

Original Article

Inhibition of miR-103 reverses epithelial-mesenchymal transition and sensitizes lung A549 cells to cisplatin *in vitro* and *in vivo*

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Abstract: Emerging evidence has demonstrated that microRNAs (miRNA) play a critical role in chemotherapy-induced epithelial-mesenchymal transition (EMT) in lung cancer. However, the underlying mechanism of chemotherapy-mediated EMT has not been fully elucidated. In the present study, we explored the role of miR-103 in regulating cisplatin (DDP)-mediated EMT in human lung adenocarcinoma A549/DDP cells. Real-time quantitative PCR revealed that miR-103 was significantly upregulated in A549/DDP cells compared with the parental cells, and inhibition of miR-103 sensitized A549/DDP cells to DDP treatment, induced cell apoptosis, inhibited cell invasion and reversed the mesenchymal features of A549/DDP cells. Moreover, inhibition of miR-103 significantly enhanced DDP sensitivity in the mouse model. Taken together, these findings suggest that inhibition of miR-103 might be a potential therapeutic approach to reverse DDP resistance in lung cancer.

Keywords: miR-103, epithelial-mesenchymal transition (EMT), lung cancer, cisplatin resistance

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. Currently, chemotherapy is one of the widely used therapeutic approaches for the treatment of lung cancer, and is beneficial to patients with lung cancer by inhibiting tumor growth and prolonging survival. However, some patients become resistant to chemotherapy after a period of time, which finally lead to failure of chemotherapy and worse treatment outcome, and the underlying mechanisms of chemoresistance in lung cancer remain largely unclear [2, 3]. Increasing studies in many kinds of human cancer have demonstrated that the chemoresistant cancer cells have increased invasion and metastasis behavior and display features of epithelial-mesenchymal transition (EMT), which was characterized by the loss of the epithelial marker E-cadherin and upregulation of the mesenchymal markers such as N-cadherin [4-10]. However, the underlying mechanism of chemotherapy-mediated EMT has not been fully elucidated.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that negatively regulate the expression of their target genes, and accumulating evidence has demonstrated that miRNAs have a key role in drug resistance and EMT in many kinds of human cancer [11-14], including lung cancer. For example, in non-small cell lung cancer, miR-129 inhibits EMT and metastasis by targeting MCRS1 [15], and miRNA 17 family regulates cisplatin (DDP)-resistant and metastasis by targeting TGF β R2 [16]. MiR-147 is reported to induce a mesenchymal-to-epithelial transition (MET) and reverses EGFR inhibitor resistance [13], while miR-23a promotes TGF- β -induced EMT by targeting E-cadherin in lung cancer cells [17]. Although these studies revealed the involvement of some miRNAs in lung cancer chemoresistance and EMT, further investigations is required to elucidate the exact molecular basis.

Recently, miR-103 has been identified as a potential biomarker for malignant mesothelioma [18] and endometrial cancer cells [19], and high expression of miR-103 was associated

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with metastasis potential of colorectal cancer cells, resulting in increased cell motility and mesenchymal features [20, 21]. Moreover, miR-103 has also been reported to modulate chemoresistance in gastric [22] and pancreatic carcinoma [23], suggesting miR-103 may play a critical role in chemoresistance. In the present study, we explored the role of miR-103 in regulating DDP-mediated EMT in A549/DDP cells. We identified that miR-103 was significantly upregulated in DDP-resistant A549/DDP cells compared with the parental cells, and found inhibition of miR-103 sensitized A549/DDP cells to DDP treatment, induced cell apoptosis, inhibited cell invasion and reversed the mesenchymal features of A549/DDP cells. In addition, inhibition of miR-103 significantly enhanced DDP sensitivity in mouse model. Our results suggested that targeting miR-103 might be a potential therapeutic approach for treating DDP-resistant lung cancer.

Materials and methods

Cell culture

Human lung adenocarcinoma A549 cells and DDP-resistant A549/DDP cells were purchased from the Academy of Military Medical Science (Beijing, China). A549 cells was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and the DDP resistant A549/DDP cells were maintained in culture medium with 10 µg/ml DDP (Caymanchem, USA).

