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miR-760 mediates chemoresistance through inhibition of epithelial mesenchymal transition in breast cancer cells

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Abstract. – OBJECTIVE: Therapeutic resistance has been a great obstacle for successful treatment of breast cancer. Our study aimed to explore the role of microRNA-760 (miR-760) in chemoresistant breast cancer cells.

MATERIALS AND METHODS: Real-time PCR was performed to measure the mRNA expression of miR-760 and Nanog. Western blot was used to determine the protein expression of Nanog and mesenchymal and epithelial markers. Cell viability was measured by the CCK-8 assay.

RESULTS: Our results showed that the expression of miR-760 was significantly reduced the doxorubicin (DOX)-resistant MCF-7/DOX cells and chemoresistant breast cancer tissues. Moreover, up-regulation of miR-760 sensitized breast cancer cells to the anti-cancer agents. The MCF-7/DOX cells exhibited increased expression of Snail, a mesenchymal marker, and decreased levels of E-Cadherin, an epithelial marker. In addition, overexpression of miR-760 suppressed the expression of Nanog, a transcriptional factor involved in chemoresistance, and resulted in the reversal of EMT in breast cancer cells.

CONCLUSIONS: Our study demonstrated that miR-760 modulated chemoresistance through the epithelial-mesenchymal transition in breast cancer cells, providing a potential therapeutic target for treatment of drug-resistant breast cancer.

Key Words:
Breast cancer, Drug resistance, miR-760, Epithelial-mesenchymal transition.

Introduction

Breast cancer is the most frequently diagnosed tumor after skin malignancies, ranking the second leading cause of cancer-related deaths in women[1]. In medical practices, it still remains difficult to make an early diagnose, which results in the poor prognosis of most breast cancer patients[2]. In addition, both de novo and acquired resistance to chemotherapeutic drugs, radiation or targeted therapy greatly hinder the successful treatment of breast cancer[3].

MicroRNAs (miRNAs) are small noncoding RNA molecules that modulate the expression of target mRNAs at a post-transcriptional level. They primarily bind to the 3'UTR of mRNA transcripts through semi-conservative binding and suppress the translation of up to 60% of mRNA transcripts[4]. Increasing studies have revealed that dysregulation of miRNA expression plays an important role in the cancer initiation, progression and prognosis, as well as acquired resistance to anticancer agents[5-7]. miR-760 has been shown to be regulated by estradiol and target multiple transcripts which belong to the estrogen-responsive gene clusters[8]. Recently, miR-760 is regarded as a potential anti-cancer miRNA with altered expression levels found in colorectal cancer[9]. In a previous study, Lv et al[10] found that miR-760 was significantly down-regulated in breast cancer

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tissues resistant to therapeutic drugs compared to tumor tissues sensitive to anti-cancer drugs. In addition, Lv et al[11] has also revealed that miR-760 is involved in the chemotherapy resistance of breast cancer cells, but its underlying mechanism has remained to be elucidated. In the current study, we explored the role of miR-760 in the chemoresistance of breast cancer cells and its underlying mechanism.

Materials and Methods

Cell Culture

Three breast cancer cell lines including Bcap-37, MCF-7, and MDA-MB-231 were obtained from the ATCC (Manassas, VA, USA). All cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained in an incubator with 5% CO_2 under controlled temperature (37°C).

Cell Proliferation Assay

Breast cancer cells were seeded at the density of $5x10^4$ per ml into 96-well plates for cell viability assay. Drugs were dissolved in complete culture medium and then added to 96-well plates. Adding of complete culture medium only set negative control. Cells were incubated at 37°C for 48 hours and then 10 μL/well CCK8 solution (Dojindo, Kumamoto, Japan) was added to each well. The absorbance of the reaction and measured with a spectrophotometer at 450 nm.

Real-time PCR

Total RNAs were extracted from tumor cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and the RNA concentration was determined by spectrophotometer. cDNA was reversely synthesized and the real-time PCR program was as follows: initial denaturation at 95°C for 1 min, denaturation at 95°C for 15 s, annealing at 60°C for 20 s, extension at 72°C for 40 s (40 cycles) and a final extension step at 72°C for 7 min. Relative gene expression was evaluated using the ΔΔCT method.

