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The effect of miR-640 on metastatic genes in breast cancer

miR-640'ın meme kanserinde metastatik genler üzerindeki etkisi

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ABSTRACT

Aim: Despite the implementation of numerous treatment protocols for breast cancer, which remains a leading cause of cancer-related mortality among women, metastasis continues to pose a major challenge during therapy. This results in prolonged treatment durations, poor prognosis, and the need to manage newly emerging cancer types due to the development of secondary tumors. This study aimed to investigate the anti-metastatic effects of miR-640 previously shown to influence tumorigenesis, angiogenesis, inflammatory responses, and metastasis in various cancers, including breast cancer—by examining its potential to downregulate metastasis-associated genes.

Materials and Methods: To assess the anti-metastatic potential of miR-640, functional assays—including wound healing, Annexin V, analyses—were conducted following lipofectamine-mediated transfection in MCF7 and MDA-MB-231 breast cancer cell lines. Additionally, expression levels of metastasis-associated genes (*WNT7A, TMSB4X, NFIB, ZEB2, IL11, AURKA, CAV1, SLC7A11, PDK1*, and *PTK6*) identified as potential targets of miR-640, were analyzed.

Results: Successful transfection was achieved in both cell lines, with a significant increase in miR-640 expression (~25-fold in MCF7, ~5-fold in MDA-MB-231). Statistically significant effects of miR-640 were observed in wound healing and Annexin V assays following transfection. Additionally, gene expression analyses revealed significant reductions in the expression of metastasis-related genes *WNT7A, TMSB4X, NFIB, IL11, AURKA, CAV1, SLC7A11, PDK1, PTK6* in the MCF7 cell line, and in *WNT7A, NFIB* and *ZEB2* genes in the MDA-MB-231 cell line.

Conclusion: This study is the first to demonstrate that miR-640 exerts an anti-metastatic effect in breast cancer by suppressing metastatic formation through the downregulation of metastasis-related genes. The findings show that cell migration is significantly inhibited in the transfected cell lines and suggest that miR-640 may serve as a potential therapeutic molecule

Keywords: Metastasis, breast cancer, gene regulation, miR-640.

ÖZ

Amaç: *Kadınlarda kansere bağlı ölümlerin başlıca nedeni olmaya devam eden meme kanseri için çok sayıda tedavi protokolü uygulanmasına rağmen, metastaz tedavi sırasında önemli bir sorun olmaya devam etmektedir. Bu durum, tedavi süresinin uzamasına, kötü prognoza ve ikincil tümörlerin gelişmesi nedeniyle yeni ortaya çıkan kanser türlerinin yönetilmesi ihtiyacına yol açmaktadır. Bu çalışma, daha önce meme kanseri dahil çeşitli kanserlerde tümör oluşumu, anjiyogenez, inflamatuar yanıtlar ve metastazı etkilediği gösterilen miR-640'ın metastazla ilişkili genleri aşağı regüle etme potansiyelini inceleyerek, anti-metastatik etkilerini araştırmayı amaçlamıştır.*

Gereç ve Yöntem: *miR-640'ın anti-metastatik potansiyelini değerlendirmek için, MCF7 ve MDA-MB-231 meme kanseri hücre hatlarında lipofektamin aracılı transfeksiyonun ardından yara iyileşmesi, Annexin V ve hücre döngüsü analizleri dahil olmak üzere fonksiyonel testler gerçekleştirilmiştir.*

Ek olarak, miR-640'ın potansiyel hedefleri olarak tanımlanan metastazla ilişkili genlerin (WNT7A, TMSB4X, NFIB, ZEB2, IL11, AURKA, CAV1, SLC7A11, PDK1 ve PTK6) ekspresyon düzeyleri analiz edildi.