Real-time quantitative polymerase chain reaction (RTq-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, CA, USA). The expression level of mature miR-103 was measured by TaqMan miRNA assays (Applied Biosystems, CA, USA) according to the protocol, and U6 snRNA was used for normalization. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed using RIPA (Beyotime) supplemented with protease inhibitors, and about 50 µg protein extractions were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Maidstone, UK) and incubated with specific primary antibodies against E-cadherin (Abcam, ab76055), Vimentin (Ab-

cam, ab92547), Twist1 (Abcam, ab180714), Slug (Abcam, ab27568) and β -actin (Santa Cruz Biotechnology Inc., CA, USA), then probed with HRP-conjugated secondary antibodies. Signals were visualized with Enhanced Chemiluminescence Plus Kit (GE Healthcare).

Cell viability assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were seeded into 96-well plates (5×10^3 cells/well) and allowed to grow for 48 h and assessed by a colorimetric assay using MTT solution (10 mg/mL) at 570 nm. All the experiments were performed three times.

Transwell invasion assay

About 1×10^5 cells were added into the upper chamber of the insert precoated with Matrigel (ECM gel, Sigma-Aldrich, St. Louis, MO). Cells were plated in medium containing 0.1% fetal bovine serum (FBS), and medium containing 10% FBS in the lower chamber. After 48 h of incubation, the non-invading cells were carefully wiped out with cotton swabs, and the invaded cells through the membrane were stained with 0.2% crystal violet solution, imaged and counted under a microscope (Olympus, Tokyo, Japan).

Cell migration assay

Cell migration was assessed by wound healing assays. Briefly, cells were seeded plates and cultured to 100% confluence, then wounds were generated by a sterile pipette tip and the cells were allowed to grow. The wound closure was assessed after 24 h and 48 h by Scion Image Software (Scion Corporation, Frederick, MD).

Colony formation assay

For the colony formation assay, Five hundred A549/DDP cells were placed in complete growth media in each 35 mm dish and allowed to grow for 6 h. Then replaced by the fresh complete growth media, and cells were allowed to grow until visible colonies formed (2 weeks). Cell colonies were fixed with methanol, stained with Giemsa, washed, air dried, photographed and counted.

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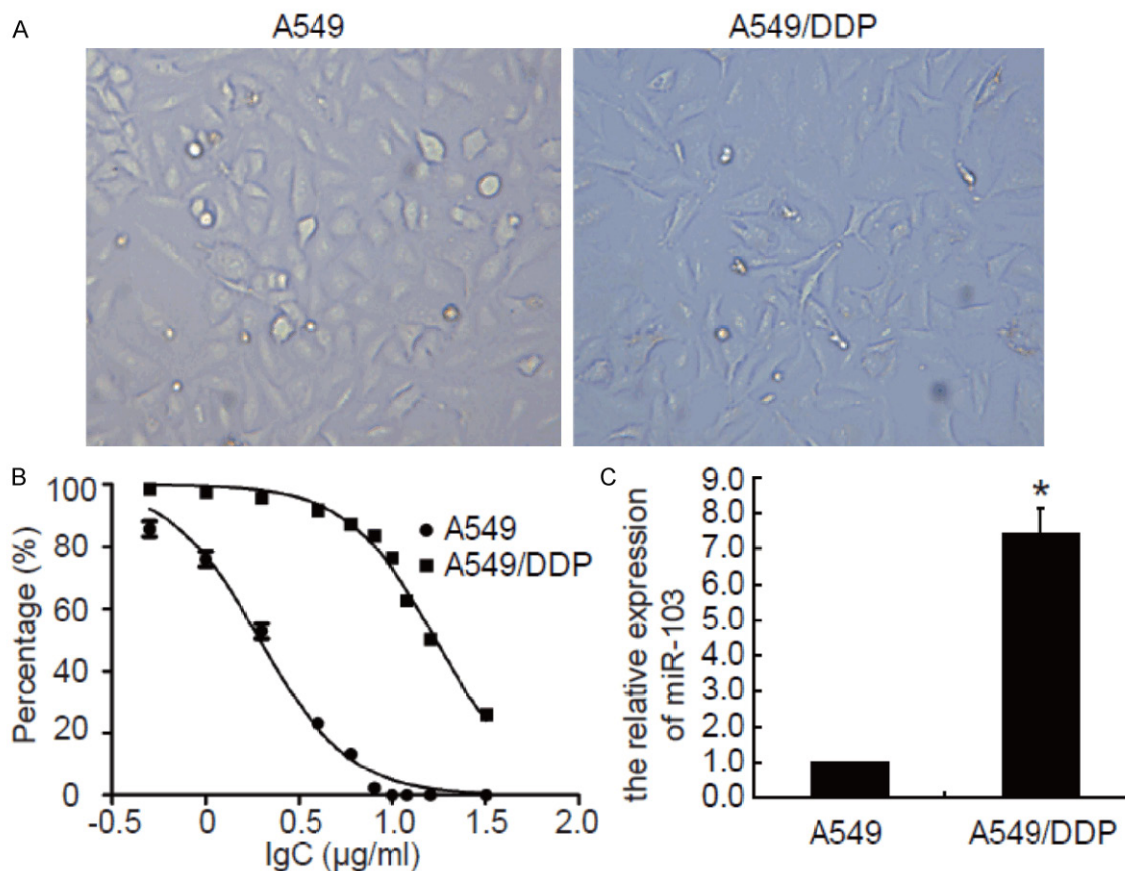