Luciferase Activity Assay

Luciferase activity was assessed using the luciferase reporter system based on the firefly luciferase-expressing vector (Ambion, Madison, WI, USA). Cells at the density of $5x10^4$ cells per well were seeded in 96-well plates the day before transfection. Then, cells were transfected with miRNA-760 mimic or control. Two days after transfection, cells were collected and the luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot

For immunoblotting, cells were homogenized in lysis buffer, and boiled for 10 minutes. Protein concentration was quantified with BCA protein quantification kit (Beyotime, Shanghai, China) according to manufacturers' instructions. Aliquots of samples were analyzed by SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). Non-specific bounding proteins were blocked with 5% fat-free milk in TBST. The membrane was incubated with the primary antibodies overnight at 4°C and indicated secondary antibodies for 1 hour at room temperature. Protein bands were visualized using enhanced chemiluminescence and protein band intensity was measured Gel-Pro32 software.

Statistical Analysis

Data were represented as mean ± SD, and subject to SPSS10 software for statistical analysis (SPSS Inc., Chicago, IL, USA). Comparison between groups was made using ANOVA and statistically significant difference was defined as $p<0.05$.

Results

Expression Level of miR-760 was Decreased in Chemoresistant Breast Cancer Cells

Firstly, the breast cancer cells line MCF-7 was incubated with DOX at a stepwise increasing concentration. Then, CCK-8 assay was performed to measure the cellular response in chemoresistant tumor cells (MCF-7/DOX) and chemosensitive cells (MCF-7) (Figure 1A and B). Results showed that the IC50 of DOX at 48 h was significantly increased in MCF-7/DOX compared to MCF-7 parental cells (Figure 1C). To explore the biological role of miR-760 in tumor cell sensitivity, we detected the expression levels of miR-760 in MCF-7/DOX and MCF-7 parental cells by real-time PCR. As a result, we observed a significant reduction of miR-760 in MCF-7/DOX cells compared to their parental cells (Figure 1D). Moreover, the miR-760 levels in chemoresistant tissues were obviously lower than those in the chemosensitive

