

Cisplatin decreases cyclin D2 expression via upregulating miR-93 to inhibit lung adenocarcinoma cell growth

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Abstract. MicroRNAs (miRNAs/miRs) serve important roles in the chemotherapeutic effect of anticancer drugs. To investigate the roles of miRNAs in cisplatin-induced suppression of lung adenocarcinoma cell proliferation, A549 cells were treated with different concentrations of cisplatin. An MTT assay demonstrated that cisplatin inhibited A549 cell proliferation in a dose-dependent manner. Cisplatin induced cell apoptosis and inhibited cell migration by increasing the levels of miR-93, miR-26a and miR-26b. Furthermore, as an upstream factor, miR-93 was proposed to regulate cyclin D2 expression in miR-93-transfected A549 cells. Cisplatin also induced Bcl-2-associated X protein expression, and decreased that of Bcl-2 and c-Myc in lung adenocarcinoma cells. *In vivo* analysis further supported that cisplatin inhibited lung adenocarcinoma cell growth by regulating cyclin D2 and miR-93 expression. In conclusion, our findings demonstrated that cisplatin could effectively inhibit lung adenocarcinoma cell proliferation by decreasing cyclin D2 expression via miR-93.

Introduction

Lung cancer is one of the most common types of cancer and has the highest mortality rates worldwide (1). Non-small cell lung cancer (NSCLC) accounts for >80% of cancer-associated mortalities (2). With the development of scientific technology, notable progress has been achieved in the early diagnosis and treatment of various tumor types. A tumor can be completely

removed in the early stage of disease, although it may recur in half of the patients who undergo the process (1). According to statistics, the majority of patients with lung cancer are diagnosed in the advanced stages, at which the prognosis is very poor and the 5-year survival rate is <16% (3). Platinum-based chemotherapy is the primary treatment modality for NSCLC. However, adverse treatment outcomes, including drug resistance, may result in failure of chemotherapy (4).

MicroRNAs (miRNAs/miRs) are single-stranded non-coding RNAs of 20-22 nucleotides, which participate in gene expression and are involved in regulating post-transcriptional expression (5). miRNAs influence the proliferation, differentiation and apoptosis of cancer cells (5). It has been reported that miRNAs participate in tumor formation, which provides opportunities for the optimization of cancer treatment (6). miRNAs function as tumor promoters or inhibitors; thus, they serve critical roles in tumor occurrence and development, which is important not only in the diagnosis and prediction of prognosis, but also in developing novel therapeutic strategies for tumor treatment (7,8). The expression of miR-21 in plasma and tissue samples from patients with NSCLC could be used to predict the survival index and chemotherapy effect of platinum-based drugs (9).

miRNAs have also reported to be relevant to the potential toxicity mechanism (10) and the antitumor activity of drugs in several tumor types, including breast cancer (6), lung, colorectal (11) and esophageal cancer (12). Blower *et al* (13) identified that overexpression of miR-7i, miR-16 and miR-21 miRNA improved the chemotherapeutic effect of an anticancer drug in NSCLC cell lines. miR-539 could increase the chemosensitivity of NSCLC cells to cisplatin by directly targeting double cortin like kinase 1 (DCLK1) (14). Zhang *et al* (15) reported that by regulating glutathione S-transferase pi gene expression, miR-513a-3p could increase the sensitivity of a lung adenocarcinoma cell line to cisplatin. These studies indicate that miRNAs serve important roles in the anticancer activity of cisplatin.

Although it is understood that miRNAs serve important roles in the chemotherapeutic effect of anticancer drugs, the roles of miRNAs in cisplatin-induced suppression of lung cancer proliferation require further investigation. Therefore, the present study aimed to investigate the effects of cisplatin on the expression of miRNAs and the proliferation of lung cancer cells.

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Materials and methods

Chemicals and supplements. The following drugs and reagents were used in the present study: Fetal calf serum (Hyclone; GE Healthcare), trypsin (Sigma-Aldrich; Merck KGaA), RPMI-1640 culture medium (Sigma-Aldrich; Merck KGaA), DMSO (Sigma-Aldrich; Merck KGaA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Merck KGaA), penicillin/streptomycin (100 x; Gibco; Thermo Fisher Scientific, Inc.), cisplatin (Qilu Pharmaceutical Co., Ltd.), SYBR Green PCR master mix quantification PCR kit (Qiagen, Inc.), 5X poly A buffer, MgCl₂, and dATP (Promega Corporation), dNTP mixture and RNase inhibitor (Takara Bio, Inc.).

