MicroRNA-126-5p Inhibits the Migration of Breast Cancer Cells by Directly Targeting CNOT7

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Abstract

**Background:** To assess the effect of microRNA-126-5p (miR-126-5p) on the migration of the breast cancer MCF7 cell line.

**Methods:** GSE143564 was downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) to identify the differentially expressed miRNAs between breast cancer and adjacent tissues. Quantitative reverse transcription PCR (RT-qPCR) was used to assess miR-126-5p levels in the normal 184A1 breast cell line and the breast cancer MCF7 cell line. The MCF7 cell line was then transfected with miR-126-5p mimics or corresponding negative control (NC-mimic). The proliferation and migration abilities of the MCF7 cell line were measured by methyl thiazolyl tetrazolium (MTT), Transwell and scratch healing assays. CCR4-NOT transcription complex and subunit 7 (CNOT7) expression levels in the NC-mimic and miR-126-5p mimic groups were measured by Western blot analysis. Bioinformatic analysis and a dual-luciferase reporter assay were performed to identify the miR-126-5p target gene.

**Results:** One hundred forty-eight differentially expressed miRNAs (downregulated = 55, upregulated = 93) were identified. MiR-126-5p expression in the MCF7 cell line was significantly downregulated relative to that of 184A1 cell line (P < 0.05). Compared with that observed in the control and NC-mimic groups, cell proliferation in the miR-126-5p mimic group was significantly decreased at 48 and 72 h posttransfection (P < 0.05). In addition, the scratch healing rate and number of membrane-piercing cells in the miR-126-5p overexpression group were lower than those detected in the control and NC groups (P < 0.05). Furthermore, miR-126-5p could reduce the luciferase activity for the wild-type CNOT7 gene 3'-untranslated region (UTR) reporter (P < 0.05) but had no effect on the mutant 3'UTR reporter (P > 0.05). Compared with that observed in the NC and control groups, the levels of CNOT7 in the miR-126-5p overexpression group decreased (P < 0.05).

**Conclusion:** Upregulation of miR-126-5p can inhibit the migration of the breast cancer MCF7 cell line, which may involve its direct targeting of the 3'UTR of CNOT7.

**Keywords**

miR-126-5p, CNOT7, breast cancer, migration

Received: June 16, 2020; Revised: October 22, 2020; Accepted: October 28, 2020.

Introduction

Breast cancer remains the primary cause of cancer-related deaths worldwide.¹,² For patients with late-stage breast cancer, the total 5-year survival rate is only approximately 15%, which is primarily due to lymph node invasion or conditions with higher transfer rates such as bone transfer and brain metastases.³ Multiple chemotherapy and radiation resistance are also significant factors.⁴ Therefore, studying the molecular mechanisms of breast cancer development and identifying new treatment targets are important for improving the treatment of patients.⁵,⁶

MicroRNAs are small fragments of endogenous single-chain noncoding RNA that are approximately 19 ~ 25 nt in length, highly conserved, and involved in all aspects of cell biological function, such as cell proliferation, differentiation,

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migration and apoptosis. MiRNAs can play a carcinogenic or tumor suppressive roles in cancer progression by specifically binding to mRNA of the target gene. It is estimated that the expression of mRNAs for more than 10,000 genes in the human genome is regulated by miRNAs. Interestingly, miR-126-5p has been shown to act as a tumor suppressor gene in many types of cancer and has also been observed to be abnormally expressed in endometriosis. Furthermore, a previous study showed that miR-126-5p downregulates BCAR3 expression to promote cell migration and invasion in endometriosis.

However, little is known regarding the mechanism of miR-126-5p in breast cancer progression.

Therefore, in the present study, we examined the role and mechanism of miR-126-5p in the regulation of migration of MCF7 cells. We observed that miR-126-5p could directly bind to CCR4-NOT transcription complex and subunit 7 (CNOT7) mRNA to regulate MCF7 cell migration.

