RESEARCH ARTICLE

miR-126 Suppresses the Proliferation of Cervical Cancer Cells and Alters Cell Sensitivity to the Chemotherapeutic Drug Bleomycin

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Abstract

In cervical cancer, one of the most common malignant tumors in women worldwide, *miR-126* has been reported to exhibit decreased expression. However, its role in cervical cancer cell proliferation and drug sensitivity has remained relatively unexplored. Here, we compared the expression of *miR-126* in cervical cancer tissues (n = 20) with that in normal cervical tissue (n = 20) using quantitative RT-PCR. The viability of Siha cervical cancer cells was further measured by MTT assay after transfection with *miR-126* mimic (Siha-*miR-126* mimic) or microRNA mimic negative control (Siha-miR mimic NC) and after treatment with various concentrations of bleomycin (BLM). IC50s were calculated, and the survival rates (SRs) of Siha cells were calculated. *miR-126* expression in cervical cancer tissue was significantly decreased compared with that in normal cervical tissue ($P < 0.01$). The relative SRs of Siha-*miR-126* mimic cells were also significantly decreased compared with those of Siha-miR mimic NC cells at 24-96 h after transfection. The IC50 of BLM in Siha-*miR-126* mimic cells (50.3 ± 2.02 μg/mL) was decreased compared with that in Siha-miR mimic NC cells (70.5 ± 4.33 μg/mL) at 48 h after transfection ($P < 0.05$). Finally, the SRs of Siha-*miR-126* mimic cells were significantly lower than those of Siha-miR mimic NC cells after cultured in medium containing 40 μg/mL BLM for 24–96 h ($P < 0.05$). These results suggest that *miR-126* is expressed at low levels in cervical cancer. Upregulation of *miR-126* inhibited cervical cancer cell proliferation and enhanced the sensitivity to BLM. Thus, *miR-126* may represent a novel approach to cervical cancer treatment.

Keywords: *miR-126* - cervical cancer - bleomycin - drug sensitivity

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Introduction

Cervical cancer is one of the most common malignant tumors in women worldwide, and much research has investigated the pathogenesis, migration, invasion, treatment, and prognosis of this disease. Previous studies have indicated that microRNAs (miRNAs) act as crucial modulators in cancer progression by targeting mRNAs through cleavage or transcriptional repression (Bartel, 2004). Various miRNAs have been shown to exhibit differential expression between cervical cancer and normal cervical tissues (Wang et al., 2008), and several reports have demonstrated the roles of miRNAs in the proliferation (Yang et al., 2009), apoptosis (Li et al., 2011), and prognosis (Hu et al., 2010) of cervical cancer. However, the role of miRNAs in mediating sensitivity of cells to chemotherapeutic drugs has not been explored in cervical cancer.

miR-126, derived from a common precursor structure located within the epidermal growth factor-like domain 7 (EGFL7) gene, has been reported to exhibit decreased expression in cervical cancer cells (Wang et al., 2008). Previous studies have reported that *miR-126* may play a role in tumorigenesis and growth by regulating the vascular endothelial growth factor (VEGF)/phosphoinositol 3-kinase (PI3K)/AKT signaling pathways in human breast cancer (Zhu et al., 2011). Additionally, this miRNA may function as a tumor suppressor, with Crk as a direct target, in gastric cancer (Feng et al., 2010) and via the regulation of ADAM9b in pancreatic cancer (Hamada et al., 2011). *miR-126* may also play a role in angiogenesis in ischemia (van Solingen et al., 2011), and has also been reported to enhance the sensitivity of non-small cell lung cancer cells to anticancer agents by targeting VEGF-A (Zhu et al., 2012). Together, these previous studies have demonstrated the important role of *miR-126* in various cancers. However, the role of *miR-126* in mediating proliferation and drug sensitivity in cervical cancer cells is still unexplored.

In the present study, we investigated the function of *miR-126* in cervical cancer cell proliferation and examined the ability of *miR-126* to enhance the sensitivity of cervical cancer cells to bleomycin (BLM). Our data may have important implications in the resistance of cervical cancer to chemotherapeutic agents.