Figure 1. MiR-103 is upregulated in DDP-resistant human lung A549/DDP cells. A. The morphological changes of DDP-resistant A549/DDP cells compared to the parental A549 cells were observed under a microscope. B. MTT assay indicated that A549/DDP cells were high resistant to DDP with the IC₅₀ value of 17.12 μg/mL, compared to the parental A549 cells with the IC₅₀ value of 1.96 μg/mL. C. The expression level of miR-103 in A549/DDP cells and the parental A549 cells by RTq-PCR. *P<0.05 versus the parental cells, data shown are means ± s.d.

Cell apoptosis assay

Cells were digested with trypsin, washed with phosphate-buffered saline (PBS) twice, and fixed in 70% ethanol at 4°C for overnight. The fixed cells were stained with annexin-V-FITC and propidium iodide (PI) labeling solution using annexin-V Apoptosis Detection kit FITC (eBioscience, Affymetrix, San Diego, CA, USA) according to the suggested procedure. The stained cells (1×10^5) were then analyzed with the flow cytometer (Beckman Coulter, Brea, CA, USA).

Lentiviral infection and miRNA mimics

Lentiviruses containing anti-miR-103 expressing vector and empty vector as control were purchased from GeneChem Company (Shanghai, China). To get stably infected A549/DDP

cells, the cells were cultured to about 70% of the plates, and then added by a concentration of 1.0×10^5 TU/well anti-miR-103 or control lentivirus. Real-time qRT-PCR was performed to validate expression levels of miR-103 after being infected for 7 days. The stably infected cells were expanded and harvested for further experiments. MiR-103 and scramble mimics and corresponding inhibitor were purchased from RiboBio (Guangzhou, China).

Animal treatment

Female nude mice of 3-5 weeks old were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). A549/DDP cells with anti-miR-103 expressing vector or empty vector as control were suspended in serum-free medium, and 100 μl (500,000 cells) of cell suspension was subcutaneously