Materials and Methods

Bioinformatic Analysis of GSE143564

GSE143564 was downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). GSE143564 was based on the Affymetrix Multispecies miRNA-4 Array [Probe Set ID version] platform and included 6 samples (3 breast tissues and 3 adjacent tissues). Quantile normalization was used to minimize inconsistencies in the GSE series. Differentially expressed miRNAs between breast and adjacent tissues were identified with the Limma package in the R software environment and were plotted using a volcano plot and heatmap using the R packages “ggplot2” and “pheatmap.” Then, we selected the most significant differentially expressed miRNAs for further analyses. Three miRNA target gene databases (TargetScan, miRanda and miRDB) were used to predict the target genes of miRNAs. Then, a Venn diagram was constructed using the online tool jvenn (http://www.bioinformatics.com.cn/static/others/jvenn/example.html) to overlap the genes obtained from these 3 databases. Subsequently, enrichment analysis was performed using the Metascape online tool (http://metascape.org). MiR-126-5p and its target gene network were constructed with Cytoscape (http://apps.cytoscape.org/).

Breast Cancer and Adjacent Tissue

Twenty primary breast cancer tissue samples and 20 corresponding adjacent normal mammary tissue samples were obtained from XuZhou Central Hospital. The diagnosis of breast cancer was pathologically confirmed, and only tumor-node-metastasis (TNM) stage III patients were included. The patients had not received hormone or adjuvant therapy prior to breast cancer surgery. All patients and their families agreed to participate in the experiment and provided written informed consent. This study was approved by the Research Ethics Committee of XuZhou Central Hospital (approval no: XZCH-2018-0515, date: 15 March 2018). All tissues were stored in RNA preservation solution and used for RNA extraction.

Cell Culture and Transfection

MCF7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin in standard conditions in a humidified CO2 incubator at 37°C and under an atmosphere with 5% CO2. When the cells reached approximately 80% confluency, they were digested with 0.25% trypsin and passaged. Cells were inoculated into 6-well plates at a density of 1×10⁵ cells per well and then changed to serum-free medium when they reached approximately 50~60% confluency. MCF7 cells were transfected with miR-126-5p mimics (overexpression group), negative control (NC group), pcDNA and pcDNA-CNOT7 using Lipofectamine® 2000 according to the manufacturer’s instructions. The assays were repeated in 3 independent assays in triplicate.

Methyl Thiazolyl Tetrazolium (MTT) Assay

MCF7 cells were cultured into a 96-well plate (1×10⁴ cells per well). After transfection, 5 µg/µl of MTT solution was added to each well for 0, 24, 48, or 72 h. After culturing for 4 h, dimethyl sulfoxide was added to stop the reaction. The absorbance values of each well at 490 nm were detected by a microplate reader, and the cell proliferation in each well was determined using a blank as the control.

Cell Scratch Experiment

MCF7 cells were cultured to monolayers in a routine manner. Then, a straight line was scratched into the monolayer with a sterilized yellow tip to form a cell-free growth zone (i.e., a scratch). The width of the scratch was approximately 500 µm, and the relative distance of the scratch zone was recorded. Then, the exfoliated cells were washed away in serum-free medium. After conventional culture in a constant temperature incubator for 4 h, the exfoliated cells were washed away, the adhered cells were observed and imaged under an inverted microscope, and the relative distance of cell migration from the injured area was analyzed.

Cell Invasion Assay

Transwell chambers (8 µm aperture) were used to assess cell migration. MCF7 cells (5×10⁴) were inoculated in serum-free medium and added to the upper chamber, while the lower chamber contained medium with 10% serum. After 48 h of culture, the cells that did not pass through the upper cavity were removed and washed. Then, the cells were fixed in 4% paraformaldehyde for 30 min, stained with crystal violet, and then observed under an optical microscope and imaged. Five fields were randomly selected for counting in each group.
 Luciferase pmirGLO reporter plasmids were viewer (http://www.bioinformatics.com.cn/static/others/jvenn/do). A Venn diagram was generated using Venn diagram and miRanda (www.microrna.org/microrna/getDownloads), miRD B (http://mirdb.org/miRDB/), miRDB (http://mirdb.org/miRDB/), and TargetScan, miRanda and miRDB databases. Twenty-one overlapping genes were identified (Figure 1B) such that miR-126-5p and target genes, respectively. Primer sequences are presented in Table 1. The quantitative results were determined using the 2^{-\Delta \Delta Ct} method.

