miR-186-5p targeting SIX1 inhibits cisplatin resistance in non-small-cell lung cancer cells (NSCLCs)

X. LIU, X. ZHOU, Y. CHEN, Y. HUANG, J. HE, H. LUO*

Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of the Army Medical University, Chongqing, China

Correspondence: luohucy888@163.com

miR-186-5p has been reported to be implicated in tumorigenesis and paclitaxel resistance in non-small-cell lung cancer cells (NSCLCs). However, it remains undisclosed whether miR-186-5p takes a part in chemoresistance against diaminodichloroplatinum (cisplatin, DDP) in lung cancers, including NSCLC. Expression of miR-186-5p and sine oculis homeobox 1 (SIX1) was detected using RT-qPCR and western blot. *In vitro*, 50% inhibitory concentration (IC50) of DDP and cell proliferation were measured by MTT assay. The rate of apoptosis and abilities of migration and invasion were evaluated with flow cytometry and Transwell assay. The target binding between miR-186-5p and SIX1 was predicted on Diana tools software and confirmed by dual-luciferase reporter assay and RNA immunoprecipitation. *In vivo* experiments, xenogeneic transplantation was conducted to monitor the tumor growth. Expression of miR-186-5p was downregulated in DDP resistant NSCLC tissues and cells (A549/DDP and H1299/DDP). Functionally, miR-186-5p overexpression could inhibit cell proliferation, migration and invasion, and promoted apoptosis rate in A549/DDP and H1299/DDP cells. Mechanically, SIX1 was identified as a downstream target for miR-186-5p and was highly expressed in A549/DDP and H1299/DDP cells. Similarly, SIX1 knockdown could also suppress DDP resistant NSCLC cell proliferation, migration and invasion, and promote apoptosis rate, which was reversed by miR-186-5p downregulation. Moreover, xenograft tumors induced by A549/DDP cells exerted cisplatin resistance, and miR-186-5p overexpression could inhibit tumor growth under DDP treatment. In conclusion, upregulation of miR-186-5p suppresses cisplatin resistance in DDP resistant NSCLC cells both *in vitro* and *in vivo* presumably by targeting SIX1.

Key words: miR-186-5p, SIX1, cisplatin resistance, NSCLC

Non-small-cell lung cancer (NSCLC) is one of the most lethal cancers worldwide and accounts for approximately 85% of all primary lung cancers [1]. NSCLC tumors are classified into three different subtypes: squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC). Chemotherapy is the major treatment approach for patients with NSCLC, and diaminodichloroplatinum (cisplatin, DDP) is one of the most effective and widely used chemotherapeutic drugs among cancers, including NSCLC [2, 3]. Following the use of cisplatin chemotherapy, many changes have been associated with the multidrug-resistance phenotype of tumor cells. However, the clinical outcomes are disappointing, largely due to a poor response to cisplatin in these patients [4]. Therefore, it is urgently important to figure out the molecular mechanism of cisplatin resistance to further prevent NSCLC progression and recurrence.

MicroRNAs (miRNAs) are a group of endogenous small non-coding RNAs with approximately 22 nucleotides in length. Functionally, miRNAs are involved in diverse cellular processes, including proliferation, apoptosis, migration, etc. [5]. Besides, it is well recognized that miRNAs are important regulators of various pathophysiological processes in carcinogenesis [6], as well as drug resistance [7]. Recent studies have reported that miRNA (miR)-186-5p functions as tumor suppressor in various malignancies. In NSCLC, miR-186-5p has been shown to have lower expression and takes part in its tumorigenesis, development and prognosis [8–10]. Moreover, accumulating evidences have suggested a link between miR-186-5p and chemoresistance with several chemotherapeutic drugs, for instance paclitaxel [11, 12], methotrexate [13], platinum [14] and DDP [12, 15, 16]. In NSCLC, miR-186-5p is declared to regulate paclitaxel resistance of NSCLC cells both *in vivo* and *in vitro* [11].

However, its role in regulating cisplatin resistance has not been investigated in NSCLC.

