Knockdown of miR-935 increases paclitaxel sensitivity via regulation of SOX7 in non-small-cell lung cancer

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Abstract. Sex determining region Y-box (SOX)7 is a member of the SOX family and is responsible for various developmental processes. As a tumor suppressor, decreased expression of SOX7 has been observed in several cancer types, including non-small-cell lung cancer (NSCLC). However, the mechanism underlying SOX7 downregulation and its role in chemoresistance in NSCLC remains poorly understood. In the present study, the inhibition of microRNA (miR)-935 increased the expression of SOX7 at the mRNA and protein levels in A549 cells. The luciferase reporter assay verified that miR-935 could directly bind to the 3'untranslated region of SOX7 mRNA to suppress its expression in A549 cells. In addition, the inhibition of miR-935 enhanced the anticancer effect of paclitaxel, i.e., induced cell growth arrest and apoptosis in A549 cells. It was further observed that the inhibition of miR-935 decreased the B cell lymphoma (Bcl)-2 and phosphorylated-RAC-α serine/threonine-protein kinase (AKT) protein levels and increased the Bcl-2 associated X, apoptosis regulator protein levels, without affecting the AKT levels in the presence of paclitaxel within A549 cells. The findings of the present study validate miR-935 as a predictor of paclitaxel sensitivity in NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for ~85% of lung cancer cases (2). Conventional chemotherapy is frequently used to treat NSCLC patients. However, the clinical outcome is unsatisfactory, due to the intrinsic and acquired drug resistance (3). Therefore, investigation of the molecular mechanisms underlying the development of drug resistance is crucial for improving patient overall survival.

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Paclitaxel is a chemotherapeutic agent that has been proven to be effective against a number of cancer types, including NSCLC (4-6). However, despite its effectiveness as an anticancer agent, tumor cells have the ability to acquire resistance against paclitaxel treatment over time. The altered expression levels of multiple genes have been implicated in the development of paclitaxel resistance (7). However, due to the heterogeneity of cancer cells, other mechanisms of paclitaxel resistance must be investigated to fulfill clinical needs.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs involved in human developmental processes (8). The dysregulation of miRNAs promotes cancer initiation and progression, and is commonly detected in cancer cells (9). As a novel miRNA, the studies on miR-935 are not very abundant. miR-935 has been reported to be a prognostic biomarker of weight loss (10); furthermore, miR-935 has also been reported to be upregulated in cluster of differentiation133+ neural stem cells when compared with medulloblastoma specimens (11); in addition, upregulation of miR-935 promotes the malignant behaviors of PANC-1 pancreatic carcinoma cells through targeting inositol polyphosphate 4-phosphatase type I gene (12). However, its specific role and target gene in NSCLC have not been reported. Consequently, the present study aimed to explore its role in NSCLC.

Notably, in 2016, miR-935 was demonstrated to promote cell proliferation of gastric cancer through targeting sex determining region Y-box (SOX)7 (13). In 2017, miR-935 was reported to promote the cell proliferation and migration of liver cancer through targeting SOX7 (14). Whether miR-935 also targets SOX7, thus influencing the progression of NSCLC, remains to be elucidated. Therefore, the present study investigated the aforementioned issue.

SOX7, a member of the SOX family of transcription factors (15), is involved in mediating developmental processes and is a well-known tumor suppressor in a number of cancer types (16,17). In lung cancer, SOX7 levels are decreased and correlate with a poor prognosis (18). In particular, SOX7 physically binds to β-catenin and transcription factor 4, thereby suppressing the Wnt pathway to repress cell stemness (19). Downregulation of SOX7 increases stemness of cancer cells and renders cells resistant to chemotherapy (20).

The aim of the present study was to investigate whether miR-935 could downregulate SOX7 by directly binding to the 3'untranslated region (UTR) of SOX7 in A549 cells, and whether inhibition of miR-935 promoted cell growth arrest

and apoptosis induced by paclitaxel treatment in A549 cells, in order to determine whether miR-935 may be a promising predictor of paclitaxel sensitivity in NSCLC.

