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MiR-146a Restoration Suppresses Triple-Negative Breast Cancer Cell Migration: A Bioinformatic and In Vitro Study

Running Title: MiR-146a and breast cancer cells

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Abstract

Purpose: Breast cancer is one of the most commonly diagnosed types of cancer worldwide. This cancer is treated with various methods like mastectomy, chemotherapy, hormone replacement therapy, and radiotherapy. Among them, targeted therapy, which specifically targets cancerous cells by using strategies like microRNA replacement therapy, is considered a new approach in treating breast cancer.

Methods: Data analysis from TCGA datasets were used to confirm the decreased expression of miR-146a in breast cancer. MTT assay was used to evaluate the viability of MDA-MB-231 cells after miR-146a restoration. A wound-healing assay was used to observe migration in the MDA-MB-231 cell line and the effect of miR-146a on the migration. Finally, qRT-PCR was used as a method to determine the effect of miR-146a on the expression of CXCR4, β-catenin, MMP2, MMP9, and Vimentin genes are known to be involved in invasion and migration in MDA-MB-231 cell lines in breast cancer.

Results: The bioinformatic study showed miR-146a expression decreased in breast tumors compared to adjusted normally. Our results indicated that miR-146a is not involved in apoptosis in the MDA-MB-231 cell line, while it is highly effective in migration inhibition. MMP9, MMP2, CXCR4, and Vimentin expressions were suppressed by miR-146a induction; however, it induced the expression of β-catenin which all result in inhibition of migration.
Conclusion: Regulatory molecules, such as miR-146a, are effective in breast cancer targeted therapy. As cancer is a complicated disorder, therefore the combination of therapies, based on these targets might lead to novel therapeutic strategies.

Keywords: MicroRNAs; Breast Neoplasms; Transfection; Cell Proliferation; Wound Healing

Introduction

Among cancer patients, breast cancer seems to be the most lethal cancer.[1,2] Based on the data from public health reports, over one million women are diagnosed with breast cancer throughout the world every year, which leads to death in 50% of the cases.[3] Also, according to reports worldwide, it seems that the incidence of breast cancer has been rising yearly.[3] In Iran, breast cancer is the first diagnosed cancer among women. Over the last few decades, several studies have been conducted on breast cancer in Iran, and the results have indicated an increased rate of breast cancer.[4] There are different therapeutic approaches to treat breast cancer such as mastectomy,[5,6] chemotherapy,[7,8] hormone replacement therapy,[9,10] and radiotherapy.[11,12] In recent decades, different treatments were used to increase the efficacy of these methods in patients such as chemotherapy, which has been used as an adjuvant therapy to mastectomy.[13] Another example is the combination of chemotherapy along with a monoclonal antibody.[7]

Among patients with breast cancer, it seems patients who are treated by chemotherapy experience more side effects. The chemotherapy drugs along with the cancerous cells also attack the healthy cells. This has become the main reason for researchers over the last 3 decades to try and find drugs like miRNA that affect cancerous cells via targeting the oncogenic mRNA at the posttranscriptional level.[14,15] The most important step in targeted therapy is to determine the right target which is only possible by a vast knowledge of the molecules and proteins involved in cancer.[16] Choosing the right target will keep the healthy cells safe and therefore, will lead to an increase in life expectancy in patients.[17] A type of small non-coding RNAs that include about 22 nucleotides, which are called microRNAs (miRNAs), are considered important regulators with crucial roles in cancer such as gene expression, metastasis, tumor suppression, and oncogenesis.[18-20] Due to the regulatory role of miRNAs, any altered expression of them may lead to cancer development.[21-23] MiR-146a is one of the microRNAs that has been reported as a molecule that could be involved in different cancers like prostate, gastric, and breast cancer due to dysregulations.[24,25] Therefore, restoring these molecules in patients is considered an important therapeutic strategy.[21,22] The studies have shown that miR-146a targets different genes including MMP9, MMP2, vimentin, β-catenin, and CXCR4,[26-29] which are known to be involved in metastasis and invasion in breast cancer.[30-33] Overall, in this research, we aimed to investigate the role of miR-146a in breast cancer by using bioinformatic analysis and in vitro assays including cytotoxicity assay, wound healing assay, and apoptosis assays. Also, we analyze the expression levels of the genes relating to tumor migration such as MMP2, MMP9, CXCR4, β-catenin and, vimentin in MDA-MB-231 cell lines after miR-146a restoration to ultimately determine the effect of miR-146a on the migration of cancer cells.