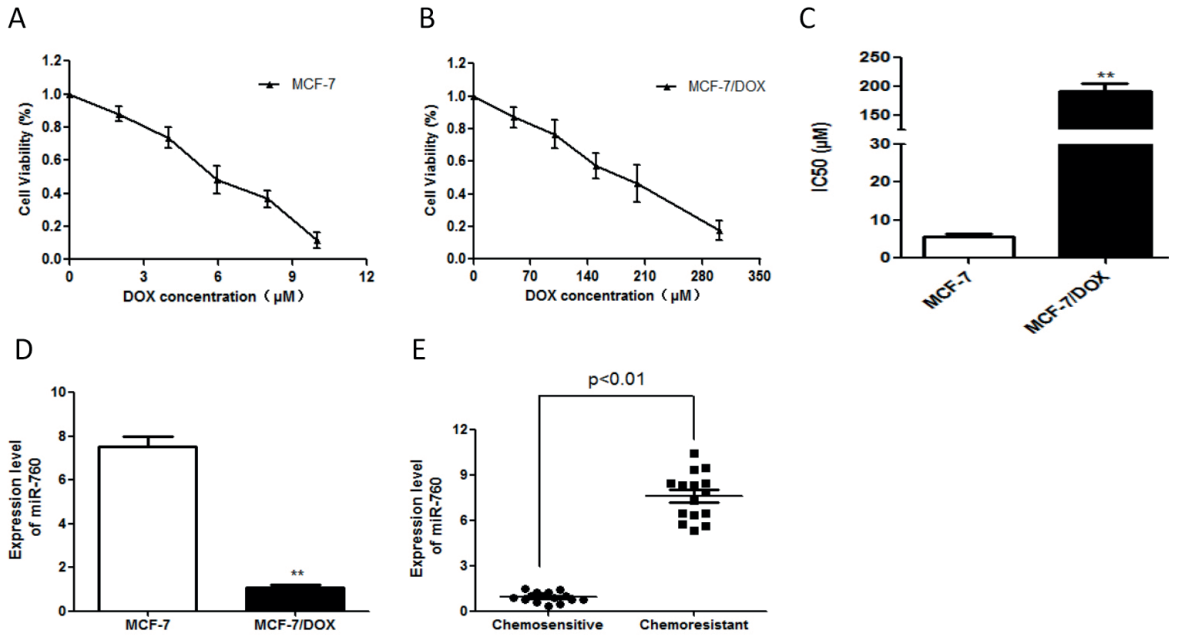

Figure 1. miR-760 expression was decreased in chemoresistant breast cancer cells. CCK-8 assay was used to measure the cell viability in MCF-7 ***(A)*** and MCF-7/DOX ***(B)*** cells treated with doxorubicin followed by determination of IC50 in each cell lines ***(C)***. Real-time PCR was performed to detect the expression of miR-760 in breast cancer cell line ***(D)*** and chemoresistant and sensitive tissues (E). $^{**}p< 0.01$.

tissues (Figure 1E). Taken together, these data implied that miR-760 might be involved in the drug sensitivity of doxorubicin in breast cancer cells.

Overexpression of miR-760 Increased Doxorubicin Sensitivity in Breast Cancer Cells

Given the potential role of miR-760 in drug resistance, we further explored the cellular responses of breast cancer cells to doxorubicin after transfection with miR-760 mimics. Real-time PCR revealed that the expression levels of miR-760 were significantly increased in Bcap-37 (Figure 2A), MCF-7 (Figure 2B), and MDA-MB-231 (Figure 2C) cells. Consequently, overexpression of miR-760 enhanced the cytotoxicity of doxorubicin in Bcap-37 cells (Figure 2D). Moreover, we observed that the sensitivity of MCF-7 and MDA-MB-231 cells was significantly increased after transfection with miR-760 mimics (Figure 2E-F). Taken together, these data suggested that up-regulation of miR-760 sensitized breast cancer cells to anti-cancer agents.

EMT was Associated with Doxorubicin Resistance in Breast Cancer Cells

A large number of studies have demonstrated that EMT program is critically important in the acquired drug resistance in cancer cells[12,13]. Therefore, we detected the expression of EMT-related markers in MCF-7/DOX cells and their parental cells. Real-time PCR showed that doxorubicin-resistant MCF-7 cells exhibited higher expression levels of Snail, a mesenchymal marker, than the doxorubicin-sensitive cells (Figure 3A). Meanwhile, the expression of E-Cadherin, an epithelial marker, was significantly reduced in MCF-7/DOX cells (Figure 3B). In addition, we found that the protein expression of Snail was increased in MCF-7/DOX cells, while E-Cadherin was decreased, which was consistent with the data from real-time PCR analysis (Figure 3C). These results showed that EMT was possibly involved in the cellular response of breast cancer cells to doxorubicin.

miR-760 Regulated Drug Resistance through EMT

miRNAs have been shown to regulate biological processes through targeting various genes; thus, it is critical to revealing the potential target of miR-760 in breast cancer cells. By bioinformatics prediction, we found that Nanog was a potential target of miR-760. Therefore, the mRNA and protein expression of Nanog were measured in doxorubicin-sensitive and resistant breast cancer cells.