Patients and methods

Patients. NSCLC patients were investigated in the present study, patients who underwent radiotherapy or chemotherapy were excluded from the present study; neoplastic and non-neoplastic lung tissue samples were obtained from 30 patients (19 male and 11 female) who were aged between 51.23±6.31-years old, treated at The Second People's Hospital of Jingmen (Jingmen, China) during surgery from November 2013 to March 2015. The tissue samples were immediately stored at -80˚C. All the patients participating in the study provided written informed consent. Ethical approval was also provided by the Ethics Committee of the Second People's Hospital of Jingmen.

Cell culture. The A549 human NSCLC and 293T cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), whereas 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37˚C at 95% humidity, in an incubator containing 5% CO_2.

Cell transfection. A549 cells were plated in 24-well plates. miR-935 inhibitor (5'-GCGGUAGCGGAAGCGGUAACUGG-3') and miR-negative control (NC) inhibitor (5'-UUGUACUACACAAAAGUACUG-3') were purchased from Shanghai GenePharma Co., Ltd (Shanghai, China), 30 μM miR-935 inhibitor and 30 μM miR-NC inhibitor were respectively transfected into A549 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h, the A549 cells were harvested for further analyses.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissues and cells was extracted using the miRNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using TransScript First-Strand cDNA Synthesis Supermix (TransGen, Beijing, China) according to the manufacturer's protocol. RT-qPCR was performed with SYBR green qPCR Supermix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The conditions were as follows: 55˚C for 10 min, followed by 40 cycles at 95˚C for 30 sec, 55-59˚C for 30 sec and 72˚C for 42 sec. Primer sequences were as followed: miR-935 forward, 5'-GCCCAGTTACCGCTTCCGCTAC-3' and reverse, 5'-AACGCTTCACGAATTGCGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; SOX7 forward, 5'-CCTCATGCTCTGAAGATTGCC-3' and reverse, 5'-GGTCAATGGTGTCATAATGG-3' and GAPDH forward, 5'-ACAAGATGGTGAAGGTCGGTGTGA-3' and reverse, 5'-AGCTTCCCATTCTCAGCCTTGACT-3'. Fold changes of genes were evaluated with the $2^{-\Delta\Delta Cq}$ method (Cq, quantification cycle) (21), and the expression levels of the miRNA and target gene were normalized to U6 and GAPDH, respectively.

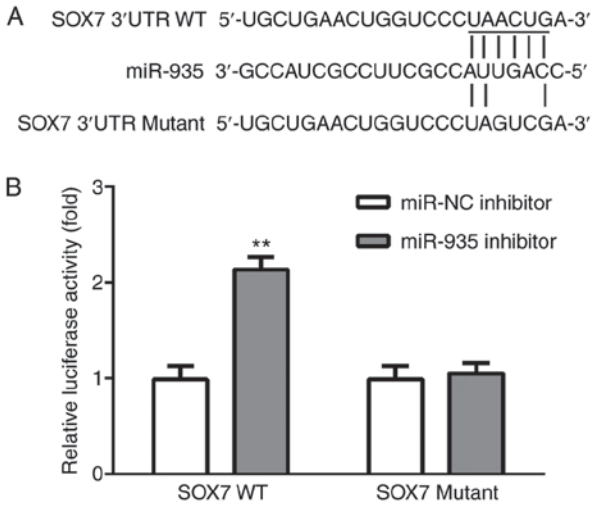

Figure 1. SOX7 is a target of miR-935. (A) Binding site between miR-935 and SOX7. (B) Inhibition of miR-935 increased luciferase activity in cells transfected with SOX7 WT, but luciferase activity was not affected following transfection with miR-NC inhibitor; there was no significant difference in luciferase activity in cells transfected with SOX7 MUT and miR-935 inhibitor or miR-NC inhibitor. **P<0.01 vs. miR-NC inhibitor group. WT, wild-type; MUT, mutant; NC, negative control; miR, microRNA; SOX7, sex determining region Y-box 7.

MTT assay. The 5-diphenyltetrazolium bromide (MTT) assay was used to assess cell proliferation. A549 cells were seeded in 96-well plates at a density of $5x10^3$ cells per well, then treated with 100 μl 0.5 mg/ml MTT for 4 h at 37˚C, and the precipitate was dissolved into 150 μl dimethyl sulfoxide. Finally, the optical density at a wavelength of 570 nm was assessed.