Cell lines and cell culture. To investigate the roles of cisplatin in the proliferation of lung cancer cells, the A549 cells were used in this study, which is a lung cancer cell line and provided by the Institute of Shanghai Cell Biology (16,17). All cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulfates at 37°C with 5% CO₂. Cells (1x10⁴) seeded in the 96-well flat bottom microtiter plates were respectively treated with 0, 3, 6 and 9 µg/ml of cisplatin for 24-72 h at 37°C as our previous report (17). Then the cells were observed under a microscope (with magnification 100 x, BX43, Olympus, Inc., Japan) at 48 h after treatment.

MTT assay. Cell proliferation assays were performed using a modified colorimetric MTT assay, according to the manufacturer's protocols. All procedures were repeated a minimum of three times, as described in our previous studies (18,19). Absorbance was measured at 570 nm using an ELISA reader (Multiskan FC; Thermo Fisher Scientific, Inc.).

Cell apoptosis assay. Apoptosis was measured using BD FACSDiva Software on a flow cytometer (BD FACSCanto™^{II}, BD Bioscience) after 48 h of incubation with 0, 3, 6 or 9 µg/ml cisplatin. Briefly, cells (1x10⁵/well of a 12-well flat-bottom microtiter plate) were stained with Annexin V-FITC/PI (Nanjing KeyGen Biotech. Co. Ltd.), according to the manufacturer's protocols.

Cell migration assay. To monitor cell migration, cells (1x10⁴ cells/well) were seeded onto the top chamber of a CIM plate (xCELLigence). The cells were incubated at room temperature for 30 min, and CIM plates were placed on an RTCA station (xCELLigence System; Roche, Diagnostics GmbH). Real-time monitoring of cell migration was conducted for 24 h by detecting the changes of electrical impedance at the electrode/cell interface.

Reverse transcription-quantitative PCR (RT-qPCR). A549 cells and tumor xenografts were harvested at 48 h following cisplatin treatment. miRNAs was isolated from the cells using mirVana™ miRNA kit (Ambion; Thermo Fisher Scientific, Inc.) and ploy (A) was added using poly (A) polymerase (2 U/µl; Ambion; Thermo Fisher Scientific, Inc.). cDNA was synthesized by incubating the reaction at 37°C for 1 h with a specific primer (5'-AACATGTACAGTCCATGGATGd(T)30N(A,G,C

or T)-3'). qPCR was performed to detect miRNA levels using TB Green® Premix Ex Taq™ kit (RR420A, Takara Bio Company), as described previously (18) on a StepOnePlus™ Real-Time PCR Systems (Version 2.3, life technologies). The following thermocycling conditions were used: 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 60°C for 20 sec and 72°C for 20 sec. The following forward primer sequences were used: miR-93, 5'-CAAAGTGCTGTTCGTGCAGGTA-3'; miR-26a, 5'-CAAGTAATCCAGGATAGG C-3'; miR-26b, 5'-CAAGTAATCCAGGATAGGT-3'; miR-29a, 5'-GCACCATCTGAAATCGGTTA-3'; miR-29c, 5'-GCACCA TTTGAAATCGGTTA-3'; and miR-125b, 5'-TCCCTGAGA CCCTAACTTGTG-3'. The reverse primer used to amplify these miRNAs was: 5'-AACATGTACAGTCCATGGATAG-3'. Human 5S rRNA was used as the control with the following primer sequences: Forward 5'-GCCATACCACCCTGA ACG-3', and reverse 5'-AACATGTACAGTCCATGGATG-3'. The data was calculated using the 2^{-ΔΔCq} method (20). All assays were performed in three triplicates.

miRNA mimics and transfection. The human miR-93 duplex mimic (miR-93; 5'-caaagugcuguucgucagguag-3') and control oligos (Mock; 5'-ccuacgccaccaauucgu-3') were obtained from Shanghai GenePharma Co., Ltd. Lung cancer cells (2x10⁵) were transfected with 1.5 µg miRNA using 2.5 µl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. miRNAs and total protein were extracted from cells 48 h after transfection.

miRNA target genes. TargetScanHuman Release 7.2 (http://www.targetscan.org/vert_72/) was used to predict the biological targets of miRNAs. The target genes and their miRNA binding sites were predicted using this online tool.