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Materials and Methods

Cervical tissue samples

All tissues were collected from the Department of Gynecology and Obstetrics at West China Second Hospital of Sichuan University. Cervical cancer tissues (20 samples; histological diagnosis: cervical squamous cell carcinoma, grade III) and normal cervical tissues (20 samples; histological diagnosis: normal cervical tissue) were collected. All cervical tissue samples were stored at -80°C. All tissues were collected after obtaining written informed consent from each patient.

Cell lines and cell culture

The human cervical squamous cancer cell line Siha was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) containing 10% newborn calf serum (HyClone), 100 U/mL penicillin sodium, and 100 μg/mL streptomycin sulfate at 37°C in humidified air containing 5% CO_2. Cells at the logarithmic growth phase were used in this experiment.

miRNA transfection

The sequence of *miR-126* was from NCBI: ucguaccgugaguaauaaugcg.

miR-126 mimic and miR mimic negative control (NC) were from RiboBio Co. (Guangzhou, China) (Hou et al., 2011). Cells in the exponential phase of growth were plated in 96-well plates (6×10^3 cells/well) and cultured in DMEM without antibiotics for 16 h. Cells were then transfected with the *miR-126* mimic or miR mimic NC at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells were harvested 5 h after transfection, and fresh DMEM was added. Each sample was evaluated in 3 replicates. Survival rates (SR) were calculated using the MTT assay as previously described ((Hong et al., 2005). Briefly, 20 μL of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Sigma, St Louis, USA) was added into each well at 0, 24, 48, 72, and 96 h after transfection. After an additional 4 h at 37°C, the culture medium was removed and 150 μL dimethyl sulfoxide (Sigma) was added into each well to dissolve the crystals. The absorbance (OD) in each well was read at 490 nm by an automated microplate reader (Bio-Rad, Hercules, USA), Calculations of SRs were performed using the following equations: $SR_{\text{Siha-miR mimic NC}}$ = mean value of ($OD_{\text{Siha-miR mimic NC}}$/mean value of OD_{Siha}); $SR_{\text{Siha-}miR\text{-}126\text{ mimic}}$ = mean value of ($OD_{\text{Siha-}miR\text{-}126\text{ mimic}}$/mean value of OD_{Siha}).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cervical cancer tissues and normal cervical tissues using TriZol Reagent (Invitrogen Life Technologies) following the manufacturer's instructions. Reverse transcription was performed on 1 μg total RNA from each sample using Bulge-Loop RT-Primer (RiboBio, Guangzhou, China) and primescript RT enzyme Mix1 (Takara, Dalian, China). Real-time PCR was carried out at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 31 s, using Bulge-Loop Forward Primers (*miR-126* special), common Bulge-Loop Reverse Primer (RiboBio), and SYBR Premix Ex Taq II (Takara, Code: DRR820S). qRT-PCR was performed on an ABI 7300 RT-PCR system (Applied Biosystems, Foster City, CA, USA). Comparative RT-PCR was performed in triplicate, including no-template controls. The internal reference gene was U6. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

In vitro drug sensitivity assay

Cells in the exponential phase of growth were seeded in 96-well plates (6×10^3 cells/well). BLM was freshly prepared before each experiment. Each condition was assayed triplicate. Drug sensitivity (as calculated using the half-maximal inhibitory concentration, or IC_{50}) was evaluated using the MTT assay at an absorption wavelength of 490 nm. Cells were divided into 3 groups (empty control: Siha; Negative control group: Siha-miR mimic NC; experimental group: Siha-*miR-126* mimic). After transfection, cells were incubated for 48 h in the absence or presence of various concentrations of BLM (10, 20, 40, 80, or 160 μg/mL) in 200 μL medium. After cells were cultured for 48 h, MTT assay was performed. SRs were calculated at each BLM concentrations using the following equations: SR_{Siha} = mean value of (OD_{Siha}-BLM / mean value of OD_{Siha}); $SR_{\text{Siha-miR mimic NC}}$ = mean value of ($OD_{\text{Siha-miR mimic NC}}$-BLM / mean value of $OD_{\text{Siha-miR mimic NC}}$); and $SR_{\text{Siha-}miR\text{-}126\text{ mimic}}$ = mean value of ($OD_{\text{Siha-}miR\text{-}126\text{ mimic}}$-BLM / mean value of $OD_{\text{Siha-}miR\text{-}126\text{ mimic}}$). The IC_{50}s were calculated using SPSS 16.0 software package. Siha cells were incubated for 0, 24, 48, 72, or 96 h in the absence or presence of BLM (40 μg/mL) in 200 μL medium after transfection, and the SRs of cells treated at each time point were calculated using MTT assays as described previously.