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Bulgular: *Her iki hücre hattında da başarılı transfeksiyon gerçekleştirildi ve miR-640 ekspresyonunda önemli bir artış gözlendi (MCF7'de ~25 kat, MDA-MB-231'de ~5 kat). Transfeksiyonun ardından yara iyileşmesi ve Annexin V testlerinde miR-640'ın istatistiksel olarak anlamlı etkileri gözlendi. Ek olarak, gen ekspresyon analizleri, MCF7 hücre hattında metastazla ilişkili genler WNT7A, TMSB4X, NFIB, IL11, AURKA, CAV1, SLC7A11, PDK1, PTK6'nın ve MDA-MB-231 hücre hattında WNT7A, NFIB ve ZEB2 genlerinin ekspresyonunda önemli azalmalar olduğunu ortaya koydu.*

Sonuç: *Bu çalışma, miR-640'ın metastazla ilişkili genlerin aşağı regülasyonu yoluyla metastaz oluşumunu baskılayarak meme kanserinde anti-metastatik etki gösterdiğini ortaya koyan ilk çalışmadır. Bulgular, transfekte edilmiş hücre hatlarında hücre göçünün önemli ölçüde inhibe edildiğini göstermektedir ve miR-640'ın potansiyel bir terapötik molekül olarak hizmet edebileceğini düşündürmektedir.*

Anahtar Sözcükler: *Metastaz, meme kanseri, gen düzenleme, miR-640*

INTRODUCTION

Breast cancer is classified as in situ carcinoma and invasive carcinoma, with the majority being invasive. Metastasis in breast cancer typically occurs in tissues such as bone (Coleman, Smith, & Rubens, 1998; Huber, Carey, & Wazer, 2009; Savci-Heijink, Halfwerk, Koster, & van de Vijver, 2016), liver (Jin et al., 2018; Yazici, Akin, Yazici, & Akin, 2019), brain (Hosonaga, Saya, & Arima, 2020; Lee et al., 2016; Witzel, Oliveira-Ferrer, Pantel, Müller, & Wikman, 2016), and lung (Friedel et al., 2002; Gennari, Conte, Rosso, Orlandini, & Bruzzi, 2005; Yazici et al., 2019). Despite optimal treatment of the primary tumor, approximately 40% of patients may develop metastasis (Yates et al., 2017). Metastasis is the main cause of cancer-related morbidity and mortality, accounting for about 90% of cancer deaths (Yazici et al., 2019).

MicroRNAs (miRNAs) play significant roles in breast cancer tumorigenesis, progression, and metastasis. miRNAs can promote oncogenesis (oncomiRs), inhibit tumor growth (tumor suppressors or miRsupps), and regulate gene targets involved in metastasis (Petri & Klinge, 2020). RNA interference (delivery of RNA into cells) is one of the most promising cancer treatment approaches due to its reliability and adaptability. The RNA interference method is advantageous because it acts at the post-transcriptional level and exhibits a strong therapeutic effect even at very low drug doses (Yoo, Ross, Pantazopoulos, & Medarova, 2021). Therefore, the clinical use of tumor suppressor miRNAs, known as "miRNA mimics," has become an attractive therapeutic strategy (Chhichholiya, Ruthuparna, Velagaleti, & Munshi, 2023).

Overexpression of miR-640 inhibits the proliferation and migration of breast cancer both in vitro and in vivo, whereas depletion of miR-640 produces the opposite effect (Tang et al., 2021). miR-640 has been confirmed as a potential suppressor and angiogenesis inhibitor in breast cancer and hepatocellular carcinoma (Pei et al., 2021). Additionally, patients with ovarian and hepatocellular cancers exhibiting higher miR-640 expression have shown better survival rates, and high miR-640 expression has also been reported to inhibit the formation of capillaries involved in the angiogenesis process (LUO et al., 2021). Angiopoietin-1 (Ang-1), which supports angiogenesis, upregulates Zinc Finger Protein 91 (*ZFP91*) expression through the transcriptional downregulation of miR-640, and ZFP91 plays important roles in Ang-1-induced endothelial cell migration and differentiation. Conversely, increased expression of miR-640 has been shown to suppress angiogenesis (Harel, Sanchez-Gonzalez, Echavarria, Mayaki, & Hussain, 2020). Another study demonstrated that Wnt7b can be directly targeted by miR-640, thereby regulating the Wnt/β-catenin signaling pathway in breast cancer. According to this study, targeting the Wnt/β-catenin signaling pathway with miR-640 in breast cancer could suppress tumor formation (Tang et al., 2021). To date, investigations have been limited to assessing miR-640 expression levels in cancer cells relative to healthy counterparts. The in vivo gene network modulated by miR-640, including its downstream targets subject to downregulation, remains unexplored.