Western blotting. The cells were lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) at 4°C on ice for 30 min. After centrifugation in 13,400 x g for 5 min at 4°C, 30 µg of protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were incubated with rabbit anti-human cyclin D2 (1:400; cat. no. 10934-1; Proteintech Group, Inc.)/Bcl-2-associated X protein (Bax; 1:400; cat. no. BS2583; Bioworld Technology, Inc.)/Bcl-2 (1:400; cat. no. 12789-1; Proteintech Group, Inc.)/c-Myc (1:400; cat. no. BS2462; Bioworld Technology, Inc.)/β-actin (1:400; cat. no. BS-0061R; Beijing Biosynthesis Biotechnology Co., Ltd.) antibodies at 4°C overnight. Membranes were washed with TBS and Tween-20 (TBST) three times. HRP-labeled goat anti-rabbit IgG (1:6,000; cat. no. BS13278; Bioworld Technology, Inc.) was then added for 1 h at room temperature. Finally, signals were captured using enhanced chemiluminescence (Wuhan Boster Biological Technology, Ltd.) after three washes with 1X TBST. The assays were performed in triplicate. Densitometry was performed using a Gel Image System 4.2 (Tanon Science & Technology Co., Ltd.).

In vivo study. In total, 6 four-week old male athymic BALB/c mice (15 g) were purchased from Beijing HFK Bioscience Co., Ltd. The mice were kept at 22±2°C with a humidity of 50±5%

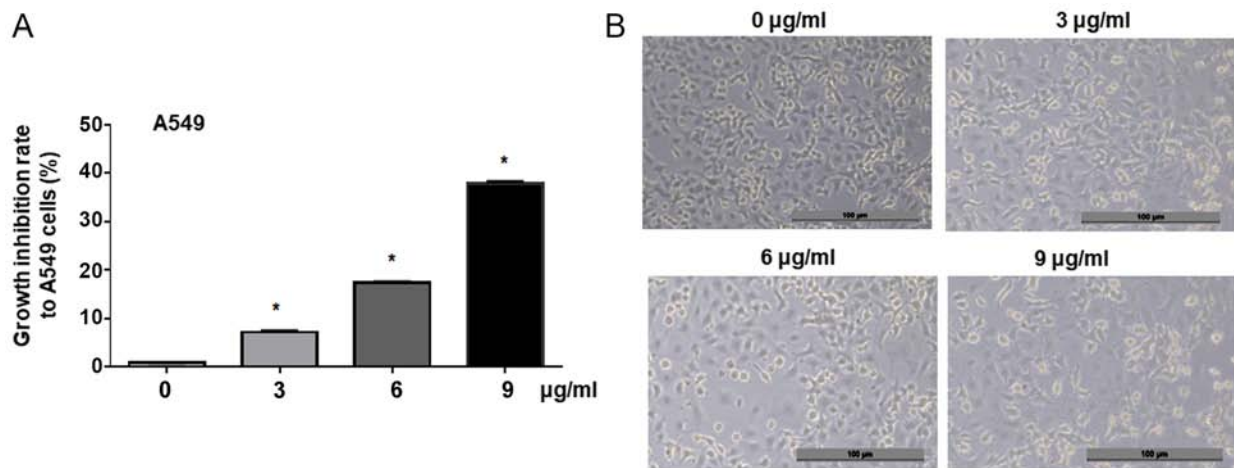


Figure 1. Effects of cisplatin on the growth of lung adenocarcinoma cells. (A) Growth inhibition rate. Cisplatin significantly inhibited A549 cell proliferation in a dose-dependent manner. * $P<0.05$ vs. 0 $\mu\text{g/ml}$. (B) Morphological changes of A549 cells. Fewer living cells were identified in the cisplatin-treated lung adenocarcinoma cultures compared with the untreated control. Scale bar, 100 μm .

in 20 x40 x60 cm cages, with a 12 h light/dark cycle and were fed with a standard diet and water. Each mouse was subcutaneously injected with an A549 cell suspensions (5×10^6 cells in 0.1 ml of PBS) into the armpit area. The experimental group ($n=3$) was treated with cisplatin (3 mg/kg) by intraperitoneal injection, while the control group ($n=3$) was treated with an equal volume of saline. All mice were sacrificed after 4 weeks and tumors were collected. All animal experiments were approved by the Ethics Committee of Animal Experiments of Binzhou Medical University.