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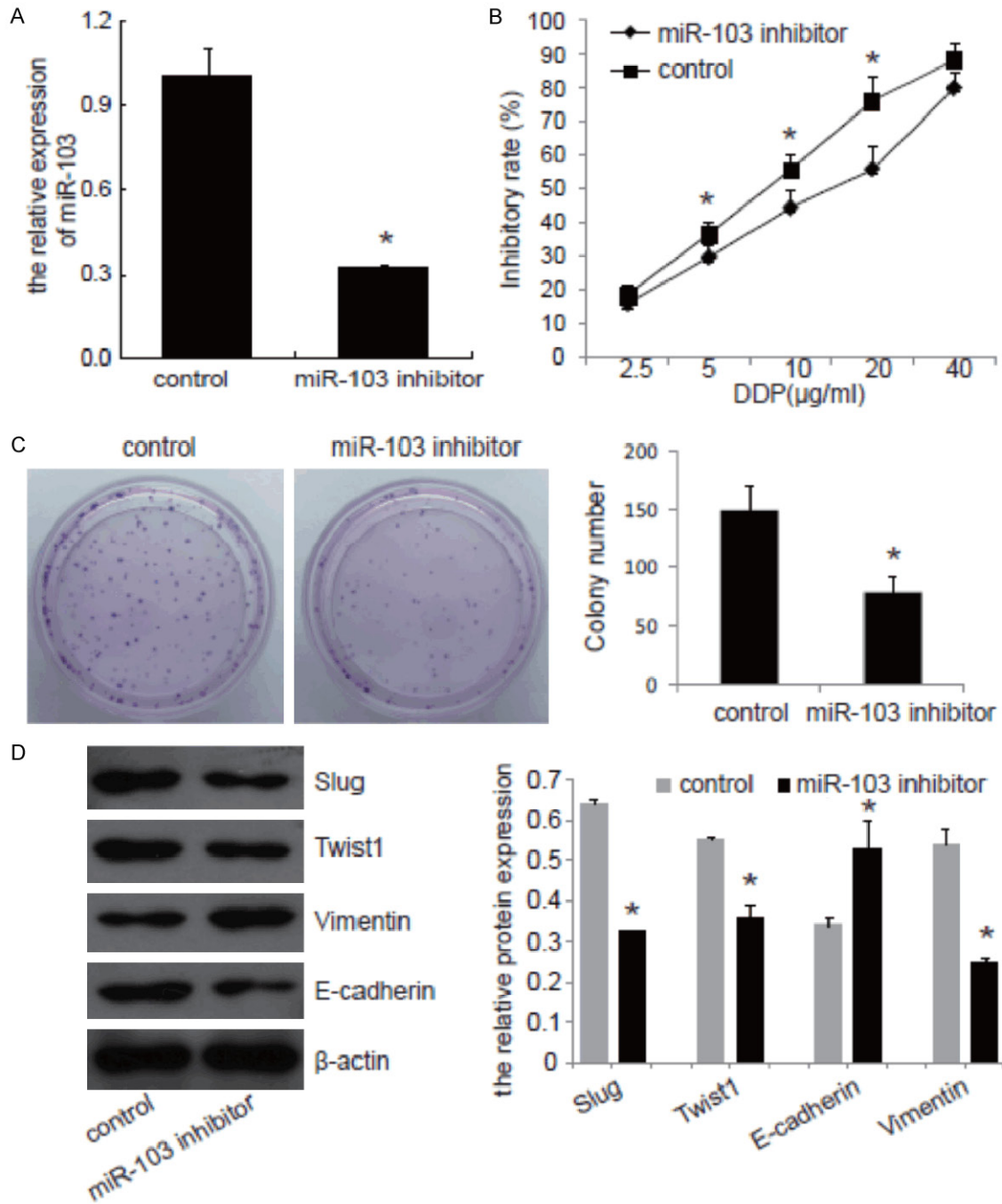


Figure 2. Inhibition of miR-103 sensitized A549/DDP cells to DDP treatment and reversed the mesenchymal features. A. A549/DDP cells were transfected with miR-103 inhibitor, and the miR-103 expression was evaluated by RTq-PCR. B. The effect of miR-103 inhibition on A549/DDP cell viability was determined by MTT assay. C. The effect of miR-103 inhibition on A549/DDP cell colony formation. D. The effect of miR-103 inhibition on EMT markers (E-cadherin, vimentin) and inducers (Slug, Twist1) were examined by western blot. *P<0.05 versus the control, data shown are means ± s.d.

injected into each anesthetized nude mouse (n=5 animals per group). The volume (mm³) of individual orthotopic tumor from each mouse

was measured according to the formula: $1/2 \times \text{length} \times \text{width}^2$. 29 days after inoculation, the DDP (10 mg kg⁻¹) was intraperitoneally injected