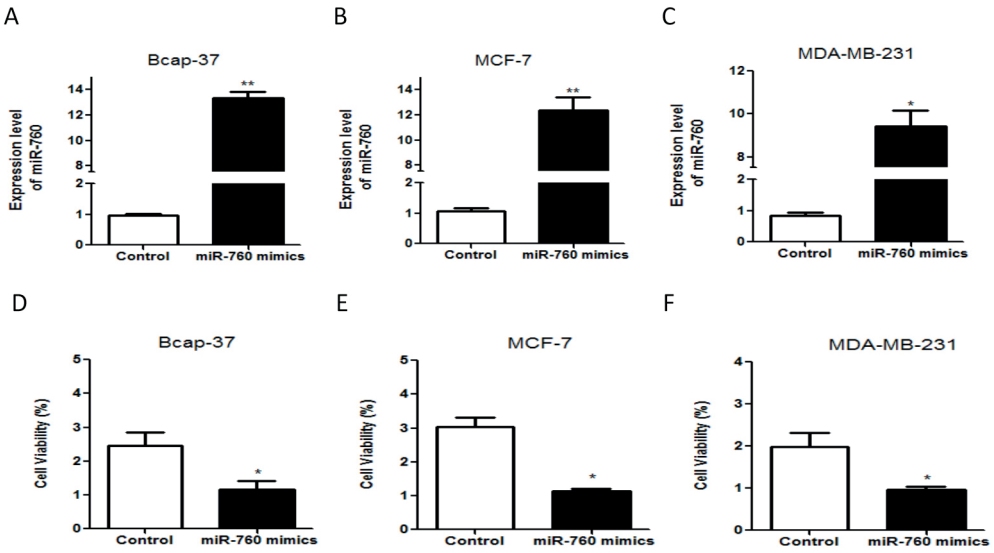

Figure 2. miR-760 regulated doxorubicin sensitivity in breast cancer cells. Three breast cancer cell lines including Bcap-37 ***(A)***, MCF-7 ***(B)*** and MDA-MB-231 ***(C)*** were transfected with miR-760 mimics or negative control. Four-eight hours later, cell viabilities in each cell line were measured by CCK-8 assay ***(D-F)***. $^{*}p<0.05$; $^{**}p< 0.01$.

Real-time PCR showed that the mRNA levels of Nanog were significantly higher in MCF-7/DOX cells compared to their parental cells (Figure 4A). In addition, transfection with miR-760 mimics repressed the mRNA and protein levels (Figure 4B-C) in MCF-7/DOX cells. Furthermore, the luciferase activity of Nanog was inhibited in MCF-7/DOX cells transfected with miR-760 mimics (Figure 4D) in. These data suggested that Nanog was a target of miR-760 in breast cancer cells.

The transcriptional factor Nanog plays an important role in the process of EMT. Thus, we further examined the expression of EMT-related markers. Overexpression of miR-760 significantly inhibited the mRNA expression of Snail, but increased E-Cadherin mRNA levels in MCF-7/DOX cells (Figure 5A-B). Western blot analysis showed that the protein expression of Snail (a mesenchymal marker) was inhibited, while E-Cadherin (an epithelial marker) was increased in chemoresistant breast cancer cells (Figure 5C). Taken together, these results demonstrated that miR-760 modulated the expression of Nanog and the EMT program in breast cancer cells.

Discussion

Currently, acquired chemoresistance has been a growing challenge for successful treatment of patients diagnosed with various cancers. Previous studies have reported the altered expression of miR-760 in breast cancer tissues[11]. However, the role of miR-760 in regulating drug sensitivity

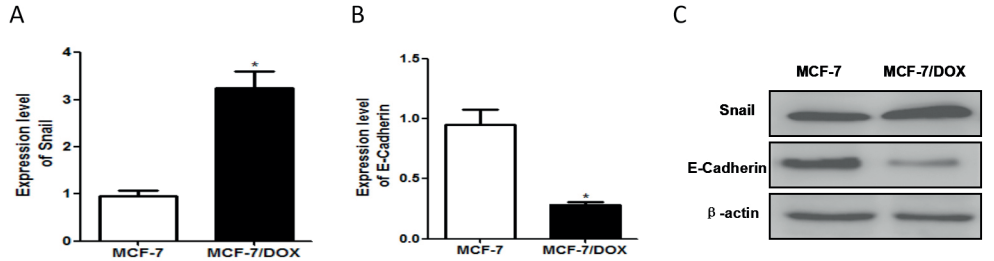

Figure 3. EMT was associated with drug resistance in breast cancer cells. Real-time PCR ***(A*** and ***B)*** and Western blot ***(C)*** were performed to measure the mRNA and protein expression of EMT markers (Snail, a mesenchymal marker; E-Cadherin, an epithelial marker) in MCF-7 and MCF-7/DOX cells, respectively. $^{*}p<0.05$.