Mechanically, miRNAs usually bind to 3' untranslated regions (3' UTR) of target genes, thus leading to their mRNA degradation or translational inhibition [17]. miR-186-5p exerts its biological functions through downregulating target genes as well [8–11]. In this study, we investigated the altered expression of miR-186-5p in DDP resistant and sensitive NSCLC tissues and cells. The role and molecular mechanism of miR-186-5p on cell proliferation, apoptosis, migration and invasion of DDP resistant NSCLC cells were evaluated *in vitro*, as well as on tumor growth *in vivo*.

Materials and methods

Acquirement of tissue samples. Before clinical sample acquirement, we had achieved the approval of Research Ethics Committee of the First Affiliated Hospital of the Army Medical University and the written informed consents from 50 NSCLC patients from 2014 to 2017. All patients received definitive chemotherapy with cisplatin after surgery. NSCLC tissue samples were obtained and divided into 2 groups: DDP resistant (n=25) and DDP sensitive (n=25). All tissue samples were immediately frozen in liquid nitrogen and then stored at –80 °C. DDP resistant NSCLC was defined as tumor progression or recurrence within 6 months after the last DDP treatment, while those recurrence or progression more than 6 months were identified as DDP sensitive NSCLC.

Cells and cell culture. Human NSCLC cell lines A549 and H1299 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO_2.

Construction of DDP resistant NSCLC cells *in vitro*. DDP-resistant A549 and H1299 cells (A549/DDP and H1299/DDP) were developed with incubation in medium containing gradually increased concentrations of DDP (Sigma-Aldrich, St. Louis, MO, USA) from 5–30 μM for 12 days. Finally, 5 μM of DDP was additionally added into the medium to maintain the resistance phenotype of A549/DDP and H1299/DDP.

Cell transfection. miR-186-5p mimic, miR-186-5p inhibitor and siRNA against sine oculis homeobox 1 (si-SIX1) were purchased from Ribobio (Guangzhou, China), along with the negative controls. Cell transfection of oligonucleotides into A549/DDP and H1299/DDP cells was performed by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were subsequently cultured for another 24 h prior for further study.

Lentivirus infection. Pre-miR-186-5p was amplified and cloned into pLV-EF1α-MCS-IRES-puro vector (Biosettia, San Diego, CA, USA). 293T cells were co-transfected with pLV-EF1α-MCS-IRES-puro-miR-186-5p/empty vector and ViraPower Lentiviral Pack aging Mix (Thermo Fisher Scientific, Waltham, MA, USA) using Lipofectamine 2000 reagent (Invitrogen). After transfection for 3 days, the cell medium supernatant was collected as lentivirus particles carrying miR-186-5p/NC (named as lenti-miR-186-5p/control). The construction of expression plasmid and the package of lentivirus particle were provided by Ribobio. A549/DDP and H1299/DDP cells were infected with lenti-miR-186-5p/control, followed by screening with 2 μg/ml puromycin lasting for 14 days.

Total RNA isolation and real-time quantitative PCR (qPCR). Total RNA from tissue samples and cultured A549/DDP and H1299/DDP cells was extracted with Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration and purity of total RNA was examined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). A reverse transcription kit (Abcam, Cambridge, UK) was used to synthesize the first strand of cDNAs. Then, amplification of cDNAs was performed by SYBR® Green Master Mix Kit (Qiagen) on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). The relative expression levels of miR-186-5p and SIX1 mRNA were calculated by $2^{-\Delta\Delta CT}$ methods with normalization to U6 small nuclear RNA (U6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. All PCR reactions were performed in triplicate. Primers were listed as following: miR-186-5p: 5'-ACACTCCAGCTGGGCAGCAGCACACT-3' (forward) and 5'-CTCAACTGGTGTCGTGGA-3' (reverse); SIX1: 5'-AAGGAGAAGTCGAGGGGTGT-3' (forward) and 5'-TGCTTGTTGGAGGAGGAGTT-3' (reverse); GAPDH: 5'-GTCAACGGATTTGGTCTGTATT-3' (forward) and 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse); U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCAT-3' (reverse).

Total protein extraction and western blot. Treated A549/DDP and H1299/DDP cells were extracted for total protein with RIPA lysis buffer (Beyotime, Shanghai, China) to measure SIX1 protein expression. Western blot was performed according to standard procedures, and β-actin on the same membrane was used as a loading control. The primary antibodies included SIX1 (sc-514441, 1:1000) and β-actin (sc-47778, 1:1000) and were purchased from Santa Cruz Biotech, CA, USA. The proteins were visualized using ECL procedure and Image J was used to analyze the gray intensity of the bands.