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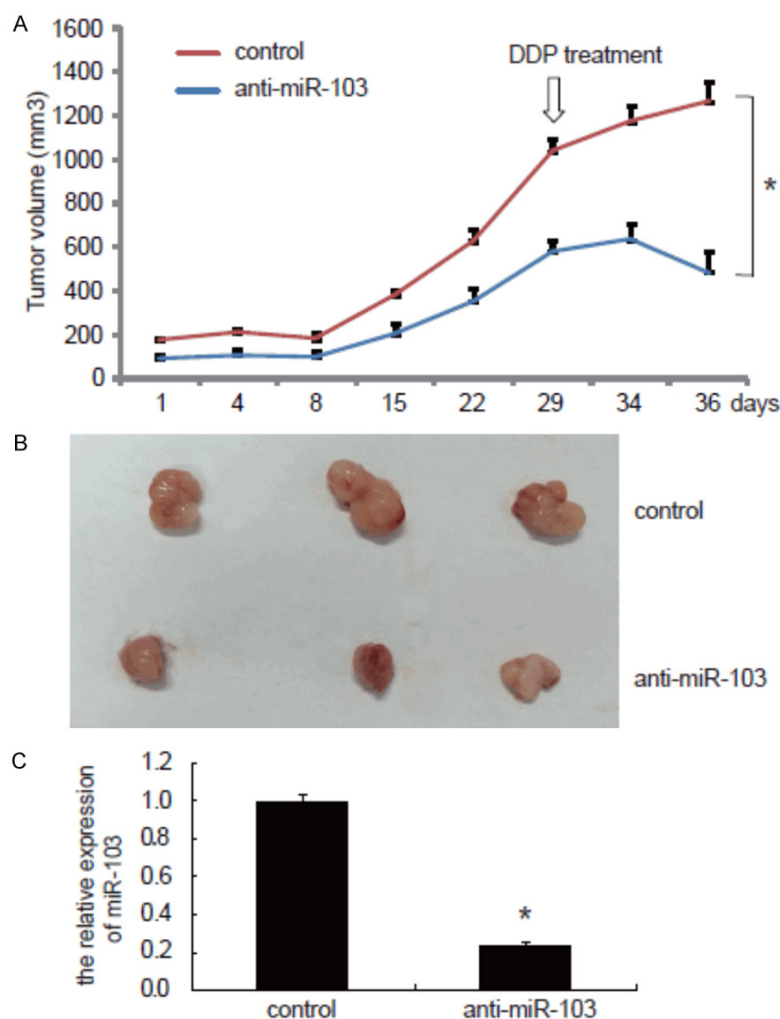


Figure 3. Inhibition of miR-103 sensitizes A549/DDP cells to DDP *in vivo*. BALB/C nude mice were subcutaneously inoculated with A549/DDP cells with anti-miR-103 expressing vector (n=5) or empty vector as control (n=5), respectively. After 29 days, DDP (10 mg kg⁻¹) was then intravenously injected into mice once a day for one week. A. Tumor volume (mm³) was calculated. B. After DDP treatments, all mice were euthanized and the tumors were excised and imaged under a light microscope. C. RTq-PCR analysis showed that miR-103 was down-regulated in transplanted tumour tissues inoculated by anti-miR-103 expressing A549/DDP cells. (*P value <0.05).

into mice once a day for one week. After DDP treatments, all of the mice were euthanized and the tumors were excised and imaged under a light microscope.

Statistical analysis

Experimental data were presented as the mean \pm s.d, and statistical analyses were carried out by SPSS19.0 software. Statistical significance between two groups was analyzed using Student t-test. P value than 0.05 was considered significant.

Results

MiR-103 is upregulated in DDP-resistant human lung A549/DDP cells

In comparison to the parental epithelial A549 cells, the DDP-resistant A549/DDP cells lost epithelial honeycomb-like morphology and obtained a spindle-like shape (**Figure 1A**). MTT assay indicated that A549/DDP cells were high resistant to DDP with the IC50 value of 17.12 μ g/mL compared to the parental A549 cells with the IC50 value of 1.96 μ g/mL. We measured the expression level of miR-103 in A549/DDP cells and A549 cells by RTq-PCR and observed miR-103 was significantly upregulated in A549/DDP cells compared with A549 cells (**Figure 1B**), suggesting that increased miR-103 might be involved in DDP resistance in lung cancer cells.

Inhibition of miR-103 sensitizes A549/DDP cells to DDP and reverses the mesenchymal features

To investigate the association of miR-103 with lung cancer chemoresistance against DDP, A549/DDP cells were transfected with miR-103 inhibitor to inhibit

the miR-103 expression (**Figure 2A**). MTT assay and colony formation assay revealed that A549/DDP cells transfected with miR-103 inhibitor displayed significantly increased sensitivity to DDP (**Figure 2B** and **2C**). Moreover, miR-103 inhibitor increased epithelial marker E-cadherin expression, and reduced mesenchymal protein vimentin and EMT inducers (Slug and Twist1) in A549/DDP cells (**Figure 2D**). Taken together, these data suggest that miR-103 might play a critical role in regulating DDP-mediated EMT in lung cancer cells.

