Abstract. This study aimed to investigate the effects of miR-93 on resistance of breast cancer MCF-7 cells to adriamycin, and to explore the possible mechanism. Expression of miR-93 in breast cancer cell lines MCF-7 and MCF-7/ADM was detected by reverse transcription-quantitative PCR (RT-qPCR). miR-93 mimics and inhibitors were transfected into MCF-7/ADM and MCF-7 cells, and MTT assay was used to detect the resistance of cells to adriamycin after transfection. Western blot analysis was used to detect the expression of anti-apoptotic protein Bcl-2 and multidrug resistance gene MDR1 related P-gp protein in MCF-7/ADM and MCF-7 cells before and after the transfection of miR-93 mimics. Expression level of miR-93 in MCF-7/ADM cells was decreased, and was 40% of that in MCF-7 cells (0.39±0.04, p<0.05). Before transfection, IC_{50} value of MCF-7 cells to adriamycin (11.02±0.95) was lower than that of MCF-7/ADM cells (21.29±1.83, p<0.05). IC_{50} value of MCF-7/ADR cells at 72 h after transfection with miR-93 mimics (13.55±0.86) was lower than that of the negative control group (24.67±1.51, p<0.05). IC_{50} value of MCF-7 cells 72 h after transfection with miR-93 inhibitor (19.88±1.28) was higher than that of negative control group (11.02±0.95, p<0.05). Expression levels of Bcl-2 and P-gp proteins in MCF-7/ADM cells were 1.63±0.24 and 1.76±0.22 times that of MCF-7 cells, respectively (p<0.05). At 72 h after transfection of miR-93 mimics, expression levels of Bcl-2 and P-gp proteins in MCF-7/ADM cells were 0.27±0.06 and 0.39±0.05, respectively, compared with the negative control group (p<0.05). At 72 h after transfection with miR-93 inhibitor, expression levels of Bcl-2 and P-gp protein in MCF-7 cells were 1.48±0.10 and 1.56±0.11 times of the negative control group, respectively (p<0.05). miR-93 can increase the apoptosis of MCF-7/ADM cells and their resistance to adriamycin by inhibiting the expression of Bcl-2 and P-gp proteins.

Introduction
Breast cancer is the most common malignant tumor in women with increasing incidence in China. Breast cancer is a serious threat to women's health and brings serious economic burdens to society and families (1). Main methods of clinical treatment of breast cancer are surgery, radiotherapy and chemotherapy, among which combination of adriamycin with other drugs is commonly used in clinical practices (1). However, for patients with advanced breast cancer, prognosis of this treatment plan is poor, and recurrence and metastasis are prone to occur. An important reason for treatment failure is resistance of cancer cells to adriamycin (2). miRNAs are single-stranded, non-coding small RNAs consisting of 18-25 nucleotides, and they exist in eukaryotes and exert their roles in the development and progression of tumors by up- or downregulating the expression of certain important proteins (3). Studies suggest that miRNAs can regulate the occurrence and development of tumor and their resistance to chemotherapeutic agents (4). For example, miR100 and miR-367 are upregulated in adriamycin-resistant tumor cells (5,6). Chu et al showed that expression of miR-93 in breast cancer cells can affect its resistance to chemotherapeutic drugs (7).

In this study, the expression of miR-93 in breast cancer cell line MCF-7 and adriamycin-resistant cell line MCF-7/ADM was detected. miR-93 was transfected into cancer cells to examine the effect of miR-93 on the resistance of MCF-7/ADM cells to adriamycin, and to explore the possible mechanism of action of miR-93. Our findings provide new insights for the clinical treatment of adriamycin-resistant breast cancer patients.

Materials and methods
Materials. MCF-7/ADM and MCF-7 cell lines were purchased from Aolushengwu (https://aolushengwu.biomart.cn, Shanghai, China). Human breast cancer azithromycin-resistant cell lines (MCF-7/ADM) were purchased from Shanghai Zhen Biotechnology (Shanghai, China). miR-93 mimics, inhibitor,
and primers were purchased from Thermo Fisher Scientific, Inc. Waltham, MA, USA. Adriamycin, fetal bovine serum, thiazolyl (MTT) and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), reverse transcription kit, real-time-quantitative PCR (RT-qPCR) kit, Bcl-2 antibody, P-gp antibody, GAPDH antibody, β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). TRIzol kit, liposomes (Lipofectamine 2000), U6 snRNA real-time PCR kit which were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA).

