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Short Communication

Mir-193a-5p Replacement Can Alter Metastasis Gene Expression in Breast Adenocarcinoma Cells *In Vitro*

Monireh Khordadmehr[1]*, Roya Shahbazi[1], Behzad Baradaran[2,3]*, Sanam Sadreddini[2], Dariush Shanehbandi[2], Khalil Hajiasgharzadeh[2], Masoumeh Firouzamandi[1]

[1]Department of Pathology, Faculty of Veterinary Medicine, University of Tabriz, 51665-1647, Tabriz, Iran.

[2]Immunology Research Center, Tabriz University of Medical Sciences, 51666-14761, Tabriz, Iran.

[3]Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, 51666-14761, Tabriz, Iran.

Article Info

Abstract

Background: Recent evidence presented the significant role of the microRNA-193 (miR-193) family in biological processes by the contribution of specific targeting, which mainly display as a tumor suppressor in various cancers. In the present study, we evaluated the effect of miR-193a-5p replacement on some metastasis gene expression in metastatic breast cancer (BC) cells.

Methods: For this purpose, firstly, the quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the miR-193a-5p expression in the MDA-MB-231 BC cell line. Subsequently, miR-193a-5p was transfected into the cells, and the expression levels of ROCK1 (Rho-associated, coiled-coil containing protein kinase 1), CXCR4 (Chemokine Receptor-4), CD44, and vimentin genes were evaluated by qRT-PCR.

Results: The expression level of miR-193a-5p strongly reduced in MDA-MB-231 cells. Interestingly, the ROCK1 ($P < 0.001$), CD44 ($P < 0.0001$), CXCR4 ($P < 0.001$) and vimentin ($P < 0.001$) expression levels significantly decreased following miR-193a-5p transfection in MDA-MB-231 BC cells.

Conclusion: To conclude, it seems that miR-193a-5p restoration can attenuate the metastatic behavior of BC cells *in vitro* through decreased expression level of metastasis-related genes and may constitute an effective novel therapeutic strategy in miRNA-replacement therapy and treatment of metastatic breast adenocarcinoma in the future.

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in women worldwide. Indeed, the American Cancer Society had predicted that there would be an estimated 266,120 and 2,550 new cases of invasive BC diagnosed in women and men, respectively, associated with 41,400 BC deaths (40,920 women, 480 men) in the US in 2018. Also, the 5- and 10-year relative survival rates for invasive BC are 90% and 83%, respectively.[1] In BC treatment, surgery (including a lumpectomy and mastectomy), radiotherapy (recommended for most patients having breast-conserving surgery), chemotherapy (before or after surgery), hormone (anti-estrogen) therapy, and targeted therapy are considered as the therapeutic strategies. However, these treatment approaches occasionally present some impediments like toxicity, relapse, different side effects, and high-cost treatment. For these reasons, in recent years, the large number of researchers has interested and focused on several replacement therapy methods, particularly microRNA replacement therapy for the treatment of BC.[2] MiRNAs are well known as an important regulator of gene expression with fundamental impacts on numerous biological functions. Likewise, emerging evidence proposes that miRNAs are often deregulated in various cancers, and carcinogenesis occurs through the dysregulation of the pivotal tumor suppressor genes or oncogenes.[3] In this regard, they have been involved in cancer biology and development, such as cell proliferation and apoptosis, angiogenesis, cell motility and migration, invasion, metastasis and EMT (Epithelial to Mesenchymal Transformation) of various tumors like BC.[2,3] In this connection, it was previously demonstrated that miR-193 family (includes has-miR-193a and has-miR-193b) which define as *MIR193a* and *MIR193b* where located on chromosome 17 (chr17: 31559996-31560083) and 16 (chr16: 14303967-14304049), respectively, contributes mainly as a tumor suppressor in both liquid and solid tumors.[4,5] Indeed, previous studies indicated the considerable role of miR-193a in various cancers such as bladder carcinoma,[6] hepatocellular carcinoma,[7] colon cancer,[8] and breast cancer.[9] Besides, several studies have

been similarly identified the inhibitory effects of miR-193b in several cancer tissues and cell lines, such as BC,[10] hepatocellular carcinoma,[11] glioma,[12] pancreatic cancer.[13]

As previously described, convincing evidence indicated that ectopic expression of miRNAs could impact on one or several steps of metastatic mechanisms comprise cell adhesion and motility, invasiveness, and EMT. In the present study, we evaluated the effect of miR-193a-5p replacement on expression levels of ROCK1, CD44, CXCR4, and vimentin potential targeting of MDA-MB-231 BC cell line, which involve in the metastatic manner of BC.