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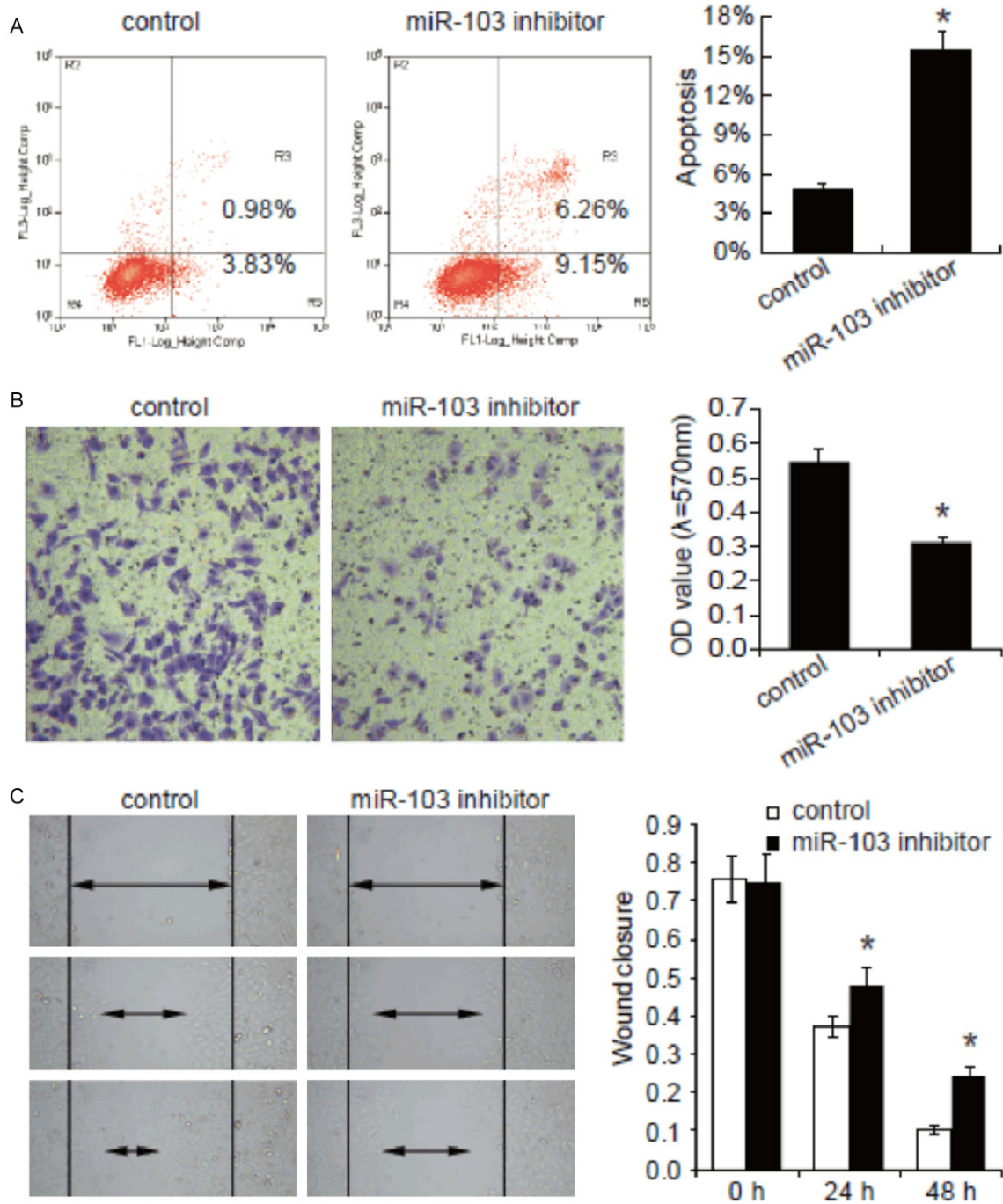


Figure 4. Inhibition of miR-103 induced cell apoptosis and inhibited cell invasion and migration of A549/DDP cells. A. The effect of miR-103 inhibition on A549/DDP cell apoptosis was evaluated by flow cytometry. B and C. The effects of miR-103 inhibition on A549/DDP cell invasion and migration were determined by transwell with matrigel invasion assay and wound healing assay, respectively. *P<0.05 versus the control, data shown are means ± s.d.