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used to analyze the data. Experiments were performed in triplicate and the data are presented as the mean \pm SD. The comparison between two groups was analyzed using an unpaired two-sided Student's t-test. The comparisons among three or more groups were analyzed by One-way ANOVA. If the results had statistical significance, the comparison between two groups was performed using Bonferonni's tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cisplatin suppresses the proliferation of lung adenocarcinoma cells. In our previous study, it was identified that cisplatin could inhibit A549 cell proliferation by upregulating the expression of MutS homolog 2 through miR-21 (17). To further investigate the mechanism of cisplatin-induced suppression of lung adenocarcinoma cell growth, the present study treated A549 cells with different concentrations of cisplatin. MTT analysis results demonstrated that cisplatin significantly inhibited the proliferation of A549 cells in a dose-dependent manner compared with the control (0, 3, 6 and 9 $\mu\text{g/ml}$, Fig. 1A). Morphological analysis revealed that the treatment with cisplatin (3, 6 and 9 $\mu\text{g/ml}$) for 24 h suppressed A549 cell viability, compared with the untreated control cells (0 $\mu\text{g/ml}$, Fig. 1B). This indicated that cisplatin could effectively suppress lung adenocarcinoma cell proliferation in a dose-dependent manner.

Cisplatin induces apoptosis and inhibits the migration of A549 cells. Next, the current study detected the roles of cisplatin in regulating the apoptosis and migration of lung adenocarcinoma cells. The results demonstrated that the percentage of apoptotic cells in 3, 6 and 9 $\mu\text{g/ml}$ cisplatin-treated cultures was 14.4, 23 and 28.7%, respectively, higher than the 8.6% determined in the control treatment. The percentage of apoptotic cells was elevated in cisplatin-treated A549 cells in a dose-dependent manner, particularly in the 9 $\mu\text{g/ml}$ cisplatin-treated cells (Fig. 2A). The migration of A549 cells was analyzed using a RTCA station, which demonstrated that treatment with 6 and 9 $\mu\text{g/ml}$ cisplatin treatment significantly suppressed A549 cell migration (Fig. 2B and C).

miR-93 is upregulated in cisplatin-treated A549 cells. Previous studies have reported that miRNAs exhibit important roles in the regulation of chemotherapy drugs and molecule-targeted drugs (6,9-12,14,15). miR-93, miR-26a, miR-26b, miR-29a, miR-29c and miR-125 have been reported to exert important roles in cancers (21-24). To further investigate the roles of these miRNAs in cisplatin-induced inhibition of lung cancer cell proliferation, RT-qPCR was used to detect changes in the expression of six miRNAs, including miR-93, miR-26a, miR-26b, miR-29a, miR-29c and miR-125b, in cisplatin-treated A549 cells. The results demonstrated that the levels of miR-29c were significantly increased following treatment with 9 $\mu\text{g/ml}$ cisplatin; compared with the control, significant increases in the expression of miR-26b were detected in response to 3 and 6 $\mu\text{g/ml}$ cisplatin, while 3 $\mu\text{g/ml}$ cisplatin significantly induced miR-29a expression. Of note, treatment with all concentrations resulted in the significant upregulation of miR-93 and miR-26a compared with the control (Fig. 3).

miR-93 regulates the expression of cyclin D2. miR-93 has been reported to promote the apoptosis and increase the percentage of human umbilical vein endothelial cells in G1 phase by targeting angiopoietin 2 (25). The present study further revealed that that cyclin D2 is a novel target gene of miR-93 using TargetScanHuman 7.2 software online

