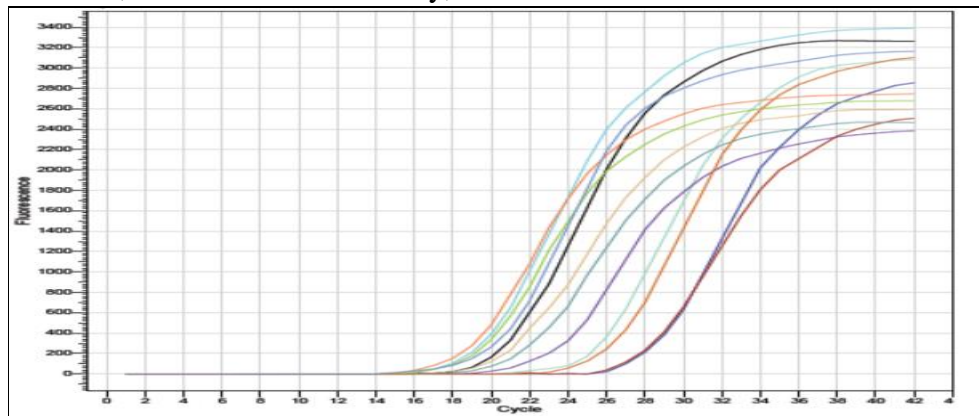


Figure (1): Amplification plots for miR199a-5p and U6 expression obtained by Real time PCR in MCF-7 cell line.

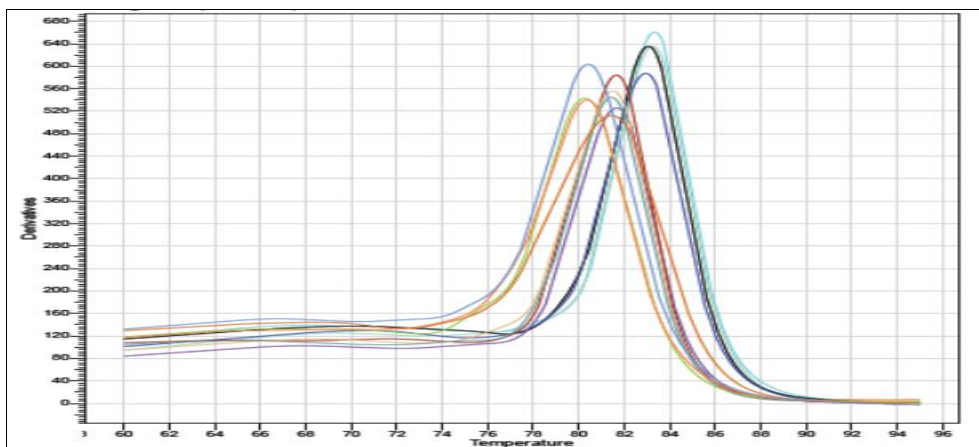


Figure (2): The miR199a-5p and U6 expression Melting Curve in MCF-7 cell line.

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 beginning template. Gene

expression initiation from (81-84) melting temperature. The expression fold of miRNA 199A-5P is displayed in table (4).

(Table 4): Fold of MiRNA199a-5p Expression in MCF-7 cell line

No.	MCF-7 cell line	MiRNA 199a-5p	U6	Δ Ct	$\Delta\Delta$ Ct	Folding
1.	miR199-6(1)	20.19	23.32	-3.13	32.35	1.82675
2.	miR199-6(1.5)	26.29	28.03	-1.74	30.96	4.78753
3.	miR199-6(2)	21.45	21.03	0.42	28.8	2.13962

For best results in transfection, it is recommended by Thermo Fisher Scientific kit to optimize the concentration of Lipofectamine 2000 in a 24-well format. Depending on the target gene's properties, it is advised to use 1, 1.5, or 2 μ l. Optimal conditions for cell transfection should also consider larger cell densities. LipofectamineTM RNAiMAX is a versatile transfection reagent that allows for the use of different cell densities and volumes due to its broad range of maximal activity. The ideal concentration and time point for transgenic expression were determined to be 1 μ l (with a folding value of 1.82675) throughout a 72-hour time course. This concentration resulted in the best transfection efficiency with the least amount of cytotoxicity. One of the most commonly used methods for delivering nucleic acid to various cell types is through lipofection (17). Non-viral vectors have been studied extensively for their ability to condense oligonucleotides. This helps protect them from nuclease degradation, improving their binding and uptake by target cells, mediating endosomal escape, and enabling their intracellular release. These vectors are designed to help the delivered oligonucleotides reach their intended site(s) of action, such as the cytoplasm for siRNA and the nucleus for DNA. The use of non-

viral vectors can help to overcome the numerous obstacles that impede gene therapy (18). When designing systems, it is crucial to ensure that they do not cause significant immunological responses or cellular damage. Synthetic or natural peptides fulfil both these requirements and offer various options for engineering such systems. These peptides can incorporate different functional species, like cell-penetrating (19), mucogenic, or cell/tissue-targeting peptides, providing ample opportunities for customization (20). (21) suggests that the complexes' higher anionic charge reduced their ability to interact with one another, resulting in fewer aggregates and smaller complexes. It is anticipated that the decreased surface charge will impede the communication between cationic complexes and cell membranes.

Conclusion

Based on the results obtained, it can be concluded that transfected mcf-7 exhibits an increase in transient transgenic expression over time. The ideal period for further study or therapeutic application is 72 hours after transfection, which is earlier than 24 hours. However, several factors that are yet to be determined, such as transgenic uptake by host cells, transgene intracellular stability, transgene access to the nucleus, and transcription

efficiency, can affect the post-transfection events in MCF-7.

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