Inhibition of miR-103 sensitizes A549/DDP cells to DDP in vivo

To further investigate the role of miR-103 in regulating DDP sensitivity *in vivo*, we constructed lentiviral vector expressing anti-miR-103

and infected A549/DDP cells. We injected subcutaneously anti-miR-103 expressing A549/DDP cells, as well as the negative control A549/DDP cells into 3- to 5-week female BALB/C nude mice, respectively. 29 days after cell injection, the mice were treated with the DDP

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(10 mg kg⁻¹) once a day for one week. As shown in **Figure 3A**, tumor growth had notable difference between anti-miR-103 expressing group and control group before DDP administration. Upon one week DDP treatment, tumor growth was more significantly inhibited in anti-miR-103 expressing group than the control group (**Figure 3A** and **3B**). We also observed that miR-103 expression was significantly decreased in tumor tissues grafted by stably anti-miR-103 expressing cells compared with the negative control (**Figure 3C**). These findings suggest that inhibition of miR-103 may enhance DDP sensitivity *in vivo*.

Inhibition of miR-103 induced cell apoptosis, inhibited cell invasion and migration

To further validate the role of miR-103 in regulating cell viability and EMT, we determined the cell apoptosis, invasion and migration in A549/DDP cells before and after miR-103 inhibitor treatment. Cell apoptosis analysis by flow cytometry revealed that inhibition of miR-103 induced A549/DDP cell apoptosis (**Figure 4A**). Further, invasion assay and wound healing assay showed that inhibition of miR-103 attenuated the cell invasion (**Figure 4B**) and cell migration of A549/DDP cells (**Figure 4C**), respectively.

Discussion

Up to date, DDP is still the most widely used first-line chemotherapeutic agent for human lung cancer. Despite tremendous efforts, DDP treatment often results in the development of drug resistance, leading to therapeutic failure. Thus, it is important to elucidate the underlying molecular mechanisms leading to DDP resistance. Recently, many studies have shown that miRNAs play an important role in modulating sensitivity and resistance to a range of chemotherapeutic agents, including DDP [24]. Here, we found that miR-103 was significantly upregulated in DDP-resistant A549/DDP cells compared with the parental cells, and inhibition of miR-103 resensitized A549/DDP cells to DDP both *in vitro* and *in vivo*, suggesting a substantial role of miR-103 in DDP resistance.

Dysregulation of miR-103 has been identified to play an important role in the initiation and progression of many kinds of human cancer, such as breast [25, 26], colorectal [21, 27],

endometrial [19], and lung cancer [28]. In these cancer cells, high miR-103 expression was reported to be correlated to advanced clinical stages, metastasis and EMT induction. Consistent with these reports, we found miR-103 inhibition inhibited cell invasion, migration and EMT of A549/DDP cells. The association between EMT and development of chemoresistance has recently been reported [5, 29, 30]. In this study, we found that in comparison to the parental epithelial A549 cells, DDP-resistant A549/DDP cells lost epithelial honeycomb-like morphology and obtained a spindle-like shape. Along with morphological alterations, the expression level of the epithelial protein E-cadherin was decreased, whereas the expression level of the mesenchymal protein vimentin was up-regulated. Taken together, the above observations prompted us to investigate the molecular mechanism underlying miR-103 regulating DDP-mediated EMT of A549 cells.

In the present study, we found that upregulation of miR-103 was significantly involved in DDP resistance in A549 cells, and inhibition of miR-103 significantly increased the sensitivity of A549/DDP cells to DDP by inducing cell apoptosis and reversing EMT in cell model, and enhanced DDP sensitivity in mouse model. Thus, our results suggest that appropriate combination of DDP application with miR-103 inhibition might be a potential therapeutic approach for lung cancer.

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Disclosure of conflict of interest

None.

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