MTT assay. Cell proliferation of A549/DDP and H1299/DDP cells was assessed by 3-(4, 5)-dimethylthiazole-2-y1)-2, 5-biphenyl tetrazolium bromide (MTT) staining. Transfected cells were plated in 96-well plates (Corning, NY, USA) at a concentration of 5,000 cells per well and incubated with DDP. As for cisplatin resistance assay, transfected A549/DDP and H1299/DDP cells were treated with different concentrations of DDP (5–30 μM) for 48 h; as for cell proliferation ability detection, transfected A549/DDP and H1299/DDP

cells were treated with 60 μM of DDP for 0, 24, 48, and 72 h. Briefly, 20 μl of MTT (5 mg/ml; Sigma-Aldrich, Louis, MO, USA) was added to each well and the cells were incubated with MTT for another 4 h. After adding 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich), the spectrophotometric absorbance of each sample was measured at 450 nm. The 50% inhibitory concentration (IC50) was calculated with GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA, USA) using the sigmoidal dose-response function. The experiments were conducted at least 3 times.

Transwell assay. For determination of abilities of migration and invasion, A549/DDP and H1299/DDP cells after transfection were evaluated with Transwell assay. Transfected cells were transferred in 24-well transwell chamber (8 μm pores; Corning) with matrigel-free (for migration) or matrigel-coated (for invasion) (BD Biosciences). Transfected cells (2×10^4 cells/ml) were re-suspended into 200 μl of serum-free medium and plated in the upper chamber, and the lower chamber was filled with 500 μL complete medium containing 10% FBS. After incubation at 37 °C for 24 h, the cells on the lower surface were stained with 0.1% crystal violet for 15 min at room temperature, followed by being photographed and counted under a light microscope.

Dual luciferase reporter assay and RNA immuno-precipitation (RIP). SIX1 3' UTR wild type and mutant type (SIX1 3' UTR-WT/MUT) was cloned into pGL4 vector (Promega, Madison, WI, USA). A549/DDP and H1299/DDP cells were co-transfected with SIX1 3' UTR-WT/MUT and miR-186-5p/NC mimic. All transfection procedures were performed by Lipofectamine 2000 (Invitrogen). 48 h post-transfection, the luciferase activity was measured using dual-luciferase reporter system (Promega). The ratio of Firefly to Renilla luciferase activity was used as the relative luciferase activity. All operations were repeated 3 times.

Cell supernatant of A549/DDP and H1299/DDP after transfection of miR-186-5p/NC mimic was collected for RIP assay. Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore, Bradford, MA, USA) was chosen to detect expression of SIX1 mRNA from the samples bound to the Ago2 or IgG antibody. All operation obeyed the standard instructions.

Flow cytometry. Apoptosis rate was analyzed by Annexin V-FITC/PI kit (Beyotime, Shanghai, China) on flow cytometry. After transfection, apoptotic cells were labelled with FITC-Annexin V and PI for 30 min in the dark and fluorescence was analyzed on CytoFLEX LX flow cytometer (Beckman-Counter Electronics, Jiangsu, China) using CytExpert software. Quadrants were positioned on Annexin V/PI plots to distinguish apoptotic cells (Annexin V+/PI–, Annexin V+/PI+). Apoptosis rate = apoptotic cells / total cells ×100%

Xenograft mouse model. Four-week-old BALB/c nude mice were obtained from Model Animal Research Center of Nanjing University. The animal experiments were approved by The Institutional Review Board of the First Affiliated Hospital of the Army Medical University and were taken in accordance with National Institutions of Health Guide for Care and Use of Laboratory Animals. Equal numbers (1×10^6) of A549/DDP cells stably infected with Lenti-miR-186-5p or Lenti-miR-NC in 0.2 ml of PBS were injected in subcutaneous area of nude mice (4 mice per group). One week later, xenograft experiments were divided into three groups: Lenti-control (Lenti-miR-NC+PBS), Lenti-miR-NC (+DDP), Lenti-miR-186-5p (+DDP). DDP or PBS was intraperitoneally injected at a dose of 5 μg/kg every 7 days for five times. The tumors were measured with a caliper once 7 days, and tumor volume was calculated using the formula: V (mm^3) = 1/2 ab^2 (a is the longest tumor axis and b is the shortest tumor axis). The mice were euthanized on day 35 after xenograft and the weight of tumors was evaluated with electronic balance. Immediately, the xenograft tumors were snap-frozen in liquid nitrogen and stored in –80 °C for further isolation of total RNA and protein.