Although there is some evidence that miR-640 suppresses angiogenesis and cancer development, which are fundamental to metastasis, there is currently no study specifically examining its role in repressing the genes responsible for metastasis in breast cancer. In this study, the silencing of genes that cause metastasis— which pose difficulty in breast cancer treatment—through miR-640 transfection was investigated, aiming to shorten the treatment

duration, achieve a treatment process with good prognosis, and eliminate the problem of recurrence.

MATERIALS and METHODS

Selection of Genes Involved in Metastasis

The list of genes targeted by miR-640 was screened in the miRDB, TargetScan Human, and TargetMiner databases. After reviewing the literature for all genes involved in or contributing to the metastasis of tumor cells to different tissues in breast cancer, these genes were filtered according to literature and selected from the miR-640 target gene list. The metastatic breast cancer genes used in the study, which could potentially be targets of miR-640, are shown in (Table-1).

Cell Culture

MCF7 and MDA-MB-231 cell lines were used to represent breast cancer. The cells were maintained in DMEM (ATCC-Catalog No. 30-2002) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (10,000 U/ml) at 37°C in a humidified incubator with 5% carbon dioxide (CO_2).

miR-640 Transfection

Transfection was carried out 24 h after plating using LipoFectMax™ 3000 Transfection Reagent (Cat. No: FP318). The miR-640 mimic was obtained from MedChemExpress (Cat. No.: HY-R01914), designed according to the mature miR-640 sequence. 50nM hsa-miR-640 Mimic was used per 6 wells for overexpression according to the manufacturer's recommended protocol. After 48 h of recovery in complete DMEM medium, cells underwent specific experimental procedures. During transfection, cells were cultured in FBS-free and antibiotic-free medium.

Total RNA isolation

Total RNA was extracted by using TRIzol as manual. Frozen TRIzol samples that had been previously suspended in 600 µL of TRIzol and stored at -20°C were thawed on ice and vortexed. Then, 200 µL of chloroform was added, followed by prolonged vortexing, and the samples were centrifuged at 10.600 rpm for 10 minutes at 4°C. The upper aqueous phase was transferred to a clean Eppendorf tube, and 500 µL of 100% ethanol was added. The samples were then centrifuged again at 10.600 rpm for 10 minutes at 4°C. The supernatant was discarded, and 1 mL of 75% ethanol was added, followed by centrifugation at 8.400 rpm for 5 minutes at 4°C. After discarding the supernatant, the tubes were left to dry at 37°C for 1-5 minutes. Finally, RNA samples were suspended in 50 µL of nuclease-free water and quantified using a Nanodrop spectrophotometer (Catalog number ND-2000/Thermo). The A260/A280 absorbance ratio was measured to assess RNA purity.