Materials and Methods
Cell culture and transfections
MDA-MB-231 cell line that originated from the human epithelial BC cells were acquired from the Pasture Institute, Tehran, Iran. Firstly, the cells were cultured in a standard cell culture condition based on our previous study.[14] Subsequently, 2×10^5 cells were seeded in a 6-well plate. When the seeded cells reached to 50-70% confluently, the cells were washed with PBS (phosphate-buffered saline), and the miR-193a-5p mimic transfection was performed using JetPEI reagent (Polyplus-transfection, Strasbourg, France). To understand the appropriate dosage of transfected miRNA and optimal time, pilot assessments were performed in three 24, 48, and 72 h time intervals,[15] and three dosages of 5, 7.5, and 10 nmol to obtain the effective time and dosage, respectively. MiRNA from *Caenorhabditis elegans* (MISSION2® miRNA, Sigma-Aldrich Co.), with no sequence homology to the human gene, was used for transfection of negative controls (Table 1).

RNA extraction, RT-PCR, and qRT-PCR for miRNA and genes expression
Total RNA was isolated from the cell pellets (approximately 1×10^6 cells) using RiboEx reagent (Gene All Biotechnology, Seoul, South Korea), and the quality and concentration of RNA were determined by Nano-Drop spectrophotometer (Thermo Fisher Scientific Life Sciences, USA). Moreover, the integrity of the RNA was checked by agarose gel electrophoresis. For miR-193a-5p (cDNA) synthesis, the miRNA Reverse Transcription Kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's protocol. In parallel, cDNA synthesis for the ROCK1, CD44, CXCR4, and vimentin genes were performed using first-strand cDNA synthesis Kit (Thermo, USA) according to the suggested protocol. A Bio-Rad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) was utilized for applying temperatures.

Quantification of miRNA and mRNA expression levels was performed using a standard SYBR Green PCR pre-mix (Ampliqon, Odense, Denmark) by a Light-Cycler 96 System (Roche Diagnostics GmbH, Mannheim, Germany). Reaction tubes comprised 5 µl of 2X SYBR green premix, 0.25 µl of 4 pmol/µl primers, and 0.5 µl of relating cDNA and nuclease-free water (up to 10 µl). The cycling carried out by 94ºC for 10 s, 59 ºC for 30 s, and 72 ºC for 20 s. Finally, the Ct values were analyzed by the $2^{-\Delta\Delta CT}$ method. Of note, all reactions were considered in triplicates. β-actin was used as a housekeeping gene for mRNA expression analysis, while U6 was employed for miR-193a-5p normalization. The sequences of primers for ROCK1, CD44, CXCR4, and vimentin are provided in Table 1, which was purchased from Bioneer, Korea.

Statistical analysis
For statistical analysis of the present data, GraphPad Prism software (GraphPad Prism 4.0, San Diego, CA) was used. The statistical significant differences between the experimental groups were evaluated by Student's t and ANOVA tests, and a $p < 0.05$ was considered statistically significant.

Results and Discussion
In the present study, qRT-PCR indicated that the miR-193a-5p mimic transfection led to the efficient restoration of miR-193a-5p in BC cells. It was found the 5 nmol in 24 h for appropriate dosage of transfected miRNA and optimal time ($P < 0.0001$), which considered for the subsequent examinations (Table 2).

Table 1. Primer sequences that were used in the present study.

Primer name	Forward/Reverse	sequences
ROCK1	F R	5′-AATCGTGTGGGATGCTACCT-3′ 5′-AAAACCCTCAGTGTGTTGTGC-3'
CXCR4	F R	5'-TCTTCCTGCCCACCATCTACTC-3' 5'-TGCAGCCTGTACTTGTCCGTC-3'
CD44	F R	5′- CAAGCCACTCCAGGACAAGG-3′ 5′- ATCCAAGTGAGGGACTACAACAG-3′
Vimentin	F R	5′-CAGGCAAAGCAGGAGTCCA-3′ 5′-AAGTTCTCTTCCATTTCACGCA-3′
β–actin	F R	5′- TCCCTGGAGAAGAGCTACG -3′ 5′- GTAGTTTCGTGGATGCCACA -3′
U6 snRNA	Target sequence	5′-UGCUCGUUCGGCAGCACACAUAUACUAAAAUUGGAACGAU ACAGAGAGAAGAUUAGCAUGGCCCCUGCGCAAGGAUGACACGCAAAUUC- GUGAAGCGUUCCAUAUUUUU-3′
Has-miR-193a-5p	Target sequence	5'-UCA UCU CGC CCG CAA AGA CCC A-3'
C. elegans miRNA	Target sequence	5'-CGGUACGAUCGCGGCGGGAUAUC-3'