Statistical analyses. Data given were the means ± standard deviation. Statistical significance was determined by two-tailed Student's t test and one-way analysis of variance using GraphPad Prism 4.0 (GraphPad Software Inc). A p-value <0.05 was considered as significant difference.

Results

Expression of miR-186-5p was downregulated in DDP resistant NSCLC tissues and cells. In order to explore the contribution of miR-186-5p on cisplatin resistance in NSCLC, altered expression of miR-186-5p was investigated. As shown in Figure 1A, miR-186-5p expression was universally downregulated in DDP resistant NSCLC tumor tissues (n=25) comparing with DDP sensitive tissues (n=25). We developed DDP resistant NSCLC cells in A549 and H1299 (named as A549/DDP and H1299/DDP) for further functional experiments. Moreover, the cultivated A549/DDP and H1299/DDP cells were identified by analyzing 50% inhibitory concentration (IC50) of DDP. As a result, IC50 of DDP in cultivated A549/DDP and H1299/DDP cells was more than 60 μM, whereas about 30 μM in the parental cells (Figures 1B and 1C), which suggested a successful construction for DDP resistant NSCLC cells *in vitro*. Expression levels of miR-186-5p were lower in A549/DDP and H1299/DDP cells than in the parental cells (Figure 1D). These findings showed the downregulation of miR-186-5p in DDP resistant NSCLC.

Overexpression of miR-186-5p inhibited proliferation, migration and invasion, and promoted cell apoptosis in DDP resistant NSCLC cells *in vitro*. We confirmed that miR-186-5p was downregulated in DDP resistant NSCLC, then miR-186-5p was highly expressed in A549/DDP and H1299/DDP cells by transfection with miR-186-5p mimic (Figure 2A). Loss-of-function experiments were conducted to figure out the role of miR-186-5p overexpression. IC50 of DDP in A549/DDP and H1299/DDP cells was dramati-
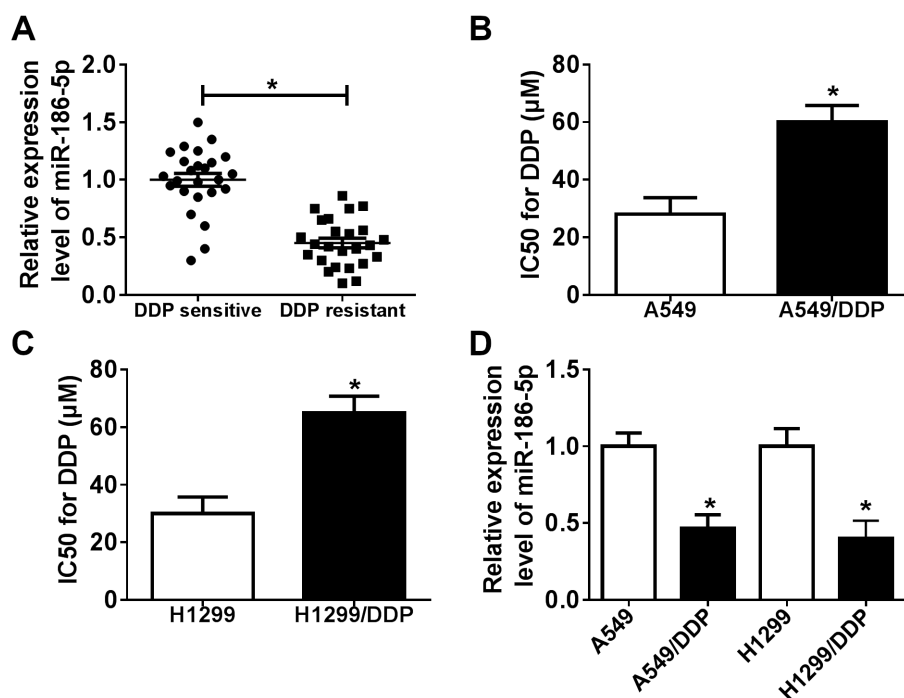
Figure 1. miRNA (miR)-186-5p was downregulated in DDP-resistant NSCLC tissues and cells. A) Levels of miR-186-5p were detected by RT-qPCR in DDP resistant (n=25) and sensitive (n=25) NSCLC tissues. B and C) IC50 of DDP was measured with cell viability assay. D) Levels of miR-186-5p were detected by RT-qPCR in A549/DDP and H1299/DDP cells, normalized to the parental cells. All experiments were carried out in triplicate, and *p<0.05.