Statistical analysis

Data were presented as the mean ± SD and were analyzed using SPSS 16.0 software. The significance of differences from the control values was determined with Student's t-tests or χ^2 tests; p-values of less than 0.05 were considered statistically significant.

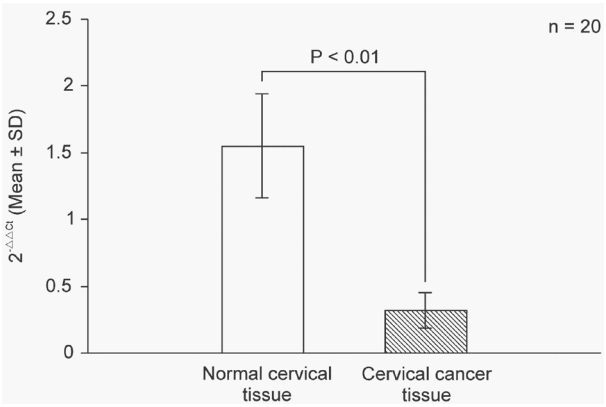

Figure 1. Results of *miR-126* Expression Using Quantitative RT-PCR ($2^{-\Delta\Delta Ct}$). Expression of *miR-126* was calculated using the $2^{-\Delta\Delta Ct}$ method by quantitative RT-PCR. The Tm was 82.1°C. Comparative RT-PCR was performed in triplicate. *miR-126* expression in cervical cancer tissue was significantly decreased versus normal cervical tissue ($P < 0.01$)

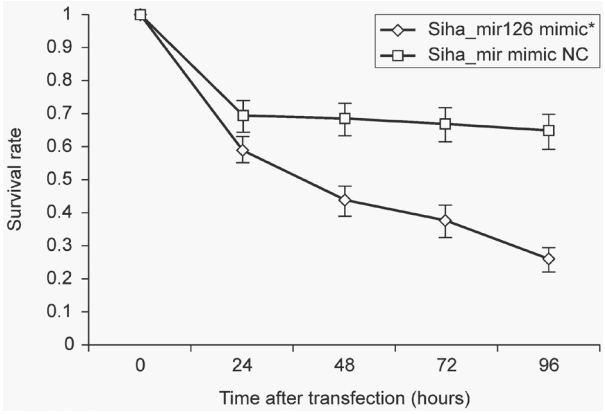

Figure 2. Upregulation of *miR-126* Suppressed the Proliferation of Cervical Cancer Cells. Cells were cultured for 24, 48, 72, or 96 h after transfection, and the survival rates (SRs) of Siha-*miR-126* mimic and Siha-miR mimic NC were calculated ($SR_{Siha\text{-}miR\ mimic\ NC} = OD_{Siha\text{-}miR\ mimic\ NC}/OD_{Siha}$; $SR_{Siha\text{-}miR\text{-}126\ mimic} = OD_{Siha\text{-}miR\text{-}126\ mimic}/OD_{Siha}$). *$P < 0.05$ versus Siha-miR mimic NC