### Western Blot Analysis

After being transfected for 48 h, cells were lysed with RIPA lysis buffer supplemented with protease inhibitors to obtain total cell protein. Then, the extracted protein concentration was determined using a BCA assay kit (Solarbio, Beijing, China). Subsequently, 20 μg of total protein for each sample was separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% nonfat dried milk, the membranes were incubated with a primary goat anti-human CNOT7 primary antibody (Abcam, 1:2000, USA) at 4°C overnight followed by an incubation with an HRP-labeled secondary antibody for 1 h. Lastly, the enhanced chemiluminescent method was used to detect the protein bands.

### Dual-Luciferase reporter Assay

A potential target gene of miR-126-5p was identified from the overlapping results of 3 databases: TargetScan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/miRDB/) and miRanda (www.microrna.org/microrna/getDownloads.do). A Venn diagram was generated using Venn diagram viewer (http://www.bioinformatics.com.cn/static/others/jvenn/example.html). Luciferase pmirGLO reporter plasmids were generated harboring the wild-type (WT) or mutated (MUT) 3′-untranslated region (3′UTR) of the CNOT7. The cells were cotransfected with the dual-luciferase reporter plasmids (WT/MUT plasmids) and miR-126-5p mimics or NC, and the dual-luciferase assay kit was used to determine the luciferase activity, where the firefly luciferase activity was normalized to that of marine kidney luciferase.

### Statistical Analysis

SPSS 20.0 was used for statistical analysis. The data are presented as the means and standard deviation (SD). One-way ANOVA was used for multigroup comparison, and the Student-Newman-Keuls (SNK) method was used for pairwise comparison. Comparison of the means between 2 groups was performed using the 2-tailed Student’s t-test (parametric) or the Mann-Whitney U test (nonparametric). Pearson correlation analysis to evaluate differences in expression between miR-126-5p and CNOT7. P < 0.05 was considered statistically significant.

### Results

#### Analysis of Differentially Expressed miRNA Expression Profiles

The box plots of the log expression values for all genes in each group (breast tissues versus adjacent tissues) before and after normalization were mapped (Figure 1A). After normalization, the median values of each sample were extremely similar, suggesting that the data should be further analyzed. After normalization, 148 differentially expressed miRNAs (downregulated = 55, upregulated = 93) were identified (Figure 1B), the top 100 of which are shown as a heatmap (Figure 1C). Among the differentially expressed miRNAs, miR-126-5p was the most significantly downregulated and was therefore chosen for an in-depth study. Twenty-one overlapping genes were identified from the TargetScan, miRanda and miRDB databases (Figure 1D). Enrichment analysis showed that these target genes were primarily enriched in pentose and glucuronate interconversions, homophilic cell adhesion via plasma membrane adhesion molecules, cellular hormone metabolic process, phenylpropanoid metabolic process, chloride transport, glycosphingolipid biosynthetic process, GPCR ligand binding, cellular response to drug, mononuclear cell migration, leukine metabolic process, negative regulation of phospholipase activity, positive regulation of T cell cytokine production, negative regulation of melobod cell differentiationation, folate biosynthesis, protein localization to cilium, positive regulation of NIK/NF-kappa B signaling, positive regulation of interleukin-23 production, alcohol metabolic process and negative regulation of response to DNA damage stimulus (Figure 1E). Figure 1F shows the interaction between miR-126-5p and its target genes.