cally decreased when miR-186-5p was overexpressed (Figure 2B). According to MTT staining, ectopic expression of miR-186-5p significantly inhibited cell proliferation ability in A549/DDP and H1299/DDP cells under 60 μM of DDP treatment (Figures 2C and 2D). Abilities of cell migration and invasion were impaired in miR-186-5p-overexpressed A549/DDP and H1299/DDP cells, as evidenced by Transwell assays (Figures 2E and 2F). On the contrary, apoptotic cells were greatly induced when miR-186-5p overexpressed (Figure 2G). These results indicated upregulation of miR-186-5p inhibited proliferation, migration and invasion, and promoted cell apoptosis in DDP resistant NSCLC cells *in vitro*.

SIX1 was upregulated and was targeted by miR-186-5p in DDP resistant NSCLC cells. In this study, we observed 4 potential binding sites between hsa-miR-186-5p and SIX1 ENSG00000126778 by computational predictions on Diana tools. As shown in Figure 3A, the potential binding site on SIX1 3' UTR 2322-2337 was further validated. Then, the target sequence on SIX1 wild type (SIX1-WT) was cloned into pGL4 vector, as well as the mutant type (SIX1-MUT). Dual-luciferase reporter assay was used to validate the target binding of miR-186-5p and SIX1. A549/DDP and H1299/DDP cells were co-transfected with SIX1 3' UTR-WT/MUT and miR-186-5p/NC mimic (miR-186-5p/NC). Relative luciferase activity of SIX1 3' UTR-WT was remarkably reduced in the presence of miR-186-5p (Figures 3B and 3C); however,

there was no difference in SIX1 3' UTR-MUT groups. RIP assay further identified this binding in A549/DDP and H1299/DDP cells. After transfection with miR-186-5p, levels of SIX1 mRNA from Ago2 immunoprecipitation were largely enriched (Figure 3D). Expression of SIX1 in DDP resistant NSCLC cells was uncovered either. Levels of SIX1 protein were higher in A549/DDP and H1299/DDP cells than parental cells (Figure 3E). Subsequently, the regulatory effect of miR-186-5p mimic and inhibitor was verified in A549/DDP and H1299/DDP cells using RT-qPCR analyzing levels of miR-186-5p (Figures 2A and 3F). Next, the regulatory effect of miR-186-5p on SIX1 expression was detected, and SIX1 expression on protein level was reduced when transfected with miR-186-5p but increased when transfected with miR-186-5p inhibitor (in-miR-186-5p) (Figure 3G). These data illustrated that miR-186-5p negatively regulated SIX1 through target binding and SIX1 was upregulated in DDP resistant NSCLC cells.