Quantitative Analysis of miR-640 Expression Using RT-PCR

miRNA cDNA synthesis was performed according to the protocol of the miRNA All-In-One cDNA Synthesis Kit (Cat. No. G898). After measuring total RNA samples with a Nanodrop, they were diluted to a concentration of 100 ng/µL. Then, miR-640 expression was analyzed by qPCR using miR-640 forward primer (ATGATCCAGGA ACCTGCCTCT) and a universal reverse primer (Cat. No. MPH00000-abm), following the instructions of the SYBR Green qPCR Master Mix (Catalog Number: D026-1) for validation of miRNA transfection (Figure-1B). U6 (CTCGCTTCGG CAGCACA) was used as the internal control. All experiments were performed in triplicate. Relative expressions of miRNAs were calculated based on the CT method, where individual CT values were normalized to the CT values of U6. Additionally, for transfection optimization, 24- and 48-hour treatments were tested, and the expression levels of two target genes of miR-640 (*ZFP91* and *MAP3K1*) were compared (Figure-1A). The 48-hour miR-640 treatment, which showed the greatest downregulation of these genes, was selected.

RT-qPCR Analysis

mRNA cDNA synthesis was performed according to the protocol of the OneScript® Plus cDNA Synthesis Kit (Cat. No. G236). After measuring total RNA samples with a Nanodrop, they were diluted to a concentration of 100 ng/µL. Then, qPCR was carried out using the primers listed in Table-2 and the BlasTaq™ 2X qPCR MasterMix (Cat. No. G891) kit following the instructions, to analyze the expression of metastasis-related genes given in Table-1 before and after miR-640 transfection. The fold change for each was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH for genes, as the internal control. CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and Bio-Rad CFX Maestro V1.1 (Bio-Rad Laboratories) were used for the RT-qPCR and analysis.

Table-1. Genes Involved in Breast Cancer Metastasis and Potential Targets of miR-640. EMT: Epithelial-Mesenchymal Transition.

Gen	Gen Name	Role in Metastasis	Referans
WNT7A	Wnt Family Member 7A	High expression is significantly associated with poor prognosis, higher incidence of metastasis, and increased tumor invasiveness in patients with breast cancer.	(Avgustinova et al., 2016; SR et al., 2016)
TMSB4X	Thymosin-ß4	Induces EMT and leads to metastasis through the TGF-ß/Tß4/MRTF signaling pathway.	(Moradpoor et al., 2020; Morita & Hayashi, 2018; J. Wang et al., 2014)
NFIB	Nuclear Factor I B	Supports EMT via the NFIB/ERO1A/VEGFA pathway.	(Wu et al., 2018; Zilli et al., 2021)
ZEB2	Zinc Finger E-Box Binding Homeobox 2	Responsible for the regulation of the EMT process.	(Castanon, Von Stetina, Kass, & Baylies, 2001; Petri & Klinge, 2020)
IL11	Interleukin 11	A strong osteoclast inducer in the bone marrow and a driver of breast cancer metastasis.	(Awolaran, Brooks, & Lavender, 2016; Müller et al., 2001)
AURKA	Aurora Kinase A	Involved in the regulation of migration and invasion.	(Cha, Lee, & Won, 2021; Weier & Mao, 2013; Whately et al., 2021)
CAV1	Caveolin-1	Participates in proliferation, apoptosis, cancer cell invasion, migration, and metastasis processes.	(Brasó-Maristany et al., 2022)
SLC7A11	Solute Carrier Family 7 Member 11	Contributes to tumor formation, proliferation, metastasis, prognosis, and chemotherapeutic resistance.	(Hosonaga et al., 2020; Li et al., 2022; Schulten et al., 2017)
PDK1	Pyruvate Dehydrogenase Kinase 1	Plays a role in tumor formation, metastasis, tumor microenvironment regulation, and drug resistance.	(N. Wang et al., 2022; Xie et al., 2006; Yeeravalli & Das, 2021)
PTK6	Protein Tyrosine Kinase 6	Regulates oncogenic phenotypes such as proliferation, migration, and invasion in breast cancer and is induced by *HIF1A*.	(Ostrander, Daniel, Lofgren, Kleer, & Lange, 2007; Paul et al., 2020)