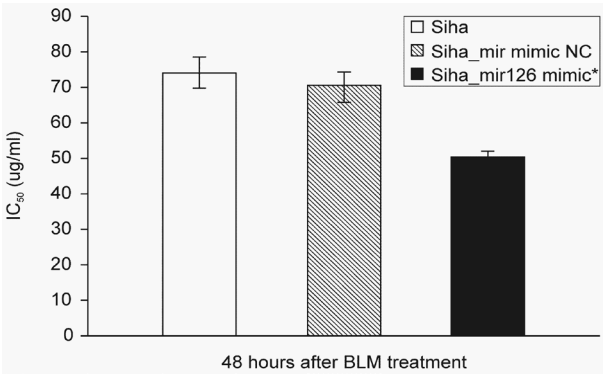

Figure 3. Upregulation of *miR-126* Reduced the IC_{50} of BLM in Siha Cervical Cancer Cells. Siha cell survival rates (SRs) were calculated after 48 h of culture in the absence or presence of various concentrations of BLM (10, 20, 40, 80, and 160 μg/mL) in 200 μL medium ($SR_{Siha} = OD_{Siha\text{-}BLM}/OD_{Siha}$; $SR_{Siha\text{-}miR\ mimic\ NC} = OD_{Siha\text{-}miR\ mimic\ NC\text{-}BLM}/OD_{Siha\text{-}miR\ mimic\ NC}$; $SR_{Siha\text{-}miR\text{-}126\ mimic} = OD_{Siha\text{-}miR\text{-}126\ mimic\text{-}BLM}/OD_{Siha\text{-}miR\text{-}126\ mimic}$). IC_{50}s were calculated using SPSS 16.0 software. *$P < 0.05$ versus Siha-miR mimic NC and Siha

Results

Expression of miR-126 in cervical cancer tissues and normal cervical tissues

First, we compared the expression of *miR-126* in cervical cancer tissues and normal cervical tissue (n = 20 samples each) by qRT-PCR (Figure 1). Our analysis revealed a Tm of 82.1°C, with a single peak in the melt curve, confirming the specificity of the primers. Interestingly, *miR-126* expression in cervical cancer tissue was significantly decreased compared with normal cervical tissue ($P < 0.01$).

Upregulation of miR-126 suppressed cervical cancer cell proliferation

Next, we investigated the role of *miR-126* in cervical cancer progression by measuring Siha cell viability following transfection with *miR-126* mimic (Siha-*miR-126* mimic) or miR mimic NC (Siha-miR mimic NC). As shown in Figure 2, *miR-126* upregulation resulted in a gradual decrease in Siha cell survival over the course of the 96-h experiment. In contrast, no changes in the rate of cell survival were seen in Siha-miR mimic NC cells. The SR of Siha-*miR-126* mimic cells was significantly decreased compared with Siha-miR mimic NC cells ($P < 0.05$).

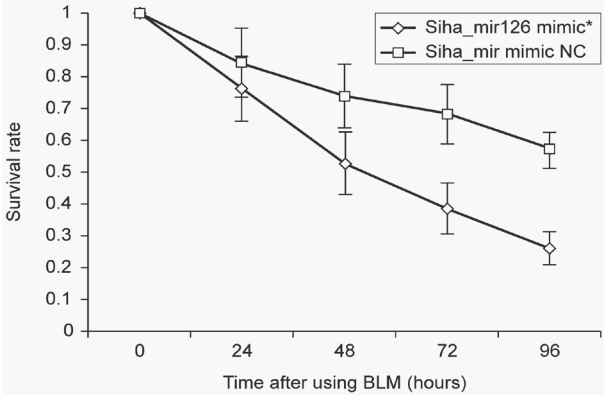

Figure 4. Upregulation of *miR-126* Enhanced the Sensitivity of Cervical Cancer Cells to Bleomycin. The survival rates (SRs) of Siha cells were calculated using MTT assays at 0, 24, 48, 72, and 96 h of treatment with BLM (40 μg/mL) in 200 μL medium ($SR_{Siha\text{-}miR\ mimic\ NC} = OD_{Siha\text{-}miR\ mimic\ NC\text{-}BLM}/OD_{Siha\text{-}miR\ mimic\ NC}$; $SR_{Siha\text{-}miR\text{-}126\ mimic} = OD_{Siha\text{-}miR\text{-}126\ mimic\text{-}BLM}/OD_{Siha\text{-}miR\text{-}126\ mimic}$). *$P < 0.05$ versus Siha-mir mimic NC

Upregulation of miR-126 enhanced the sensitivity of cervical cancer cells to BLM

Next, we sought to investigate the role of *miR-126* in mediating chemosensitivity in cervical cancer cells. As shown in Figure 3, the IC_{50} of BLM in Siha-*miR-126* mimic cells (50.31 ± 2.02 μg/mL) was significantly decreased compared with those of Siha (74.37 ± 4.51 μg/mL) and Siha-miR mimic NC (70.54 ± 4.33 μg/mL) cells ($P < 0.05$ for each). The IC_{50}s of BLM in Siha-miR mimic NC and Siha cells were not significantly different (Figure 4, $P > 0.05$).