Flow cytometry assay. A549 cells were removed from plates via trypsinization, collected by and centrifugation at 4˚C with the speed of 23,200 x g for 5 min, washed in ice-cold PBS, and fixed in 80% ice-cold ethanol PBS at 4˚C overnight, 48 h after transfection with miR-935 inhibitor. Next, A549 cells were re-suspended in 500 μl 1X binding buffer, followed by the addition of 5 μl Annexin V-fluorescein isothiocyanate (FITC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 2.5 μl propidium iodide (PI, Sigma-Aldrich; Merck KgaA). Cell apoptosis was evaluated via a FACSAria Sorter (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed with Cell Quest software (version FCS2.0, BD Biosciences).

Luciferase activity assay. SOX7 was predicted as a target gene of miR-935 using a publicly available online algorithm, TargetScan (http://www.targetscan.org/vert_71/). Oligonucleotides containing the SOX7 cDNA fragment, including binding sites with miR-935, were cloned into pmirGLO plasmid (Promega Corporation, Madison, WI, USA). Mutant SOX7 (pmirGLO-SOX7-MUT) was generated by site-directed mutagenesis PCR with Platinum Pfx DNA polymerase (Thermo Fisher Scientific, Inc.)

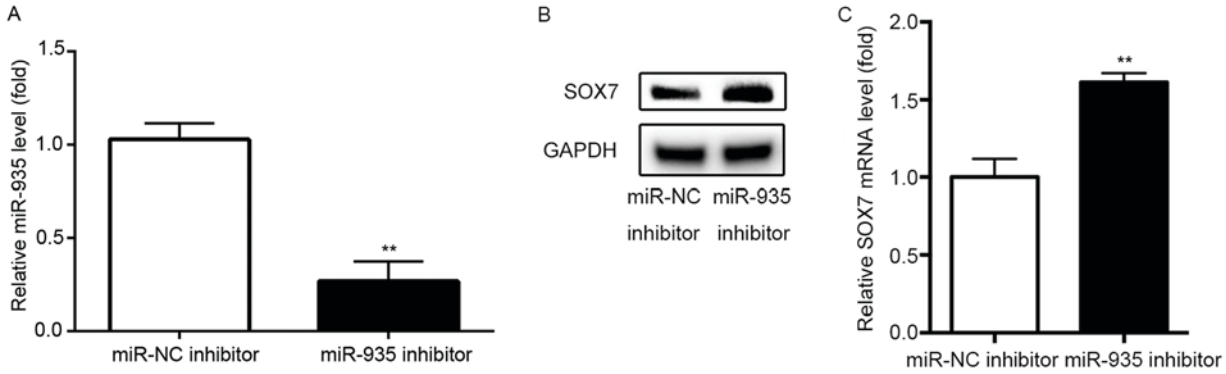

Figure 2. miR-935 inhibitor upregulates expression of SOX7. (A) miR-935 inhibitor was successfully transfected into A549 cells, as confirmed by RT-qPCR. (B) miR-935 inhibitor upregulated the protein level of SOX7, as evidenced by western blotting. (C) miR-935 inhibitor upregulated the mRNA level of SOX7, as evidenced by RT-qPCR. **P<0.01 vs. miR-NC inhibitor group. NC, negative control; miR, microRNA; SOX7, sex determining region Y-box 7. Reverse transcription-quantitative polymerase chain reaction.

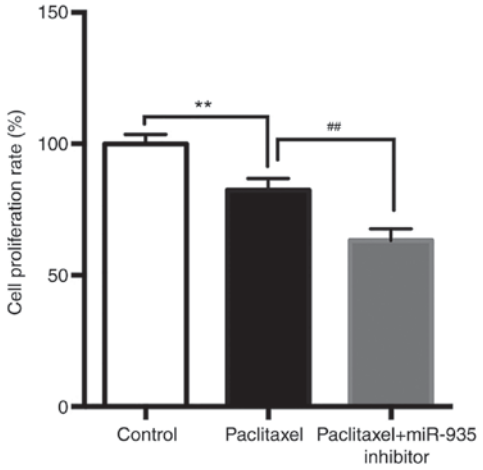

Figure 3. miR-935 inhibitor enhances paclitaxel-induced arrest of cell proliferation. MTT analysis demonstrated that, compared with control cells, paclitaxel significantly induced A549 cell growth arrest, which was further enhanced by the knockdown of miR-935. **P<0.01, paclitaxel group vs. control group, ##P<0.01, paclitaxel+miR-935 inhibitor group vs. paclitaxel group. miR, microRNA.