Table-2. Primers Used for the Quantification of Gene Expression by RT-qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
WNT7A	TGCCCGGACTCTCATGAAC	GTGTGGTCCAGCACGTCTTG
TMSB4X	ACAAACCCGATATGGCTGAG	GAAGGCAATGCTTGTGGA
NFIB	AAAGCATGAGAAGCGAATGTC	ACTCCTGGCGAATATCTTTGC
ZEB2	GCCGTTAGCTCCCAACAGTA	AGCCTGAGAGGAGGATCACA
IL11	CGAGCGGACCTACTGTCCTA	GCCCAGTCAAGTGTCAGGTG
AURKA	TGTGCCTTAACCTCCCTATTC	AACCTTGCCTCCAGATTATGA
CAV1	CCAAGGAGATCGACCTGGTCAA	GCCGTCAAAACTGTGTGTCCCT
SLC7A11	GCTGTGATATCCCTGGCATT	GGCGTCTTTAAAGTTCTGCG
PDK1	CTGTGATACGGATCAGAAACCG	TCCACCAAACAATAAAGAGTGCT
PTK6	TACTTTGGGGAGGTCTTCGAG	TGCCGCAGCTTCTTCATG
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

Annexin V Assay

After the cells were collected, they were washed with 1× PBS, resuspended in binding solution, and then stained with 2.5 µL Annexin V-fluorescein isothiocyanate (AV) and 2.5 µL propidium iodide (PI) according to the manufacturer's protocol (Apoptosis Detection Kit II; BD Pharmingen, Franklin Lakes, NJ). The stained cells were analyzed using the BD Accuri™ C6 Plus flow cytometer.

Wound healing assay

Cells were grown to confluence in a 6-well tissue culture plate. Cells were treated with 50 nM miR-640, and for the control group, FBS-free DMEM medium was used. After 24 hours, A scratch was made on the monolayer by dragging a p200 pipette tip across the well. The well was rinsed with PBS to remove the dislodged cell debris and culture media was added to the well. The wound gap was photographed at 0 h, 24 h and 48 h at a specific predefined point in the well. The relative wound area was measured using ImageJ software (LOCI, University of Wisconsin).

Statistical analysis

All data were obtained from at least three independent experiments and are presented as mean ± standard deviation (SD). Statistical analyses and figure generation were performed using GraphPad Prism software (version 9; GraphPad Software, La Jolla, CA, USA). The comparison between miRNA-treated samples and non-treated samples was performed using the paired Student's t-test. For the statistical analysis of cell data, a one-way ANOVA. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Quantitative Validation of miRNA Delivery via Transfection for Functional Studies

Following miR-640 transfection, a consistent downregulation of the investigated target genes was observed across both cell lines (Figure-1A). Overall, miR-640 administration resulted in a significant suppression of gene expression in MCF7 and MDA-MB-231 cells, with the magnitude of repression exhibiting a treatment duration-dependent effect. Notably, robust downregulation was evident after 48 hours of miR-640 exposure, suggesting that the inhibitory impact of miR-640 on its gene targets undergoes dynamic temporal regulation.

A marked elevation in miR-640 levels was observed in transfected cells relative to untreated controls, thereby confirming the efficient delivery and intracellular uptake of miR-640 in both cell lines (Figure-1B). The observed variability in the magnitude of miR-640 upregulation between cell lines may reflect intrinsic differences in miRNA stability and transfection efficiency specific to each cellular context. At the same time, the inter-cell-line difference in miR-640 levels should be considered when comparing the extent of target suppression and functional responses between MCF7 and MDA-MB-231 cells, since variable miRNA uptake or persistence can modulate downstream molecular and phenotypic effects.

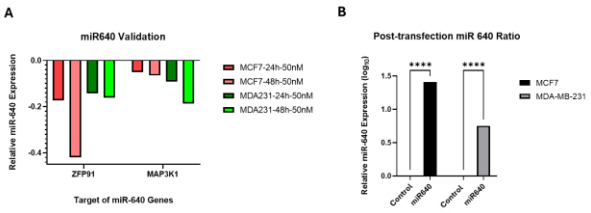

Figure-1. A) Silencing efficacy of miR-640 on target genes at different time points.
B) miR-640 levels post-transfection in MCF7 and MDA-MB-231 cell lines (Control: no treatment; miR-640: miRNA transfection only).