Materials and Methods

Bioinformatic analysis

The breast cancer clinical data extracted from The Cancer Genome Atlas-Breast Cancer (TCGA-BRCA) project. MiR-146a expression level in the breast cancer tissues compared to adjusted normal analyzed by R software with limma and ggplot packages.

Breast cancer cell culture

The MDA-MB-231 cell line was purchased from Pastor Institute in Iran (Iran, Tehran) and cultured in RPMI-1640 medium (Gibco, Lot No. 1703986X), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Lot NO. 42F8160K, USA). The medium was later added 1% antibiotic (penicillin 100 IU/ml, streptomycin 100 µg/ml) and sterilized by 0.22-micron filters. After de freezing, cells were transferred to a T25 cell culture flask. The flask was kept in the incubator at 37 °C, 5% CO_2, and 60% humidity. The media was changed every 2 days until the cells entered the logarithm phase. Then they were ready to be used for the tests according to our previous study.[34]

MiR-146a restoration

Cells were seeded into a 6 well plate; 2×10^5 cells in each well, and incubated for 24 hours (h). After 24 h and as the first step, cells were transfected by miR-146a in a 6 well plate to set the optimum concentration.[35] We used 4 µl transfection reagent (TR) (JetPrime, PolyPlus, France) for each well with different doses of miR-146a and optimum media, which was 1 pmol miRNA and 99 µl media for first well, 5 pmol miRNA plus 95 µl media for the second well, 10 pmol miRNA and 90 µl media in triplicate. As the second step, the cells were seeded in a 6 well plate and transfected by 10 pmol miR-146a, which was determined as the optimal dose before this test. The cells were incubated for 24, 48, and 72 h to determine the optimum time, which was indicated as 24 h.

Quantitative real-time PCR

RNA extraction (isolation)

The total amount of RNA was isolated from each well using RiboEx reagent (Gene All ، Lot NO. REX15J12014, Korea) and transferred into a microtube. Based on the protocol, 120 µl chloroform (Merck, Germany) was added to each microtube and was kept at -20 °C for 10 min. Then the mixture was centrifuged for 20 min at 12000 rpm and 4 °C. The surface layer, which is the aqueous phase and contains RNA was collected and mixed with 250 µl cold isopropanol. Then, it was left at -20 °C for 45 min. The mixture was centrifuged again for another 20 min, at 4 °C, and 13000 rpm. The surface layer was thrown out, and the residue was added 500 µl cold 75% ethanol and was centrifuged for 10 min, at 7800 rpm and 4 °C. The last step was repeated one more time. As the final step, the surface layer was thrown out, and the microtubes were dried out in the dry bath at 56 °C for 10 min after it was added 30 µl Nuclease free water solution (Lot No.00181198, EXIQON, Denmark). Extracted RNA was stored at -80 °C upon analysis.

cDNA synthesis

First, the OD (quality and concentration) of RNA samples were calculated using NanoDrop 2000c (Thermo, USA). After that, cDNA was synthesized by the EXIQON kit. Based on the protocol, 5 µg/µl RNA was needed for cDNA synthesis. Afterward, 2µl 5x reaction buffer (EXIQON Lot NO.176643, Denmark), 1 µl nucleic mix, and 1 µl reverse transcriptase was added to a microtube. The temperature protocol including 42 °C for 60 min, 95 °C for 5 min, and 4 °C for storage. cDNA for mRNA performed by biofact kit (Seoul, South Korea) according to manufacturer protocols. Briefly, 5 µg/µl total RNA, 1 µl Oligo (dT), 1 µl Random hexamer primers and 10-enzyme mixture were added to the 0.1 µl microtube. The temperature protocol including 25 °C for 5 min, 42C for 60 min, 85 °C for 5 min, and 4 °C for storage. The

microtubes were later transferred to a thermal cycler (BioRad model T100 Thermal Cycle, SN 621BR141187, USA).