according to the manufacturer's protocol and served as a negative control. Luciferase reporter plasmids pmirGLO (Promega Corporation) and target miR-935 inhibitor or miR-NC inhibitor were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h after cells were transfected with the corresponding plasmid, the relative luciferase activity was assessed with the Dual-Luciferase Reporter Assay System (Promega Corporation); luciferase activity was normalized to that of *Renilla* luciferase activity.

Western blot analysis. Proteins were extracted with radioimmunoprecipitation assay lysis buffer (Roche Diagnostics, Basel, Switzerland). Protein quantification was performed via BCA method (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (10 μg protein/lane) were separated by 8-12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes with an iBlot Gel Transfer Device (Thermo Fisher Scientific, Inc.). The PVDF membranes were blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature for 2 h, then incubated with primary antibodies at 4˚C overnight. The primary antibodies used were anti-SOX7 (1:1,000; ab49163, Abcam, Cambridge, UK), anti-B cell lymphoma (Bcl)-2 associated X, apoptosis regulator (Bax; 1:1,000; 14796, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Bcl-2 (12789; 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA), anti-RAC-a serine/threonine-protein kinase (AKT; 1:1,000; ab81283, Abcam), anti-phosphorylated (p)-AKT (1:1,000; 4060), and GAPDH (1:1,000; 5174; both Cell Signaling Technology). Subsequently, the PVDF membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000, 7074, Cell Signaling Technology, Inc., Danvers, MA, USA). Finally, the bands were treated with enhanced chemiluminescence reagents (GE Healthcare Life Sciences, Little Chalfont, UK) and the intensity of the bands were quantified by ImageQuant TL 7.0 (GE Healthcare Life Sciences).

Statistical analysis. Analyses were performed with SPSS software, version 13 (SPSS Inc., Chicago, IL, USA). All data were expressed as the mean ± standard deviation. Comparisons between two groups were performed using the Student's t-test, and comparisons among multiple groups by one-way analysis of variance followed by a Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

SOX7 is targeted by miR-935. SOX7 was the predicted target gene for miR-935 as determined by publicly available algorithms online, including TargetScan (Fig. 1A). Luciferase analysis found that, in cells transfected with SOX7 WT, the inhibition of miR-935 significantly increased luciferase activity, whereas in cells transfected with SOX7 MUT, luciferase activity did not significantly change when either miR-935 inhibitor or miR-NC inhibitor was transfected (Fig. 1B).

Thereafter, RT-qPCR results confirmed the successful transfection of miR-935 inhibitor into A549 cells (Fig. 2A) and western blot analysis was performed to determine the