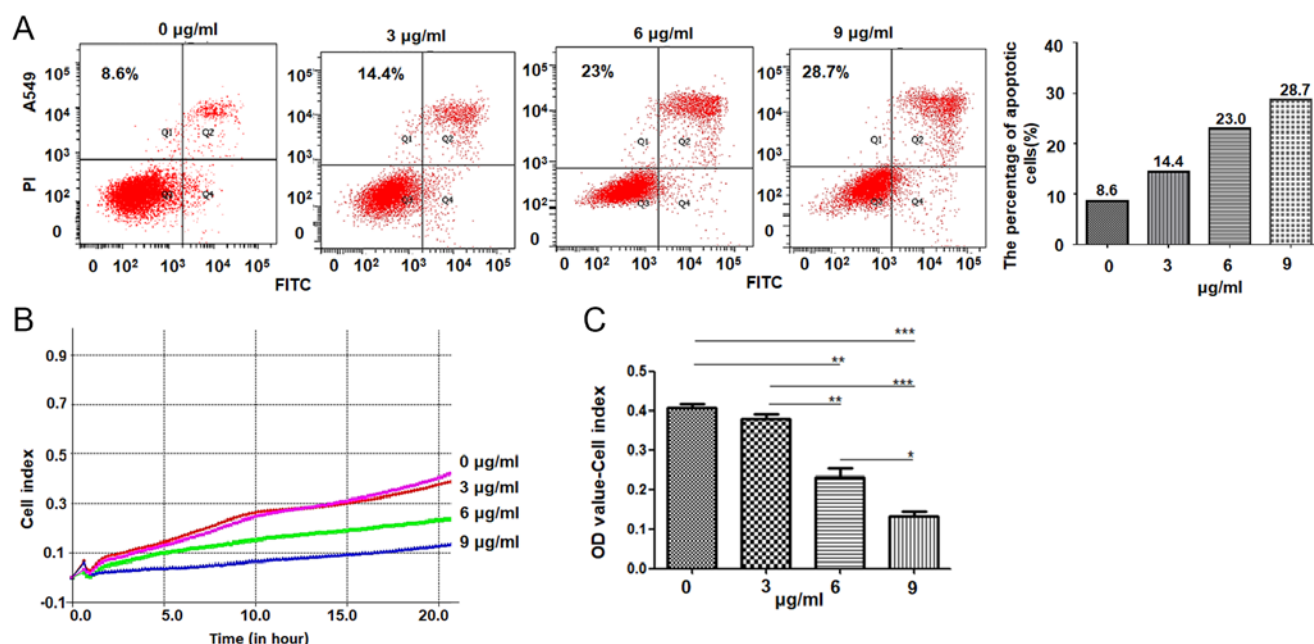


Figure 2. Cisplatin induces apoptosis and suppresses cell migration. (A) Apoptosis of A549 cells. More apoptotic cells were detected in the cisplatin-treated groups compared with the control. * $P < 0.05$ vs. 0 $\mu\text{g/ml}$. (B) Real-time monitoring of cell migration using a CIM plate. (C) Quantification of the number of migrated cells. Treatment with 6 and 9 $\mu\text{g/ml}$ cisplatin treatment effectively inhibited lung cancer cell migration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. FITC, fluorescein isothiocyanate; OD, optical density; PI, propidium iodide.

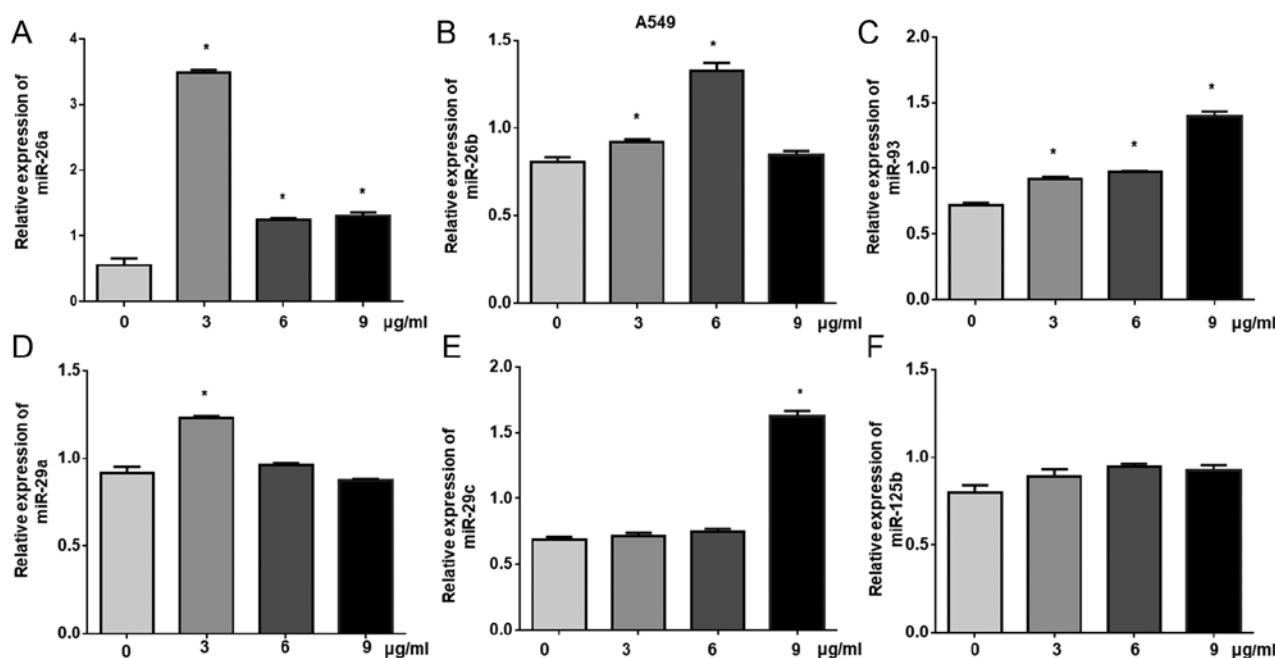
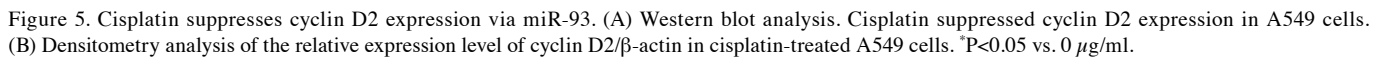
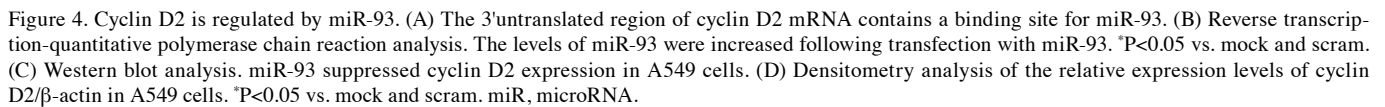