With regard to chemosensitivity over time, the differences between the SRs of Siha-*miR-126* mimic and Siha-miR mimic NC were apparent at all time points, becoming greater as the experiment progressed. Again, the SR of Siha-*miR-126* mimic cells was significantly reduced compared with that of Siha-miR mimic NC cells ($P < 0.05$).

Discussion

miR-126 is relevant in many tumors, and studies have shown that *miR-126* is significantly downregulated in gastric cancer tissues (Feng et al., 2010), non-small cell lung cancer cell lines (Zhu et al., 2012), prevalent diabetes mellitus (Zampetaki et al., 2010), cystic fibrosis airway epithelial cells (Oglesby et al., 2010), human breast cancer (Zhu et al., 2011), invasive ductal adenocarcinoma (Hamada et al., 2011), and cervical cancer tissues (Wang et al., 2008), as compared with relevant normal tissues. Using qRT-PCR analysis, we found that the expression of *miR-126* in cervical cancer tissues was significantly decreased compare to that in normal cervical tissues, consistent with a previous report (Wang et al., 2008).

miR-126 has been reported to affect cancer progression through signaling pathways that control tumor cell proliferation, migration, invasion, and survival (Feng et al., 2010; Hamada et al., 2011; van Solingen et al., 2011; Zhu et al., 2011; Zhu et al., 2012). Some studies have also demonstrated that *miR-126* has roles in regulating adhesion molecule expression (Harris et al., 2008), providing additional control of vascular inflammation (Harris et al., 2008), regulating the VEGF/PI3K/AKT signaling pathway (Hu et al., 2010), regulating the translation and invasiveness of vascular endothelial cell sprouting (Musiyenko et al., 2008), and governing the integrity and angiogenesis of the vasculature (Kuhnert et al., 2008; Wang et al., 2008b). Using MTT assays, our research showed that the SR of Siha-*miR-126* mimic was significantly reduced compared to that of control cells and that the differences between the 2 cell groups increased over time. Thus, our results indicated that *miR-126* blocked the proliferation of Siha cervical cancer cells, suggesting that *miR-126* may be applicable to the treatment of cervical cancer.

miR-126 has also been reported to enhance the sensitivity of non-small cell lung cancer cells to adriamycin and vincristine (Zhu et al., 2012). We chose BLM to test the effects of *miR-126* in mediating the sensitivity of cervical cancer cells to chemotherapeutic drugs because BLM belongs to a family of aminoglycopeptide antibiotics that shows high antitumor activity and is used as a first-line antitumor drugs in the clinical setting, especially in cervical squamous cancer. Interestingly, our data demonstrated that upregulation of *miR-126* significantly reduced the IC_{50} of BLM in Siha cells, as compared to the controls. Moreover, this effect (i.e., increased sensitivity of Siha cells to BLM) was maintained throughout the entire 96-h experiment, and the SR of Siha-*miR-126* mimic cells was dramatically reduced compared to control cells. These results indicated that *miR-126* could enhance the sensitivity of cervical cancer cells to BLM, again supporting that *miR-126* may be applicable to enhance chemotherapeutic efficacy in the treatment of cervical cancer.

In conclusion, our data have supported three novel ideas. First, *miR-126* expression in cervical cancer tissue was significantly decreased compare with normal cervical tissue. Second, the upregulation of *miR-126* suppressed the proliferation of cervical cancer cells. Finally, *miR-126* enhanced the sensitivity of cervical cancer cells to BLM. Based on these results, *miR-126* may represent a novel target for cervical cancer treatment, and further analyses of the biological mechanisms of *miR-126* function in cervical cancer chemotherapy may deepen our understanding of these pathways.

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