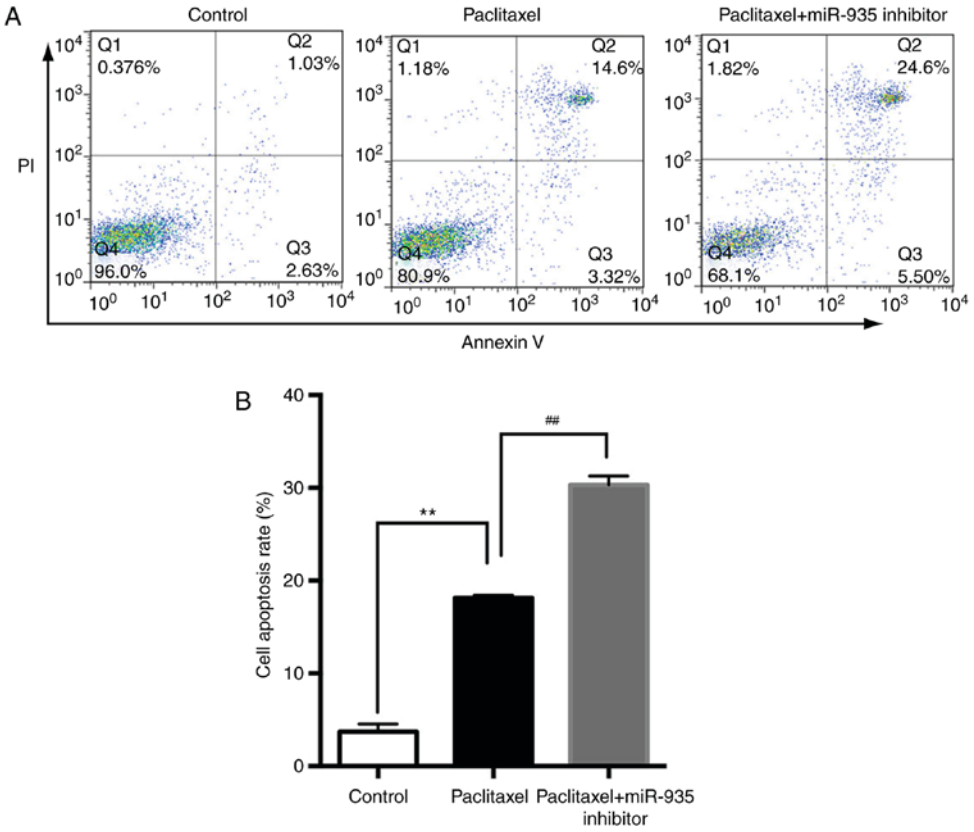

Figure 4. miR-935 inhibitor promotes paclitaxel-induced cell apoptosis. (A) Flow cytometry analysis indicated that, compared with control cells, paclitaxel significantly induced A549 cell apoptosis, and the downregulation of miR-935 further promoted cell apoptosis. (B) Quantitative representation of flow cytometry results. **P<0.01, paclitaxel group vs. control group, $^{\#\#}$P<0.01, paclitaxel+miR-935 inhibitor group vs. paclitaxel group. miR, microRNA; PI, propidium iodide.

effect of miR-935 on SOX7 expression in A549 cells. It was demonstrated that miR-935 inhibitor upregulated the protein level of SOX7 (Fig. 2B). Furthermore, the RT-qPCR results revealed that miR-935 inhibitor upregulated the mRNA level of SOX7 (Fig. 2C). These results confirmed SOX7 as a direct target for miR-935 in human NSCLC cells.

miR-935 inhibitor enhances paclitaxel-induced cell proliferation arrest. miR-935 inhibitor was transfected into A549 cells. MTT analysis revealed that, compared with control cells, paclitaxel significantly resulted in A549 cell growth arrest, which was further enhanced by the knockdown of miR-935 (Fig. 3). It may therefore be inferred that miR-935 contributes to cell proliferation *in vitro*.

miR-935 inhibitor enhances paclitaxel-induced cell apoptosis. Furthermore, flow cytometry analysis indicated that, compared with control cells, paclitaxel significantly induced A549 cell apoptosis, and the downregulation of miR-935 further promoted cell apoptosis (Fig. 4A). The corresponding statistical data are presented in Fig. 4B. It may be inferred that miR-935 represses cell apoptosis *in vitro*.

miR-935 inhibitor enhances paclitaxel-induced Bax upregulation and Bcl-2 and p-Akt downregulation. The present study detected the alterations of p-Akt, Bax and Bcl-2 protein levels in A549 cells following treatment with miR-935 inhibitor. The results indicated that, compared with control cells, there was marked upregulation of Bax (Fig. 5A and B), and downregulation of Bcl-2 (Fig. 5A and C) and p-Akt (Fig. 5A and D), following treatment with paclitaxel. Whereas following transfection with miR-935 inhibitor, the aforementioned changes were further enhanced (Fig. 5). These results suggested that miR-935 acts through regulating apoptotic-associated pathways and molecules via targeting SOX7.

miR-935 is upregulated and SOX7 is downregulated in NSCLC tissues. To determine the role of miR-935 and SOX7 in NSCLC tissues, the present study first tested miR-935 expression levels in primary NSCLC tissues and the matched adjacent normal lung tissues from 30 patients, and observed that miR-935 was significantly upregulated in NSCLC tissues (Fig. 6A). SOX7 expression was then tested in primary NSCLC tissues and matched adjacent normal lung tissues; SOX7 was revealed to be downregulated in NSCLC (Fig. 6B).