qRT-PCR

After that, samples were analyzed by qRT-PCR. First, 4 µl cDNA which was diluted 1 to 80 were transferred to PCR microtubes. Second, a 5 µl master mix was also added to microtubes. Master mix is a premixed concentrated solution of SYBER green (EXIQON Lot No. 203421, Denmark), dNTP, 1 µl primer, and U6. U6 is used as the internal control. 1 µl has-miR-146a (EXIQON ID: 204483, Denmark) was added to the mixture as well. Finally, we put the microtubes in Light Cycle 96 (Roche REF: 05815916001 J SN: 11769 Germany). All three steps were programmed according to the protocol, which was 95 ºC for 10 seconds in the first step, 60 ºC for 60 seconds in the second step, and melting in the final step. According to the results, the optimum dosage of miR-146a was 10 pmol, and the optimum time was 24 h. We also used qRT-PCR to determine which of the genes involved in migration were affected by miR-146a (Microcynth, Switzerland) restoration. MMP9, MMP2, vimentin, B-catenin, and CXCR4 (Sinaclon, Iran) were the genes in question. We used 18s rRNA (Sinaclon, Iran) as an internal control. The sequences of the primers were represented in table 1.

Cytotoxicity assay

MTT assay was used to evaluate the viability of cells. In this method, we used Tetrazolium salt. We seeded 15×10^3 MDA-MB-231 cells in a 96 well plate and incubated them for 24 h. After the passing time, cells were transfected in a triplicate way for more accuracy with three dosages of miRNA-146a (1 pmol, 5 pmol, 10 pmol) for 24 h. Then, 50 µl MTT solution (2mg/ml Bio Basic‹Lot No. DU21373R2, Canada) was added to each well, and plates were incubated for 4 h. After 4 h, the surface layer was thrown out and the cells were washed by 100 µl PBS (Gibco ‹Lot No. 2259817 ‹USA). Next, 200 µl Dimethyl sulfoxide (DMSO) was added to each well, and plates were incubated at 37 °C for 30 min. The values of the optical density of the cells were evaluated at 570 nm with an ELISA Reader (Sunrise RC, Tecan, Switzerland).

Scratch test (wound healing assay)

Cells were seeded in 2 wells of a 12 well plate, approximately 15×10^4 cells in each well. Cells were then incubated for 72 h to reach the right confluency. One well was transfected by 10 pmol (optimum dosage) of miR-146a. Before transfection, using the tip of a yellow micropipette we created a wound gap in the bottom of the plate. Then started taking photos at once. The first photo was taken immediately after transfection (time point 0), and the second one was taken after 6 h. Then, more photos were taken at 12, 24, and 48 h after transfection to determine the cell migration in the MDA-MB-231 cell line induced by miR-146a.

DAPI staining

First, 5000 cells were seeded in a 96 well plate. Later, the cells were transfected with the optimum dosage of miR-146a (10 pmol resulted from q-RTPCR). 24 h later, the media was thrown out, and cells were washed with PBS. Next, 200µl paraformaldehyde 4 % was added to each well, and plates were incubated for 60 min to fix the cells. After that, the cells were washed with PBS and then added 200 µl triton 0.1% and were left at room temperature for 10 min to reduce the surface tension. Finally, the cells were washed with 200 µl PBS and stained with DAPI (4′, 6-diamidino-2-phenylindole). This fluorescent color that binds to DNA in adenine- thymine enriched regions, enters the cells through the cell membrane; thus, it is an efficient way to observe the viable cells. 10 min later, the solution was aspirated and 200 µl PBS was added, and the plate was taken to the imaging system (citation 5; Biotek).

Flow cytometry

After miR-146a restoration annexin V/propidium iodide (PI) assay was used to estimate apoptosis in MDA-MB-231 cell lines. The microtubes were divided into two groups: control

groups and transfected by the miR-146a group. In brief, the detached cells were centrifuged at 1300 rpm for 5 min. Then, the cells were stained with an Annexin V- FITC/PI staining assay kit according to the manufacturer's protocol (Roche). After that, microtubes were kept at RT for 15 min in the dark. Both groups were evaluated by the flow cytometer instrument (MACS Quant 10; Miltenyi Biotec, GmbH, Germany). Later, FlowJo software (Tree Star, San Carlos, CA) was used to evaluate the apoptosis rate.

Statistical analysis

All data are shown as the mean ± SEM. GraphPad Prism 6 software (San Diego, CA, USA) was applied for statistical analysis. One-way analyses of variance were done to demonstrate statistical differences among groups, followed by the Tukey test. The P values smaller than 0.05 were considered statistically significant.

Results and discussion

To the extent of our knowledge from this research and other related researches, miR-146a has been known to be a metastasis suppressor[36,37,38] in different cancers like prostate and gastric cancer.[39,40] However, the effects of miR‐146a‐5p on cancer-related properties in the MDA-MB-231 breast cell line are unclear. Here, we conducted a comprehensive bioinformatic analysis and a set of different in vitro experiments to determine its role in breast cancer development.