Table 2. The results to determine the appropriate dosage of transfected miRNA and assessment time, showed that 5 nmol in the 24 h was the most effective dosage at the most effective time. The data represent mean ± SD (n = 3).

Parameters	Groups	Related miR-193a-5p expression	P value
Concentration	Mimic 5 nM	17.03 ± 0.072	
	Mimic 7.5 nM	15.06 ± 0.097	
	Mimic 10 nM	14.47 ± 0.106	0.0001
Time interval	Control	1 ± 0.00	
	24 h	0.937 ± 0.006	
	48 h	0.604 ± 0.004	0.0001
	72 h	0.751 ± 0.006	
	Control	1 ± 0.00	

To evaluate the expression levels of ROCK1, CD44, CXCR4, and vimentin after transfection of miR-193a-5p mimic in MDA-MB-231 BC cells, qRT-PCR was also used. These data demonstrated the considerable decrement in the expression levels of ROCK1 ($P < 0.001$), CD44 ($P < 0.0001$), CXCR4 ($P < 0.001$), and vimentin ($P < 0.001$) genes compared to the control group (Figure 1). Therefore, it was suggested that the miR-193a-5p transfection could attenuate metastases manner in BC cells *in vitro* by modulation of related metastasis genes.

The present study exhibited the impact of miR-193a-5p transfection on BC cells, which subsequently decreased expression levels of some related genes involved in BC metastases, including ROCK1, CD44, CXCR4, and vimentin. In this regard, increasing evidence indicated the effective function of miR-193a-3p on BC migration and invasion.[16,17] However, little was previously known about the effect of miR-193a-5p on the biological behavior of BC cells. Indeed, the present findings were consistent with previous data which confirmed the tumor-suppressive function of miR-193a in tumor biology.[16,17]

In the present study, we observed the marked decreased expression levels of ROCK1, CXCR4, CD44, and vimentin, which are involved in invasion and metastasis biology of BC cells. ROCK1 functions as a substantial modulator of focal adhesion formation, cancer cell motility, and invasion.[18] A recent study indicated that the down-regulation of ROCK1 by miR-340 could repress the invasion and metastasis of BC cells.[19] Recently, it was established miR-335 reduced metastasis of osteosarcoma by functional ROCK1 targeting.[20] Moreover, it was clarified that miR-148a suppresses tumor cell invasion and metastasis by down-regulating ROCK1 in gastric cancer.[21] Also, it was reported that miR-1280 decreases invasion and metastasis by targeting ROCK1 in bladder cancer.[22] Very recently, it was also demonstrated that miR-448 inhibits the progression of
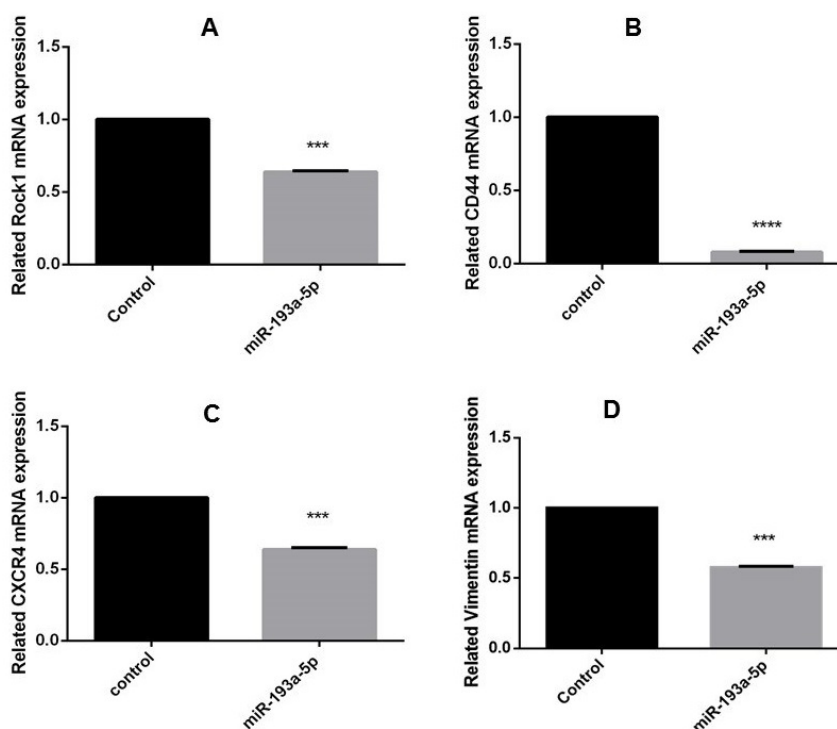
Figure 1. The effect of miR-193a-5p replacement on ROCK1 (A), CD44 (B), CXCR4 (C), and vimentin (D) expression levels in breast cancer MDA-MB-231 cell line. Q-RT-PCR assay exhibited that ROCK1, CD44, CXCR4, and vimentin expression markedly reduced ($P < 0.001$, $P < 0.0001$, $P < 0.001$, $P < 0.001$, respectively) in miRNA replaced cells compared with the control group. The data represent mean ± SD, n = 3.