Quantitative PCR (qPCR) Analysis Results

Figure-2 illustrates the fold changes in the expression of target genes in MCF7 and MDA-MB-231 cells following miR-640 transfection, relative to untreated control groups. The majority of genes in both cell lines exhibited negative fold changes, indicative of a predominant downregulatory effect exerted by miR-640 on its targets. Nonetheless, the extent of repression varied among individual genes as well as between the two cellular contexts.

In MCF7 cells, pronounced downregulation was observed in several genes, including *WNT7A, TMSB4X, AURKA, CAV1, SLC7A11*, and *PTK6*, whereas other genes exhibited more modest reductions. Conversely, in MDA-MB-231 cells, robust repression was evident for *WNT7A*, with weaker inhibitory effects detected for *NFIB* and *ZEB2*. These differential responses may reflect not only the inherent target specificity of miR-640 but also variable transfection efficiencies between the two cell lines (Figure-1B). Nonetheless, miR-640 consistently exerts a suppressive impact on

metastasis-associated genes across both cellular models, although the magnitude of this effect appears to be modulated by cell-type specific responses and transfection efficacy.

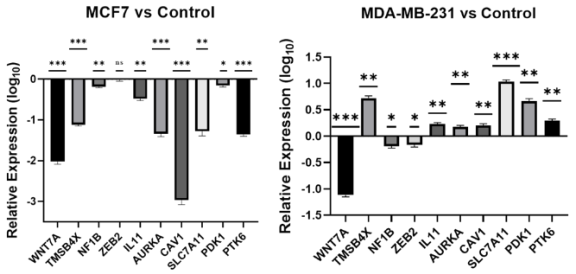

Figure-2. Comparative Analysis of Gene Expression in MCF7 and MDA-MB-231 Cells Post miR-640 Transfection Relative to Untreated Controls.

Annexin V Assay

The apoptotic effects of miR-640 mimics were evaluated in MCF7 and MDA-MB-231 breast cancer cell lines compared to control groups. The graphs on the right depict the percentage distribution of apoptotic cells, as determined by Annexin V staining. In MCF7 cells, transfection with miR-640 mimics elicited a significant increase in both early and late apoptotic cell populations, indicating that miR-640 promotes apoptotic induction and enhances cell death in this context (Figure-3A). Similarly, miR-640 mimic transfection in MDA-MB-231 cells resulted in markedly elevated early and late apoptotic rates relative to controls (Figure-3B). Concomitantly, the proportion of viable cells was reduced in both cell lines following miR-640 mimic treatment.

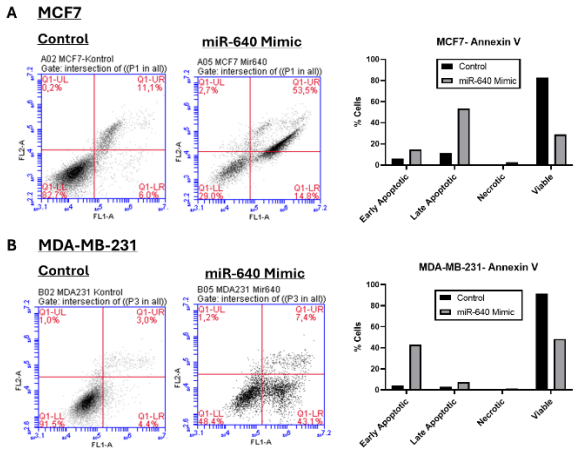

Figure-3. Comparative Analysis of Apoptotic Effects Following miR-640 Transfection in MCF7 and MDA-MB-231 Cells Relative to Untreated Controls.