Discussion

The present study successfully transfected the miR-935 inhibitor into A549 cells and revealed that miR-935 down-regulated SOX7 by directly binding to the 3'UTR of SOX7 mRNA in A549 cells. Additionally, the inhibition of miR-935 enhanced cell growth arrest and apoptosis which were induced by paclitaxel treatment in A549 cells. Inhibition of miR-935 reduced the Bcl-2 and p-AKT protein levels, increased the Bax protein levels, but did not alter the protein levels of AKT. The data suggested that miR-935 may be a promising predictor of paclitaxel sensitivity in NSCLC.

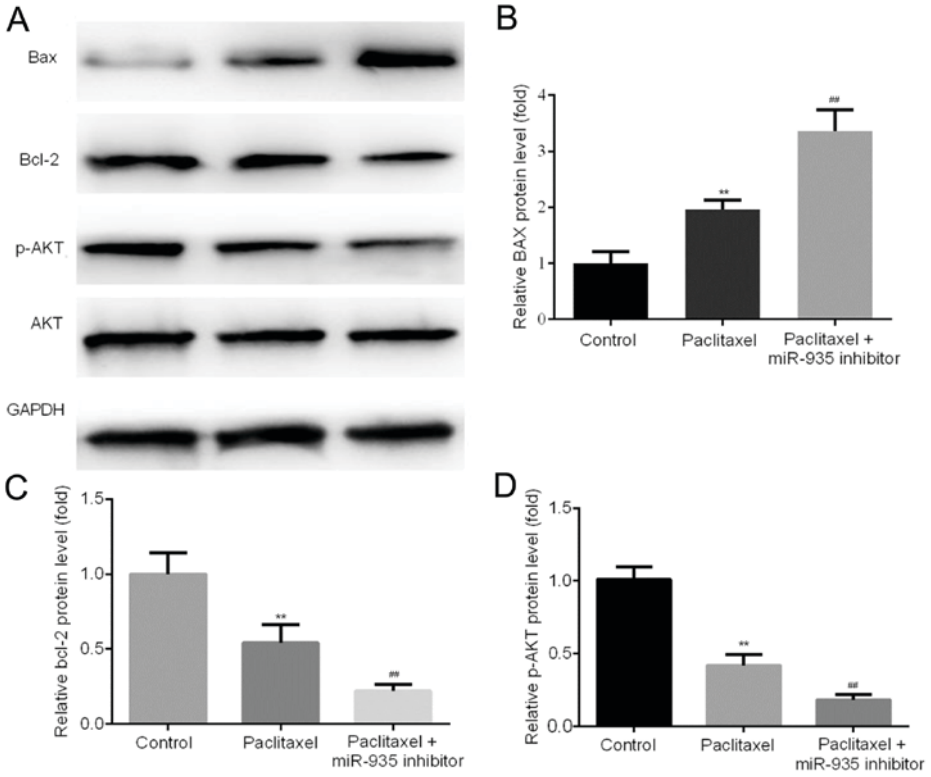

Figure 5. miR-935 inhibitor enhances paclitaxel-induced upregulation of Bax and downregulation of Bcl-2 and p-Akt. Compared with control cells, there was marked upregulation of (A and B) Bax and downregulation of (A and C) Bcl-2 and (A and D) p-Akt following paclitaxel administration. miR-935 inhibitor further enhanced these changes. **P<0.01, paclitaxel group vs. control group, ##P<0.01, paclitaxel+miR-935 inhibitor group vs. paclitaxel group. Bcl-2, B cell lymphoma; Akt, RAC-a serine/threonine-protein kinase; Bax, Bcl-2 associated X, apoptosis regulator; p, phosphorylated; miR, microRNA.