Bioinformatics analysis of miR-146a expression in breast cancer samples

According to the data extracted from the TCGA dataset BRCA project, the expression level of miR-146a was decreased in primary breast cancer tissues (n=749) compared to adjusted normal tissues (n= 76) (pvalue= 5.78×10^{-5}) (Figure 1).

Optimal miR-146a restoration was the dose-dependent manner in 48 h

Quantitative real-time PCR was used after transfecting cells with1 pmol, 5 pmol, and 10 pmol miR-146a to determine the optimum dosage based on the expression of miR-146a. The optimum dosage of miR-146a was determined10 pmol. The optimum time was 48 h (Figure 2).

MiR-146a was not able to decrease cell viability

The MTT assay was done to show cell viability. We realized miR-146a restoration has no effect on cell viability in the MDA-MB-231 cell line (Figure 3).

MiR-146a is not involved in cell apoptosis

DAPI staining and AnnexinV/PI assays were performed to show the effect of miR-146a on apoptosis in the MDA-MB-231 cell line. According to their results, miR-146a restoration does not induce apoptosis, therefore nucleus fragmentation was not visible in the images (Figure 4A) and There was not any change between the number of events in early and late apoptosis flow cytometric quadrants (Figure 4B).

MiR-146a suppressed breast cancer cell migration

A wound-healing assay was performed to measure migration in the MDA-MB-231 cell line. The results were gathered by images that were taken by citation 5 instruments. Based on the results, breast cancer cell migration was inhibited after 48 h of miR-146a transfection (Figure 5).

MiR-146a decreased MMP9, MMP2, and CXCR4 expression

MiR-146a downregulated the expression of MMP9, MMP2, CXCR4, vimentin, and overexpression of β -catenin. MMP9 was reduced to 0.5 fold, CXCR4 to 0.6 fold, MMP2 to 0.8 fold, and vimentin to 0.2 fold; However, B-catenin was increased to 1.5 fold. Therefore, a reduction in cell migration was observed (Figure 6).

Similar to our results, in a study by Hurst, D.R, et al. on the MDA-MB-231 cell line, miR-146a was upregulated by breast cancer metastasis suppressor 1 (BRMS1) as an inhibitor of metastasis in cancerous cells in breast tissue. This research has indicated miR-146a, an inhibitor of invasion and migration and metastasis in MDA-MB-231 cell line in breast cancer into the lung tissue.[41,42] Tao, et al. reported the induction of apoptosis by miR-146a upregulation under the effect of quercetin in the MDA-MB-231 cell line in 2015[41] however, the results from this research showed that miR-146a is not involved in the process of apoptosis in MDA-MB-231 cell lines. It was also shown that transfecting MDA-MB-231 cells that were treated with quercetin bymiR-146a inhibit cell growth.[43] In contrast, we concluded in this study that cell viability is not affected by miR-146a restoration. In the present study, we investigated the expression level of MMP9, MMP2, CXCR4, B-catenin, and vimentin, genes involved in metastasis in breast cancer, and indicated a significant relationship between the expression of these genes and the level of expression of miR-146a. Based on the data, CXCR4 expression is downregulated by the overexpression of miR-146a.[44] The previous studies on CXCR4 have pinpointed that CXCR4 is a metastasis inducer thus, its down-regulation leads to inhibition of metastasis.[45,46] MMP2 and MMP 9 are both considered important factors in the induction of metastasis.[47,48] In this research, our findings were inlined with other studies on metastasis in breast cancer, which detected an increase in the expression of MMP2 and MMP 9 in breast cancer.[33,49] As stated, transfection of MDA-MB-231 cells by miR-146a reduced the expression of MMP2 and MMP9.[50]

As reported in other studies, loss of expression in B-catenin induces metastasis in MDA-MB-231 cancerous cells.[51] In line with our results, a lower expression of miR-146a leads to a lower expression of β-catenin and results in metastasis. Thus, miR-146a induction upregulates the expression of β-catenin and therefore, inhibits metastasis in the MDA-MB-231 cell line. Vimentin overexpression is known to be an important factor in increased metastasis in different cancers[52,53] including breast cancer.[54] Among all the proteins, vimentin was reduced the most, which led us to conclude that its expression is affected by miR-146a induction. Overexpression of MMP2, MMP9, CXCR4, and decrease in b-catenin expression level induces expression of vimentin[30] (Figure 7).