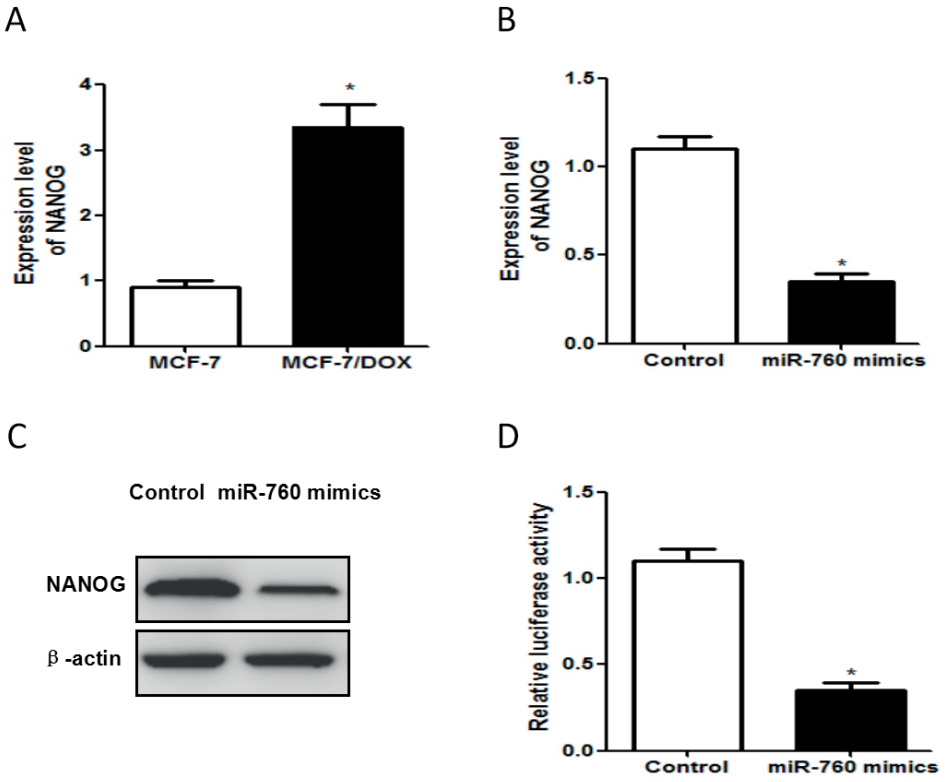

Figure 4. miR-760 inhibited the expression of Nanog. Determination of the mRNA levels of Nanog in MCF-7 and MCF-7/DOX cells ***(A)***. The suppressive effect of miR-760 on Nanog was measured by real-time PCR ***(B)***, Western blot ***(C)***, and luciferase activity assay ***(D)***. $^{*}p<0.05$.

still remains unexplained. In the current study, we aimed to measure the expression pattern of miR-760 in chemosensitive and resistant breast cancer cells and further investigated the sensitizing role in breast cancer cells.

A large number of studies have demonstrated that miRNAs play critical roles in regulating the proliferation, migration and invasion of cancer cells through targeting various genes involved in the oncogenic signaling pathways[14-16]. Of note, miR-760 is a recently identified miRNA, which is thought to play an important role in the initiation and progression of cancers. For example, Wang et al[9] have suggested that plasma miR-760 may serve as a potential biomarker for the early detection of colorectal cancer. Another study has demonstrated that miR-760, together with several other miRNAs, induces cellular senescence via targeting a subunit of protein kinase CKII in colorectal cancer[17]. In addition, the biological relevance between miR-760 and the replicative senescence has been reported in human lung fibroblast cells[18]. Altered expression of miR-760 has been reported in breast cancer[10,11]. However, the role of miR-760 in drug resistance has not been fully elucidated. Our study used an *in vitro* model, doxorubicin-resistant cell line MCF-7/DOX, to investigate the role of miR-760 in chemoresistant breast cancer cells. We found that the expression levels of miR-760 were significantly decreased in MCF-7/DOX cells compared to their parental cells. Similarly, miR-760 expression was also reduced in chemoresistant breast cancer tissues compared to the chemosensitive tissues. In addition, up-regulation of miR-760 was capable to sensitizing breast cancer cells to anti-cancer agents, suggesting that miR-760 was involved in the acquired drug resistance.