Wound Healing Assay

Microscopic images of wound areas at 0, 24, and 48 hours post-wounding are presented for control and miR-640 transfected MCF7 cells. While the initial scratch widths (0 h) were comparable between both groups, a markedly delayed wound closure was observed in miR-640 transfected cells at 24 and 48 hours (Figure-4A). Quantitative analysis of wound area changes over time (measured in square micrometers) revealed a significant reduction in wound size in the control group at 24 and 48 hours, whereas the decrease in wound area was notably attenuated in the miR-640 treated group. These findings indicate that miR-640 significantly inhibits cell migration ($p < 0.0001$) (Figure-4B). Approximately 80% wound closure was observed in control cells, in contrast to a significantly impaired closure rate of around 30% in miR-640-transfected cells (Figure-4C). Collectively, these results demonstrate that miR-640 potently suppresses migratory capacity in MCF7 cells, thereby potentially diminishing their invasive and metastatic potential. Similar results apply to MDA-MB-231 cells (Figure-5).

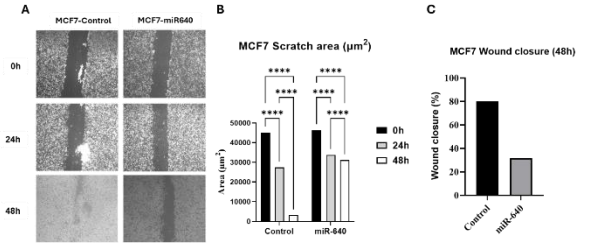

Figure-4. Analysis of cell migration in MCF7 cells after miR-640 transfection.
A) Representative microscopic images of scratch wounds at 0, 24, and 48 hours,
B) Measurement of wound area over time,
C) Percentage of wound closure.

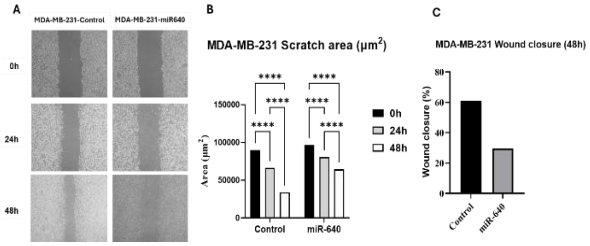

Figure-5. Analysis of cell migration in MDA-MB-231 cells after miR-640 transfection.
A) Representative microscopic images of scratch wounds at 0, 24, and 48 hours,
B) Measurement of wound area over time,
C) Percentage of wound closure.

DISCUSSION

In this study, we comprehensively examined the functional role of miR-640 in regulating metastasis-associated gene expression and key cellular behaviors in two distinct breast cancer cell lines, MCF7 and MDA-MB-231. Our results demonstrate that efficient transfection of miR-640 leads to a significant increase in its intracellular levels, thereby confirming successful miRNA delivery across different breast cancer cell models. Although Lipofectamine is a widely used reagent for intracellular delivery of RNA and its derivatives, various formulations exist, and transfection efficiency can differ among these versions (Yamano, Dai, & Moursi, 2010). Taking these factors into account, it is expected that the amount of transfected miRNA differs between cell lines.

Notably, miR-640 transfection induced widespread downregulation of metastasis-related genes, including EMT markers and oncogenes such as *WNT7A, TMSB4X, AURKA, CAV1*, and *PTK6* in the MCF7 Cell line. Additionally, *NFIB, IL11, SLC7A11* and *PDK1* genes were downregulated by miR-640. It shows that metastasis can be inhibited when these genes are downregulated via miRNA-mediated gene silencing. In the MDA-MB-231 cell line, *WNT7A, NFIB* and *ZEB2* genes were downregulated. In addition, while Samaeekia et al. reported that the IL11 (Samaeekia et al., 2017) gene is downregulated via miR-206, Liu et al. reported that the SLC7A11 (Liu et al., 2011; Yadav et al., 2021) gene is downregulated via miR-26b, Yadav et al. reported that the *SLC7A11* gene is downregulated via miR-5096, and Zhang et al. reported that the *ZEB2* (Zhang et al., 2017) gene is downregulated via miR-205, no studies were found for the other genes we investigated. In this situation, the degree of suppression varied depending on both the gene and the cell line. The pronounced downregulation observed in MCF7 and MDA-MB-231 cells, particularly in genes critically involved in EMT and metastatic progression, may be attributed to cell line-specific differences such as miRNA stability, basal expression levels of target genes, and transfection efficiency. These observations highlight the context-dependent effects of miR-640 and suggest differential sensitivity across cell types.