Figure 3. Cisplatin affects the expression of miRs in A549 cells. (A-F). The expression levels of miR-26a, miR-26b, miR-93, miR-29a, miR-29c and miR-125b in cisplatin-treated A549 cells were detected by reverse transcription-quantitative polymerase chain reaction. The levels of miR-93, miR-26a, miR-26b, miR-29a and miR-29c revealed significant increases in expression in response to certain concentrations of cisplatin. * $P < 0.05$ vs. 0 $\mu\text{g/ml}$. miR, microRNA.

(Fig. 4A). Subsequently, A549 cells were transfected with miR-93 to investigate whether miR-93 could regulate cyclin D2 expression as an upstream factor (Fig. 4B). Western blot analysis showed that overexpression of miR-93 significantly reduced the expression of cyclin D2 in miR-93-transfected A549 cells compared with control cells (Fig. 4C and D), which indicated that miR-93 decreased the expression of cyclin D2 as an upstream factor.

Cisplatin suppresses cell growth by decreasing cyclin D2 expression via miR-93. The aforementioned results indicated that cisplatin suppresses lung cancer cell growth by upregulating miR-93 expression, which negatively regulates cyclin D2 expression. To further investigate whether cisplatin suppresses cell growth by decreasing cyclin D2 expression via miR-93, cyclin D2 levels were detected by western blotting following cisplatin treatment. Our results revealed that



Cisplatin suppresses A549 cell growth in vivo. To evaluate the roles of cisplatin and miR-93 in regulating cell proliferation *in vivo*, A549 lung cancer xenografts were established in BALB/c nude mice. The results demonstrated that tumor volumes and weights were markedly decreased in cisplatin-treated xenografts compared with controls (Fig. 7A-C). RT-qPCR revealed that miR-93 expression was significantly increased in cisplatin-treated xenografts compared with the control treatment (Fig. 7D). Cyclin D2, the predicted target of miR-93, was notably downregulated in tumors treated with cisplatin compared with in control tumors (Fig. 7E), which supports the hypothesis that cisplatin suppresses A549 cell growth *in vivo* via miR-93 and cyclin D2.

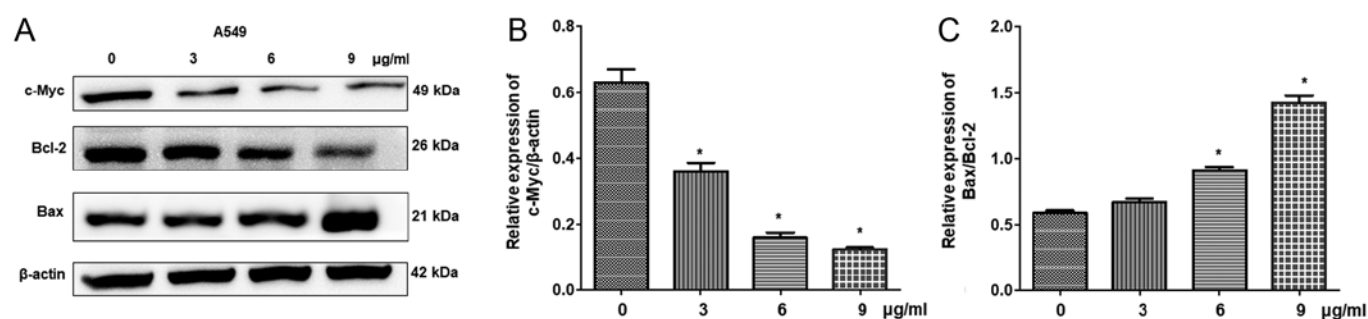


Figure 6. Cisplatin regulates the expression of apoptosis-associated genes. (A) Western blot analysis. (B) Densitometry analysis of the relative expression level of c-Myc/β-actin. (C) Densitometry analysis of the relative expression level of Bax/Bcl-2. Cisplatin decreased c-Myc and Bcl-2, and increased Bax levels in A549 cells. *P<0.05 vs. 0 μg/ml. Bax, Bcl-2-associated X protein.