#### MiR-126-5p Is Downregulated in MCF7 Cells

As shown in Figure 2A, miR-126-5p expression was decreased in breast cancer tissue relative to that observed in adjacent tissues (t = 41.53, degree of freedom [df] = 38, P < 0.0001). In contrast, CNOT7 expression was significantly increased in breast cancer tissue relative to that observed in adjacent tissues (t = 25.19, df = 38, P < 0.0001, Figure 2B) such that miR-126-
Figure 1. miR-126-5p is downregulated in breast cancer tissues. (A) Comparison of the expression values before and after normalization. (B) Volcano plot of the differentially expressed miRNAs between breast cancer and adjacent tissues. Green dots represent downregulated miRNAs, and red dots represent upregulated miRNAs in breast tissues; black dots represent normally expressed miRNAs. (C) Heatmap showing the profiles of the top 100 differentially expressed miRNAs between breast and adjacent tissues. Green and red indicate low and high expression, respectively. (D) Venn diagram results showing the overlapping predicted genes from the TargetScan, miRanda and miRDB databases. (E) Top 20 enriched gene ontology and KEGG pathway terms for the overlapping predicted genes. (F) Regulatory network between miR-126-5p and its target genes.
5p expression was negatively correlated with that of CNOT7 in breast cancer tissue \( (r = -0.772, P = 0.040, \text{Figure 2C}) \).

In addition, miR-126-5p expression was observed to be decreased in MCF7 cells relative to that detected in 184A1 cells \( (t = 37.46, df = 10, P < 0.0001, \text{Figure 2D}) \). In contrast, the expression of the target gene of miR-126-5p, CNOT7, was increased in MCF7 cells relative to that detected in 184A1 cells \( (t = 41.88, df = 10, P < 0.0001, \text{Figure 2E}) \). Further study revealed that the relative expression of CNOT7 and miR-126-5p were negatively correlated \( (r = -0.734, P = 0.002, \text{Figure 2F}) \).

The MTT results showed that the proliferation of cells in the miR-126-5p overexpression group was significantly decreased at 48 and 72 h relative to that observed in the control group \( (P = 0.0001) \). There was no significant difference in the proliferation between the control and NC groups at 0 and 24 h \( (P > 0.05) \), as shown in Figure 2G.

**Effects of miR-126-5p Overexpression on MCF7 Cell Migration**

Transwell assay results showed that the number of membrane-penetrating cells in the control, the NC and miR-126-5p overexpression groups was 100 ± 12, 105 ± 8 and 65 ± 4 cells/vision, respectively. Compared with that observed in the control and NC groups, the number of membrane-penetrating cells in the miR-126-5p overexpression group was significantly decreased \( (F = 132.8, P < 0.0001, \text{Figure 3A}) \).
The wound healing assay results showed that the migration distance of cells in the control, the NC and the miR-126-5p overexpression groups was $5.0 \pm 1.0$, $5.2 \pm 0.6$ and $1.1 \pm 0.4$ μm/24 h, respectively. Compared with the control and NC group, the migration distance in the MCF7 cell line in the miR-126-5p overexpression group was significantly decreased ($F = 314.6$, $P < 0.001$, Figure 3B).

**MiR-126-5p Can Directly Bind to CNOT7**

TargetScan predicted that the 3’UTR of the CNOT7 gene harbors a complementary site that could bind to miR-126-5p (Figure 4A). Subsequently, dual-luciferase reporter gene assay results showed that miR-126-5p mimics could reduce the luciferase activity of the WT CNOT7 3’UTR reporter ($P < 0.0001$, Figure 4B) but had no effect on the reporter with the mutant 3’UTR ($P = 0.971$, Figure 4B).

**Effects of miR-126-5p Overexpression on CNOT7 in MCF7 Cells**

To assess the effects of CNOT7 on MCF7 cell migration, MCF7 cells were transfected with miR-126-5p mimic alone or combined with pcDNA-CNOT7. The inhibitory effect of miR-126-5p on the migration of MCF7 cells was partially reversed by cotransfection with CNOT7 (Figure 5A). In addition, the wound healing assay results were similar to those of the Transwell assay (Figure 5B).

Finally, we measured the protein expression of CNOT7 under various conditions. CNOT7 expression in the miR-126-5p mimic group was significantly downregulated. However, the downregulation of CNOT7 in the miR-126-5p group was partially reversed by cotransfection with pcDNA-CNOT7 (Figure 5C).