The study was approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University (Jinan, China).

Cell culture. MCF-7/ADM and MCF-7 cells were cultured in RPMI-1640 medium (containing 10% fetal bovine serum) at 37°C with 5% CO₂, and 1 µg/ml of adriamycin was added to MCF-7/ADM culture medium. Subculture was performed every 3 to 4 days.

Detection of miR-93 expression by RT-qPCR. U6 snRNA was used as the endogenous control to measure the relative expression level of miR-93. MCF-7 and MCF-7/ADM cells were collected, and TRIzol reagent was used to extract total RNA. Total RNA was reversely transcribed into cDNA and the reaction conditions were: 37°C for 40 min, 85°C for 40 sec, using cDNA as a template and using miR-93 primers for PCR amplification. PCR reaction conditions were: 94°C for 2 min, followed by 50 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Cq values were processed using 2-ΔΔCq method to calculate the relative expression level of miR-93 (8). miR-93 primer sequences were: 5'-UUCUCCGAACGUGUCACGU-3' (forward) and 5'-ACGUGACACGUUGCAGAATT-3' (reverse).

Cell transfection. MCF-7 and MCF-7/ADM cells were seeded into 6-well plates and miR-93 mimics, inhibitor, and their negative controls were transfected into MCF-7 and MCF-7/ADM, respectively, using Lipofectamine 2000. After 6 h, cells were cultured for 48-96 h (37°C, 5% CO₂) in fresh culture medium and harvested. RT-qPCR was used to detect mRNA expression, and reduced expression level of miRNA indicated the successful transfection.

MTT assay to detect the resistance of cancer cells to adriamycin. Transfected tumor cell suspension was inoculated into the microtiter wells of the test plates. After cell adherence, adriamycin was added to final concentrations of 5, 10, 20, 40, and 80 µg/ml, and control and blank control wells were set. Cells were cultured at 37°C with 5% CO₂ for 72 h, then 20 µl MTT solution was added to each well, followed by cell culture for another 4 h. Finally, 100 µl DMSO was added to each well, and crystals were dissolved by shaking for 15 min. Absorbance of each well at a wavelength of 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell growth inhibition rate = (absorbance of the control group - absorbance of the experimental group)/absorbance of the control group x100%. IC₅₀ value of adriamycin was calculated.

Figure 1. Expression of miR-93 in MCF-7 and MCF-7/ADM cells. RT-qPCR showed that the expression level of miR-93 in MCF-7/ADM cells was 40% (0.39±0.04) of that MCF-7 cells, and the difference was statistically significant, P<0.05. RT-qPCR, reverse transcription-quantitative PCR.

Western blot analysis to detect Bcl-2 and P-gp protein expression. MCF-7 and MCF-7/ADM cells were seeded into 6-well plates. MCF-7/ADM cells were transfected with miR-93 mimics and negative control groups, and total protein was extracted after 72 h of cell culture. Protein concentration was measured by BCA method. Then, 10% PAGE was performed to separate proteins, followed transmembrane to PVDF membrane. Blocking was performed using 5% skimmed milk powder (BSA). After washing with TBST (TBS + Tween) 3 times, rabbit polyclonal Bcl-2 antibody (dilution: 1/500; cat. no. ab59348) and rabbit polyclonal P-gp antibody (dilution: 1/500; cat. no. ab103477) were added and incubated for 2 h at 4°C. After washing with TBST 3 times, secondary goat anti-rabbit (HRP) IgG antibody (dilution: 1/2,000, cat. no. ab6721) was added and incubated for 2 h at room temperature. All the antibodies were purchased from Abcam (Cambridge, MA, USA). After washing with TBST 3 times, ECL reagent was added to develop signals, and ImageJ software was used to normalize the expression level of each protein to endogenous control GAPDH.