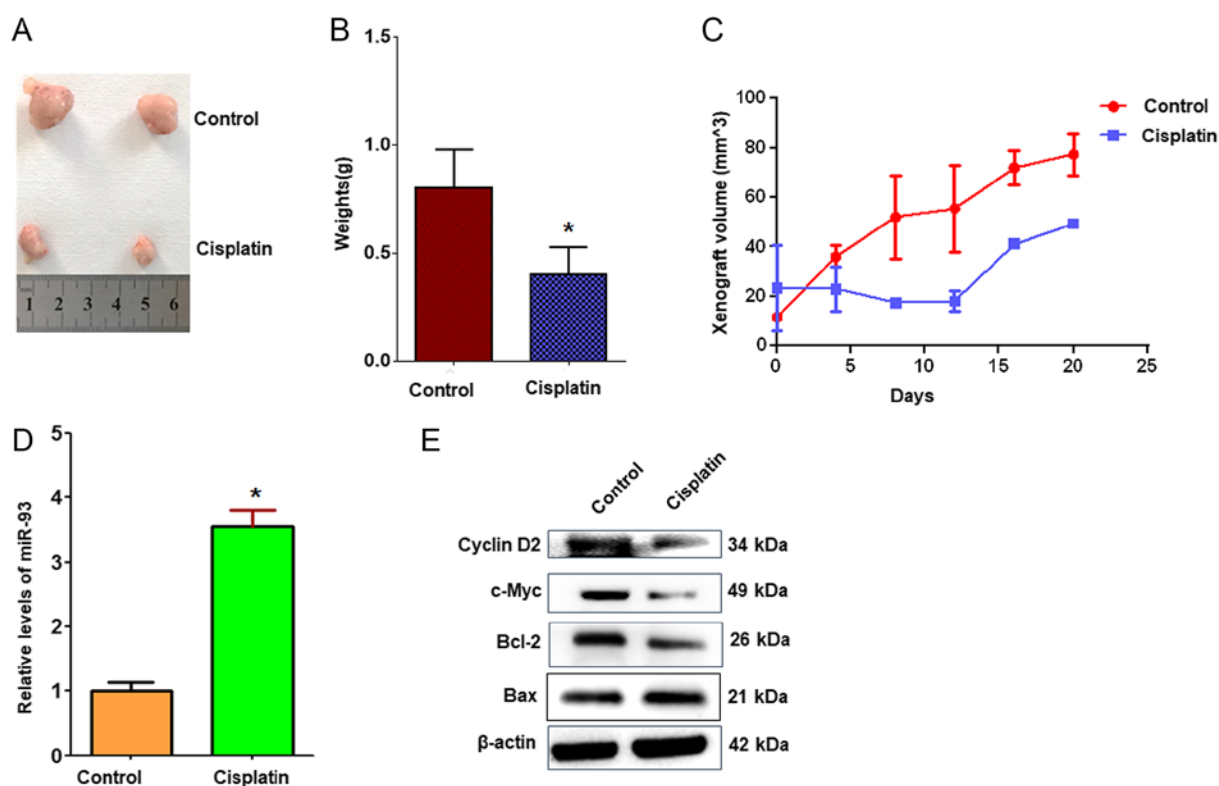


Figure 7. Cisplatin suppresses the growth of lung cancer cell xenografts *in vivo*. (A) Lung cancer xenografts. Cisplatin suppressed the growth of A549 lung cancer xenografts in BALB/C-nude mice. (B) Tumor weight. Tumor weight was significantly reduced in cisplatin-treated xenografts compared with the controls (*P<0.05 vs. control, n=3). (C) Alterations in tumor volume. The volume of A549 lung cancer xenografts were reduced in BALB/C nude mice following cisplatin treatment. (D) Reverse transcription-quantitative polymerase chain reaction analysis. The levels of miR-93 increased in cisplatin-treated cell xenografts (*P<0.05 vs. control, n=3). (E) Western blot analysis. The levels of cyclin D2, c-Myc and Bcl-2 decreased, while Bax increased, in cisplatin-treated cell xenografts. Bax, Bcl-2-associated X protein.