As a transcriptional factor, Nanog has been reported to be essential for self-renewal, differentiation, and proliferation of embryonic stem cells[19,20]. Altered expression of Nanog has been observed in several types of cancers, and these studies have shown that increased expression of Nanog is associated with advanced stages of cancer and predicts a poor prognosis[21-23]. In addition, a functional analysis reveals that Nanog plays an important role in tumorigenesis, tumor transformation, and acquired drug resistance[24-26]. Therefore, inhibi-

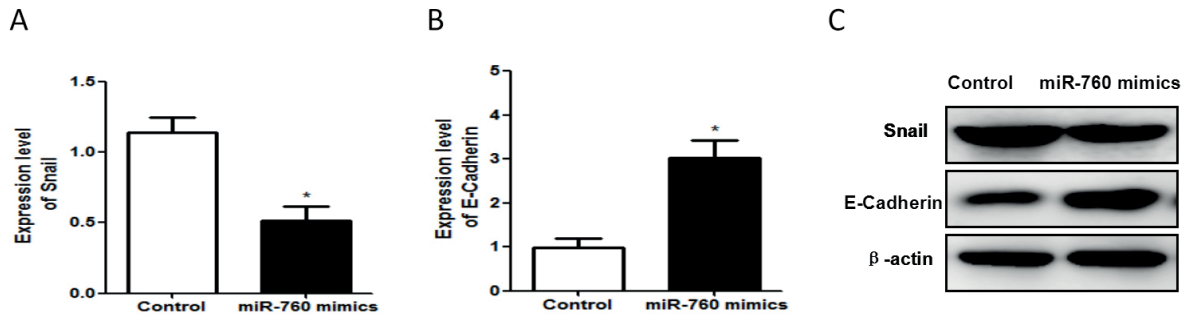

Figure 5. miR-760 modulated the expression of EMT-related markers. MCF-7/DOX cells were transfected with miR-760 mimics or negative control. Then, the mRNA ***(A*** and ***B)*** and protein ***(C)*** expression of Snail (a mesenchymal marker) and E-Cadherin (an epithelial marker) were measured by real-time PCR, respectively. $^{*}p<0.05$.

tion of Nanog is a potential therapeutic strategy for treatment of cancers. The functional relevance between miRNAs and Nanog has been found in several studies, suggesting that Nanog is required for the suppressive effects of miRNAs on tumor cell behaviors, such as cell growth, differentiation, and chemoresistance[25,27]. Our study found that Nanog was significantly elevated in chemoresistant breast cancer cells, and suppressed by transfection of miR-760 mimics, suggesting that miR-760 sensitized breast cancer cells though down-regulation of Nanog.

The pathogenesis of malignant tumors is a complex process involved in dysregulation and mutation of a variety of genes[28]. The EMT is regarded to be an important step for the progression of the neoplasm to malignancy, which is characterized by the conversion of epithelial cells to mesenchymal cells in cellular morphology[29]. Nanog has been reported to modulate the EMT and chemoresistance in several cancers[28,30]. Our work found that up-regulation of miR-760 reversed EMT as shown by decreased expression of Snail (a mesenchymal marker) and increased E-Cadherin (an epithelial marker) in MCF-7/DOX cells. These data suggested that miR-760 mediated the expression of Nanog and EMT in breast cancer cells.

Conclusions

The present study loss of miR-760 is a common event in chemoresistant breast cancer cells, and miR-760 overexpression could increase drug sensitivity through regulation of EMT. Further investigation of miR-760 in preclinical models of breast cancer will contribute the understanding of its therapeutic values for cancer treatment.

Conflicts of interest

The authors declare no conflicts of interest.

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