When examining the temporal dynamics of gene silencing, it was observed that the suppressive effects of miR-640 increased following 48 hours of exposure, emphasizing the necessity of sustained miRNA expression for effective target modulation. This time-dependent downregulation is consistent with miRNA-mediated mRNA degradation and translational inhibition mechanisms (Grigaitis et al., 2020; Ihle et al., 2015; Park, Surendran, Kamendulis, & Morral, 2011).

In functional analyses, miR-640 treatment significantly induced apoptotic processes in both cell lines. Flow cytometric analysis of Annexin V and propidium iodide staining revealed marked increases in early and late apoptotic cell populations, accompanied by a reduction in the percentage of viable cells. These findings indicate that miR-640 plays a critical role in impairing tumor cell survival by activating programmed cell death pathways, thereby negatively affecting cell viability. This is consistent with the literature supporting the crucial involvement of miRNAs in cancer progression and therapy resistance (Jafari et al., 2024; Rasoolnezhad, Safaralizadeh, Hosseinpourfeizi, Banan-Khojasteh, & Baradaran, 2021; Venkatadri, Muni, Iyer, Yakisich, & Azad, 2016; Zadeh, Motamed, Ranji, Majidi, & Falahi, 2016).

Additionally, wound healing (scratch) assays revealed that miR-640 markedly impairs the migratory capacity of MCF7 and MDA-MB-231 cells. In the transfected cells, the rate of scratch closure was significantly delayed, and the reduction in wound area remained limited over time. Considering that cell migration is a prerequisite for invasion and metastasis, this functional effect of miR-640 suggests its potential to inhibit metastatic dissemination. Consistent with the observed downregulation of genes such as *WNT7A* (Avgustinova et al., 2016; SR et al., 2016) and *TMSB4X* (Moradpoor et al., 2020; Morita & Hayashi, 2018; J. Wang et al., 2014), this anti-migratory effect is likely mediated through direct targeting of key regulators involved in cytoskeletal remodeling and EMT pathways.

CONCLUSION

Our findings identify miR-640 as a potent tumor-suppressor microRNA in breast cancer that exerts multifaceted anti-cancer effects. miR-640 broadly represses the expression of metastasis-promoting genes, induces apoptotic cell death, and inhibits cell migration. Notably, this study is the first to investigate the relationship between miR-640 and metastatic processes, revealing its critical role in suppressing metastasis in breast cancer. These results underscore the therapeutic potential of

miR-640 mimics as promising RNA-based agents to slow breast cancer progression, particularly in aggressive subtypes, and provide a solid foundation for the development of novel treatments aimed at controlling metastatic disease.

Future studies should include the evaluation of these in vitro findings in in vivo breast cancer models; the molecular targets of miR-640 and the downstream signaling pathways should be thoroughly elucidated, and correlation analyses with clinical patient samples should be conducted to investigate its prognostic and predictive values. Additionally, conducting RNA-Seq using high-throughput next-generation sequencing (NGS) techniques to examine the precise molecular targets and downstream signaling cascades modulated by miR-640 will be critically important for illuminating its mechanistic framework and optimizing its clinical translation. Correlative analyses involving patient-derived samples can further clarify the prognostic and predictive significance of miR-640, supporting its integration into personalized therapeutic strategies.

Conflicts of interest: Authors declared no conflict of interest.

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