**Discussion**

In the present study, GSE143564 was used for bioinformatic analyses of differentially expressed miRNAs between breast cancer and normal adjacent tissue. We observed that miR-126-5p expression was decreased in breast cancer tissue relative to that detected in normal adjacent tissues, which was further analyzed using the MCF7 cell line. Additionally, miR-126-5p was shown to potentially be involved in the regulation of breast cancer cell migration. MiR-126-5p and CNOT7 expression are negatively correlated. Moreover, miR-126-5p could significantly inhibit the migration of MCF7 cells, as demonstrated by Transwell and scratch experiments. MiR-126-5p mimics could significantly decrease the migration distance and migratory cell number. Furthermore, TargetScan and
dual-luciferase reporter analyses revealed that miR-126-5p could significantly bind to the CNOT7 3’UTR.

Abnormal miR-126-5p has been shown to be associated with the pathological processes of various diseases. Wang et al. reported that miR-126-5p binds to the Becl21 mRNA 3’-UTR and promotes cell apoptosis in cervical cancer. Thus, miR-126-5p plays an inhibitory role in human cervical cancer progression and may be a potential therapeutic target for cervical cancer. In addition, Sun et al. observed that the miR-126-5p/YAP1/MALAT1 axis can control angiogenesis and the epithelial-mesenchymal transition in colorectal carcinoma.

Recently, miR-126-5p has been shown to be useful in different types of cancer as a tumor suppressor. Fiala et al. found that miR-126-5p expression was significantly associated with progression-free survival and overall survival in metastatic colorectal cancer. In another study, Gu et al. explored the relationship between miR-126-5p and esophageal cancer cells and observed that miR-126-5p could target CDK6 and has a positive role in inhibiting the proliferation and migration of esophageal cancer cells.

However, studies on miR-126-5p in breast cancer are scarce. In the present study, miR-126-5p expression in breast cancer and normal adjacent tissue was assessed. The results showed that miR-126-5p expression in MCF7 cells was significantly downregulated compared to that observed in the normal breast cell line 184A1. MTT, Transwell and scratch experiments were used to assess the effect of miR-126-5p on the proliferation and migration of MCF7 cells. MiR-126-5p mimics reduced breast cancer cell migration, indicating that miR-196b-5p may serve as a novel therapeutic target for breast cancer. MiR mimic therapy is an emerging modality for cancer treatment and has some advantages over siRNA therapy, as miR mimics target multiple genes. However, miR mimics have low serum stability, and developing an effective delivery system is of great importance to realize the full potential of miR therapy. Further experiments will be conducted in the future using in vivo animal models, which will further reveal the effects of miR-126-5p for the treatment of breast cancer.

CNOT7 expression is altered in acute leukemias and may represent a promising biomarker and drug target for these diseases. Maragozidis et al. revealed that CNOT7 is differentially expressed in squamous cell lung cancer clinical samples. Furthermore, CNOT7 was identified as a diagnostic marker for lung cancer patients. In the present study, we also observed that CNOT7 was highly expressed in MCF7 cells relative to that detected in the normal breast cell line. Therefore, we performed a dual-luciferase reporter assay to confirm that miR-126-5p could directly target the 3’UTR of CNOT7.

The results of the present study predicted and verified that CNOT7 is a target gene of miR-126-5p and that CNOT7 exhibited the opposite expression pattern as miR-126-5p. Upregulating miR-126-5p significantly inhibited CNOT7 expression. CNOT7 has been shown to be overexpressed in a variety of human cancers and to play a carcinogenic role in tumor development. For example, Maragozidis et al. showed that PARN, CNOT6, CNOT7 and NOC, 4 deadenylases, are differentially expressed in lung cancer clinical samples. In the present study, we showed that miR-126-5p directly binds to the 3’UTR of CNOT7 to inhibit breast cancer progression.
In summary, miR-126-5p may serve as a tumor suppressor and inhibit the migration of breast cells by directly binding to CNOT7. Therefore, miR-126-5p may act as a potential diagnostic marker and therapeutic target of breast cancer. However, how miR-126-5p regulates the epithelial-mesenchymal transformation and angiogenesis of breast cancer through other signaling pathways and complex regulatory networks requires further research.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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