Figure 2. Comparison of adriamycin resistance of MCF-7 and MCF-7/ADM cells. Before transfection, IC₅₀ value of MCF-7 cells to adriamycin (11.02±0.95) was lower than that of MCF-7/ADM cells (21.29±1.83), and the difference was statistically significant, P<0.05.

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Quantitative data were analyzed by t-test, and results were expressed as mean ± standard deviation. P-values indicate a two-sided probability, and test standard α was 0.05.
Results

Expression of miR-93 in MCF-7 and MCF-7/ADM cells. Results showed that expression level of miR-93 decreased in MCF-7/ADM cells, and the expression level was 40% (0.39±0.04) of that in MCF-7 cells, and the difference was statistically significant (p<0.05, Fig. 1).

Effect of miR-93 transfection on adriamycin resistance of MCF-7 and MCF-7/ADM cells. Before transfection, IC_{50} value of MCF-7 cells to adriamycin (11.02±0.95) was significantly lower than that of MCF-7/ADM cells (21.29±1.83, p<0.05, Fig. 2). IC_{50} value of MCF-7/ADM cells at 72 h after transfection of miR-93 mimics (12.71±0.54) was significantly lower than that of the negative control group (25.16±1.28, p<0.05, Fig. 3). IC_{50} was significantly higher in MCF-7 cells (19.88±1.28) at 72 h after transfection with miR-93 inhibitor than in negative control group (11.02±0.95, p<0.05, Fig. 4).

Effect of miR-93 transfection on the expression of Bcl-2 and P-gp proteins in MCF-7 and MCF-7/ADM cells. Before transfection, expression levels of Bcl-2 and P-gp proteins in MCF-7/ADM cells were 1.63±0.24 and 1.76±0.22 times that...
of MCF-7 cells, respectively. The differences were statistically significant (p<0.05, Fig. 5). At 72 h after transfection of miR-93 mimics, the expression levels of Bcl-2 and P-gp protein in MCF-7/ADM cells were 0.27±0.06 and 0.39±0.05 times of those in the negative control group, and the differences were statistically significant (p<0.05, Fig. 6). At 72 h after transfection with miR-93 inhibitor, expression levels of Bcl-2 and P-gp protein in MCF-7 cells was 1.48±0.10 and 1.56±0.11 times of that of the negative control group, respectively, and the differences were statistically significant (p<0.05, Fig. 7).

Discussion

The combinations of adriamycin and other drugs are widely used in the clinical treatment of breast cancer (1). Adriamycin is a kind of chemotherapeutic drug widely used in clinical practices (9). Due to the emergence of adriamycin resistance in breast cancer cells, patients with advanced breast cancer are prone to recurrence or metastasis after treatment, which reduces the therapeutic effect. Mechanism of development of drug resistance in tumor cells is complex and may be related to the expression and dysfunction of multiple genes (10).

miRNA is a non-coding RNA and plays an important role in the occurrence and development of tumors (5). Studies have shown that dysfunction of different miRNAs is closely related to tumor recurrence, metastasis, and drug resistance, suggesting that miRNAs can be used as gene targets for cancer therapy (7). Li et al (10) found that miR-106b can reverse the resistance of breast cancer cell lines. miR-93 expression in non-small cell lung cancer, gastric cancer, esophageal cancer, colon cancer tissue exist down phenomenon (11-14), in breast cancer cells, patients with advanced breast cancer is associated with the occurrence and prognosis of various tumors, and the expressed proteins have the functions of inhibiting apoptosis, promoting tumor cell proliferation and metastasis (19). The binding of BAG3 protein to anti-apoptotic protein Bcl-2 can regulate the apoptosis process and play an important role in development of drug resistance in malignant tumor cells (20,21).

This study shows that miR-93 is downregulated in adriamycin-resistant breast cancer cell lines. Upregulation of miR-93 can reduce the resistance of cells to adriamycin, and the mechanism of action may be related to the downregulated Bcl-2 and P-gp proteins expression. Therefore, our findings provide new insights for the treatment of breast cancer, and miR-93 may serve as a potential target for gene therapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QW drafted this manuscript. QW and CS were mainly devoted to cell transfection and MTT assay. CW and JL contributed to western blot analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University (Jinan, China).

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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