Conclusion

In summary, in this study, we indicated that miR-146a is a metastasis inhibitor in the MDA-MB-231 cell line. Consequently, it could be an alluring therapeutic strategy to inhibit metastasis in breast cancer by targeting the genes above. The clinical analysis through the TCGA dataset showed miR-146a is decreased in primary breast cancer tissues compared to normal breast tissues. These results are consistent with the results of Yongqing et al., which showed both miR-146a and miR-146b, were decreased in breast cancer tissues.[55] In the other study, Li et al. showed the lower expression of miR-146a in pancreatic cancer tissues compared to normal samples.[39] Our findings also recommend miR-146a as an apt candidate for future microRNA replacement therapy, here the objective to inhibit the metastasis in breast cancer.

Conflict of Interest

The authors have no conflicts of interest to declare.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research (Ethical code: IR.TBZMED.REC. 1398.321).

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Figure legends

Figure 1. MiR-146a was decreased in breast cancer primary tumors. The data extracted from the BRCA project from the TCGA dataset.

Figure 2. MiR-146a restoration in a dose-dependent manner after 48h. The highest miR-146a restoration was displayed at 10 pmol concentration. Besides, the optimal time for miRNA induction was approved 48 h after transfection. **P-value <0.01, ***P-value <0.001, and ****P-value <0.0001.

Figure 3. MiR-146a did not have cytotoxicity effects on breast cancer cells.

Figure 4. MiR-146a does not induce apoptosis in breast cancer cells. DAPI staining did not show any significant change in MDA-MB-231 nucleus fragmentation between miR+142a transfected cells and negative control (NC CTRL) group (A), AnnexinV/PI apoptosis assay did not show any significant change in early and late apoptosis (B).

Figure 5. MiR-146a inhibited breast cancer cell migration. Transfection of miR-146a into MDA-MB-231 cells decreased breast cancer migration. (A) Images of scratched areas of control and transfected with miR-146a areas respectively undergone wound healing assay, 0, 24, and 72 h after the scratch mark. (B) Numbers of the migrated cells in the scratched area. ****P-value <0.0001.

Figure 6. MiR-146a reduced metastatic related genes in breast cancer cells. MiR-146a transfection decreased MMP2, MMP9, Vimentin, and CXCR4 mRNA expression. Besides, this miRNA increased β-catenin mRNA after transfection. **P-value <0.01, ***P-value <0.001, and ****P-value <0.0001.

Figure 7. MiR-146a restoration reduces the expression of MMP2, MMP9, and CXCR4 and results in inhibiting metastasis. However, overexpression of B-catenin leads to metastasis suppression. Downregulation of vimentin is due to the induction of miR-146a and overexpression of B-catenin and lower expression of MMP2, MMP9, and CXCR4 causes suppression in metastasis.

Table 1. miR- 146a and primer sequences in Real-Time PCR.

Target Gene	Strand	Sequence
Hsa- miR- 146a		5′-UGAGAACUGAAUUCCAUGGUU-3′
U6	Forward	5′-GGCAGCACATATACTAAAATTGG-3′
	Reverse	5′-AAAATATGGAACGCTTCACGA-3′
MMP2	Forward	5′-GCCCTCCTGGGAATGAAGCAC-3′
	Reverse	5′-GCATTGCCTCTGGACAACACA-3′
MMP9	Forward	5′-ATTCATCTTCCAAGGCCAATCC-3′
	Reverse	5′-CTTGTCGCTGTCAAAGTTCG-3′
CXCR4	Forward	5′-TCTTCCTGCCCACCATCTACTC-3′
	Reverse	5′-TGCAGCCTGTACTTGTCCGTC-3′
Vimentin	Forward	5′-CAGGCAAAGCAGGAGTCCA-3′
	Reverse	5′-AAGTTCTCTTCCATTTCACGCA-3′
Vimentin	Forward	5′-CACAAGCAGAGTGCTGAAGGTG-3′
	Reverse	5′-GATTCCTGAGAGTCCAAAGACAG-3′
β-catenin	Forward	5′-CACAAGCAGAGTGCTGAAGGTG-3′
	Reverse	5′-GATTCCTGAGAGTCCAAAGACAG-3′
18s	Forward	5′-GCTTAATTTGACTCAACACGGGA-3′
	Reverse	5′-AGCTATCAATCTGTCAATCCTGTC-3′