retinoblastoma by directly targeting ROCK1.[23]

The role of CXCR4 in BC metastasis was previously clarified and demonstrated that overexpression of the CXCR4 receptor in BC is closely associated with increasing of cancer metastasis phenotype and is necessary for cellular migration of BC cells.[24] Recently, it was indicated that miR-145 and miR-203 could regulate the invasiveness of BC cells by CXCR4 targeting.[25] A previous study revealed that miR-302a inhibits the metastatic feature of BC cells by down-regulating of CXCR4.[26] The present results indicate that overexpression of miR-193a-5p can reduce the expression level of CXCR4 in BC cells, and would be probably inhibited migration and metastasis features of BC.

Some miRNAs like miR-373 and miR-520c promote cell migration and invasion by targeting CD44 in BC cells.[27,28] Also, it was investigated that HER2 interacts with CD44 to CXCR4 up-regulation through epigenetic silencing of miR-139 in gastric cancer cells.[29] On the other hand, it is believed that cancer cells that undergo EMT gain stem cell-like properties and exhibit an enhanced in CD44 expression.[30] Besides, cancer cells with an EMT phenotype present augmented invasiveness and occasionally are more resistant to chemotherapy.[31] The contribution of CD44 in cancer progression proposes that CD44 may be a molecular target for cancer therapy. According to the present findings, it seems that the down-regulation of CD44 induced by the restoration of miR-193a-5p may be related to the CXCR4 expression level.

EMT is a remarkable index of cellular plasticity that contributes to the epithelial-tight-junctions dissolution, adherence-junctions intonation, and cytoskeleton remodeling.[32] In this way, some scientists indicated the critical role of vimentin as one of the main EMT-related proteins in the EMT process. It was provided that miR-214 contribute to the EMT process in BC cells by vimentin presentation.[33] Besides, miR-138 suppresses metastasis and EMT phenotype in BC cells by potentially targeting vimentin.[34] Likewise, it was believed that miRNA-30a inhibits cell migration and invasion by down-regulating vimentin expression in BC.[35] Interestingly, it was previously indicated that overexpression of CD44 up-regulated vimentin expression, which led to the cell invasion and migration.[36] Very recently, it was also reported that miR-193a-5p can repress migration and invasion of colorectal cancer cells via directly targeting vimentin.[37] Interestingly, these results are consistent with the present findings. Collectively, current data indicate an important role of miR-193a-5p as an inhibitory miRNA for breast adenocarcinoma metastasis.

Conclusion

To conclude, we present here that miR-193a-5p functions as a tumor-suppressive miRNA, which conventionally down-regulate in BC cells, and can negatively modulate BC cell migration, metastases, and EMT by targeting ROCK1, CD44, CXCR4, and vimentin. Taken together, these findings provide that restoration of miR-193a-5p may consider as a beneficial therapeutic strategy for miRNA-replacement therapy and treatment of metastatic breast adenocarcinoma in the future.

Acknowledgments

Conflict of Interest

The authors declare they have no conflict of interest.

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