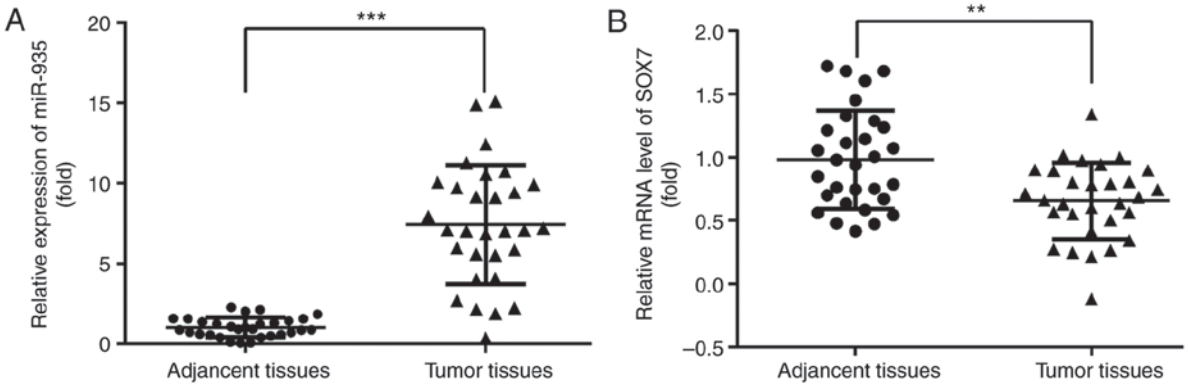

Figure 6. miR-935 is upregulated and SOX7 is downregulated in NSCLC tissues. (A) miR-935 was significantly increased and (B) SOX7 was decreased in NSCLC tissues compared with the matched adjacent normal lung tissues. **P<0.01, ***P<0.0001, tumor tissues vs. adjacent tissues. NSCLC, non-small-cell lung cancer; miR, microRNA; SOX7, sex determining region Y-box 7.

SOX7, together with SOX17 and SOX18, belongs to the SOX-F subfamily (22). SOX7 mediates various developmental processes and is a well-known tumor suppressor in several cancers (16,17). In lung cancer, SOX7 levels are decreased and have been demonstrated to be closely associated with poor prognosis (18). Downregulation of SOX7 increases the stemness of cancer cells and renders cells resistant to chemotherapy (20). Notably, in 2016, miR-935 was revealed to promote cell proliferation of gastric cancer through targeting SOX7 (13). Later, in 2017, miR-935 was reported to promote the cell proliferation and migration of liver cancer through targeting SOX7 (14). Whether miR-935 also targeted SOX7, thus resulting in the progression of NSCLC was unknown. Therefore, the present study investigated the aforementioned issue.

The PI3K/Akt signaling pathway is crucial in modulating cell proliferation, migration, apoptosis and angiogenesis (23). The Bcl-2 protein family, including Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic), is involved in the apoptotic process (24). In the present study, the protein levels of Bax and Bcl-2 were measured following treatment with paclitaxel and miR-935 inhibitor combined with paclitaxel.

miR-935 was demonstrated to be markedly upregulated in NSCLC tissues compared with the adjacent non-cancerous

tissues and SOX7 was verified as a direct target gene of miR-935. Knockdown of miR-935 increased the expression of SOX7 at both the mRNA and protein levels, and further promoted the cell proliferation arrest and cell apoptosis of A549 cells induced by paclitaxel *in vitro*. The precise underlying mechanism may be as follows: Inhibition of miR-935 reduced the Bcl-2 protein level and activity of p-AKT, whereas it increased the Bax protein level.

However, there are several limitations that need to be addressed in future studies: the authors will aim to apply more than one cell line in future studies; the function of SOX7 and the combined effect of miR-935 and SOX7 in NSCLC chemo-sensitivity will be investigated; experiments on cell proliferation/apoptosis under paclitaxel treatment using miR-935 inhibitor and small interfering-SOX7 or miR-935 mimics and SOX7 overexpressing vector may be conducted; in addition to investigating how SOX7 influences the expression of the downstream pathway molecules Bax, Bcl-2, and p-AKT. In conclusion, the results of the present study suggested that miR-935 may serve a key role in regulating the proliferation and apoptosis of NSCLC cells, indicating the potential value of miR-935 as a therapeutic target for NSCLC.

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Availability of data and materials

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

Authors' contributions

GC made substantial contributions to the concept and design of the present study, and wrote the paper. BP, CL and PC performed the experiments and analyzed the data. LY and BZ collected samples for analysis. All authors read and approved the final manuscript.

Ethic approval and consent to participate

The present study was approved by the ethics committee of the Second People's Hospital of Jingmen (Jingmen, China). Written informed consent was obtained from all the patients who participated in the research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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