Discussion

Noncoding RNAs, including miRNAs, are involved in a number of pathological conditions of cancer (26). miRNAs are responsible for the development of resistance to anticancer drugs as they affect drug resistance-associated genes, and induce alternative signaling pathways and the DNA damage response (26). miR-205 can significantly induce apoptosis and enhance chemotherapeutic effects in prostate cancer cells (27). Furthermore, ethanol extract of *Antrodia cinnamomea* can inhibit the growth of breast cancer cells by increasing the expression levels of miR-21-5p, miR-26-5p and miR-30-5p (28). The

interaction of phosphoinositide 3-kinase with seven in absentia homolog 2 is regulated by the miRNA-30-5p family and is considered as a potential treatment target in NSCLC (29). The aforementioned miRNAs serve important roles in enhancing tumor sensitivity toward targeted therapies. The present study selected miR-93, which was significantly increased in lung adenocarcinoma cells after cisplatin treatment. Additionally, it was demonstrated that cyclin D2 is a direct target of miR-93, and miR-93 and cyclin D2 were reported to serve important roles in cisplatin-induced lung adenocarcinoma cell apoptosis.

Platinum-based drugs, particularly cisplatin, are first-line chemotherapy drugs (30). miRNAs are involved into the

mechanism of cisplatin for the treatment of cancer (14,31,32). The epigenetic regulation of insulin like growth factor 1 receptor via miR-1294 is important for cisplatin resistance in ovarian cancer (32). miR-539 can increase the chemosensitivity of lung cancer cells to cisplatin treatment by directly targeting DCLK1 (14). miR-363 may be a biomarker for predicting responsiveness to cisplatin-based chemotherapy by snail-induced epithelial-mesenchymal transition in epithelial ovarian cancer cells (31). Although these studies support that miRNAs participate in the anticancer roles of cisplatin for the treatment of cancer, the effects of cisplatin on the proliferation of lung cancer cells require further investigation. Previous studies found that miR-93, miR-26a, miR-26b, miR-29a, miR-29c and miR-125 play important roles in cancer (22-24). The present study investigated the roles of these miRNAs in the cisplatin-induced suppression of lung cancer and demonstrated that cisplatin could effectively inhibit lung adenocarcinoma cell proliferation in a dose-dependent manner. The mechanism by which cisplatin inhibits lung adenocarcinoma cell proliferation is proposed to be associated with the expression of miR-93 and miR-26a.

miRNAs are involved in numerous biological processes associated with cancer, including carcinogenesis, cell proliferation, invasion and migration, which serve crucial roles in the regulation of tumor development and progression (33,34). Through binding with the 3'-untranslated region of target mRNAs, miRNAs inhibit targeted gene expression to a certain degree (33). miR-9600, a novel molecule, has been identified to impair its target expression, which inhibits the growth of lung cancer cells (35). miR-93 has been reported to promote the apoptosis and the percentage of cells in the G1 peak of human umbilical vein endothelial cells by regulating its target gene, angiopoietin 2 (25). As miR-93 levels were significantly increased in cisplatin-treated A549 cells, the present study further investigated the targeted gene of this miRNA to evaluate the mechanism of cisplatin-induced lung cancer cell apoptosis. It was proposed that cyclin D2 is a novel target of miR-93, which serves important roles in cisplatin-induced apoptosis and migration.

D-type cyclins, including D1, D2 and D3, serve important roles in the G1 to S phase transition (36). Cyclin D2 acts as a proto-oncogene in several types of cancer (37). Cyclin D2 cytoplasmic localization may reflect an important physiological role in tumor progression (38). Cyclin D2 is overexpressed in 53% of colon tumors, and correlates to the high metastatic degree of tumors (39). Overexpression of cyclin D2 also correlates with progression and poor prognosis in gastric cancer (38). Aberrant cyclin D2 expression has been demonstrated in human ovarian granulosa cell tumors and testicular germ cell tumor cell lines (40). As a cell cycle regulator, cyclin D2 is regulated by let-7 in lung cancer (41). Similarly, the present study demonstrated that cisplatin inhibited lung cancer cell proliferation by decreasing cyclin D2 levels.

In summary, the current study further investigated the mechanism of cisplatin in the inhibition of lung cancer cell proliferation, which demonstrated that cisplatin could inhibit lung adenocarcinoma cell proliferation and migration in a dose-dependent manner. Furthermore, cisplatin was proposed to effectively inhibit lung adenocarcinoma cell growth by downregulating cyclin D2 via miR-93.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SYX conceived and designed the study. NX, YRL, YML, YNY, LP, YBW, PYW, and YJL performed the experiments. NX, YRL and SYX wrote the paper. PYW and SYX reviewed and edited the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Binzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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