Knockdown of SIX1 suppressed proliferation, migration and invasion, and promoted cell apoptosis in DDP resistant NSCLC cells depending on miR-186-5p upregulation. We wondered whether miR-186-5p exerted suppressive effect on cisplatin resistance in NSCLC through inhibiting its target SIX1 expression. Thus, rescue experiments were performed. A549/DDP and H1299/DDP cells were transfected with si-SIX1/NC or co-transfected with si-SIX1 and in-miR-186-5p/NC. Expression levels of SIX1 were measured
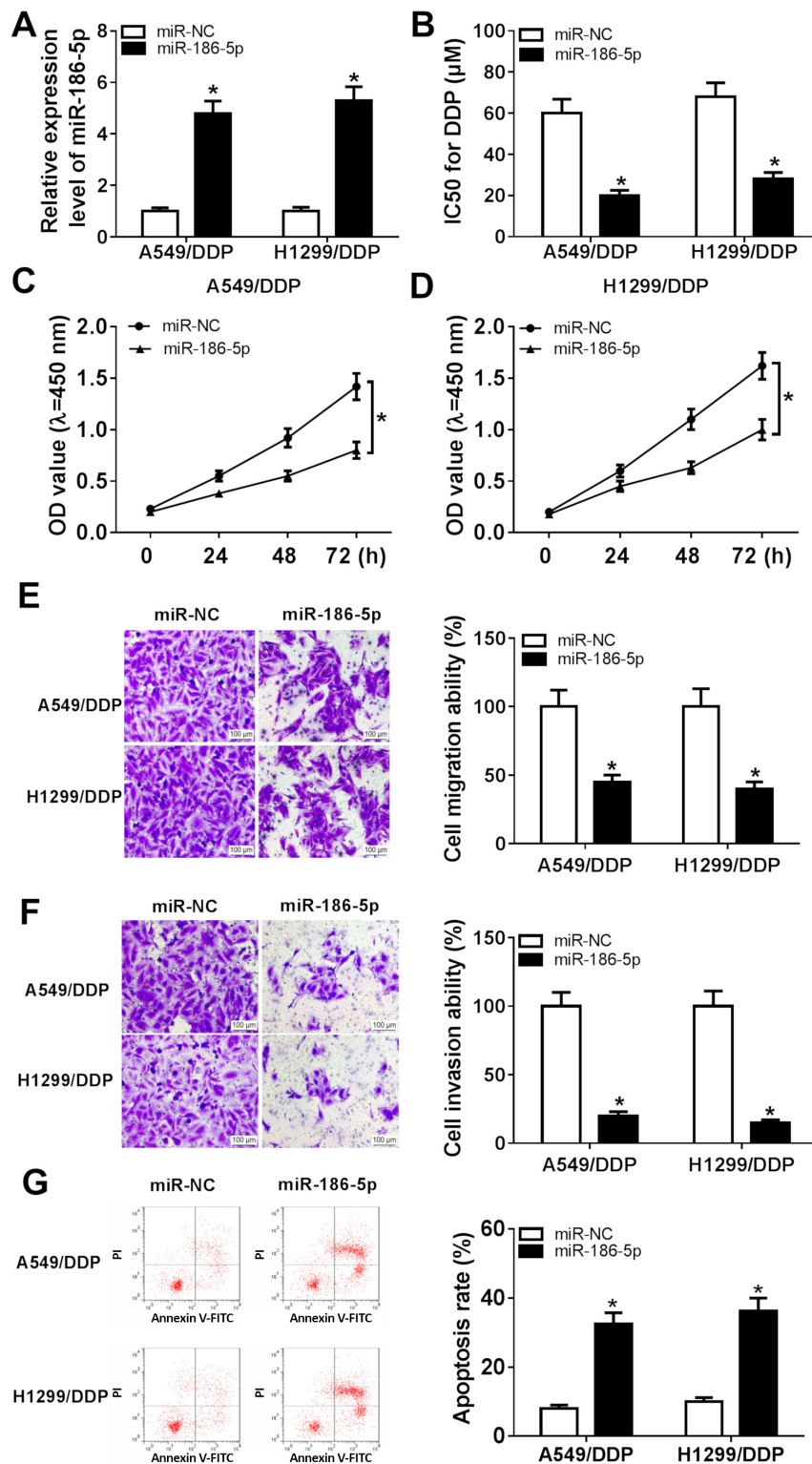
Figure 2. Effect of miR-186-5p overexpression on DDP resistant NSCLC cells *in vitro*. A549/DDP and H1299/DDP cells were transfected with miR-186-5p mimic (miR-186-5p) or miR-NC mimic (miR-NC). A) Levels of miR-186-5p were detected with RT-qPCR after transfection. B) IC50 of DDP was measured with cell viability assay (with MTT) after transfection. C and D) Cell viability was measured in transfected A549/DDP and H1299/DDP cells after 60 µM DDP treatment for 0, 24, 48 and 72 h. E and F) Ability of cell migration and invasion was examined with Transwell assay after transfection. G) Apoptosis was measured with apoptosis rate using flow cytometry. The percentage of cells in quadrants of Annexin V+/PI− and Annexin V+/PI+ was statistically recorded. All experiments were carried out in triplicate, and *p<0.05 versus miR-NC.
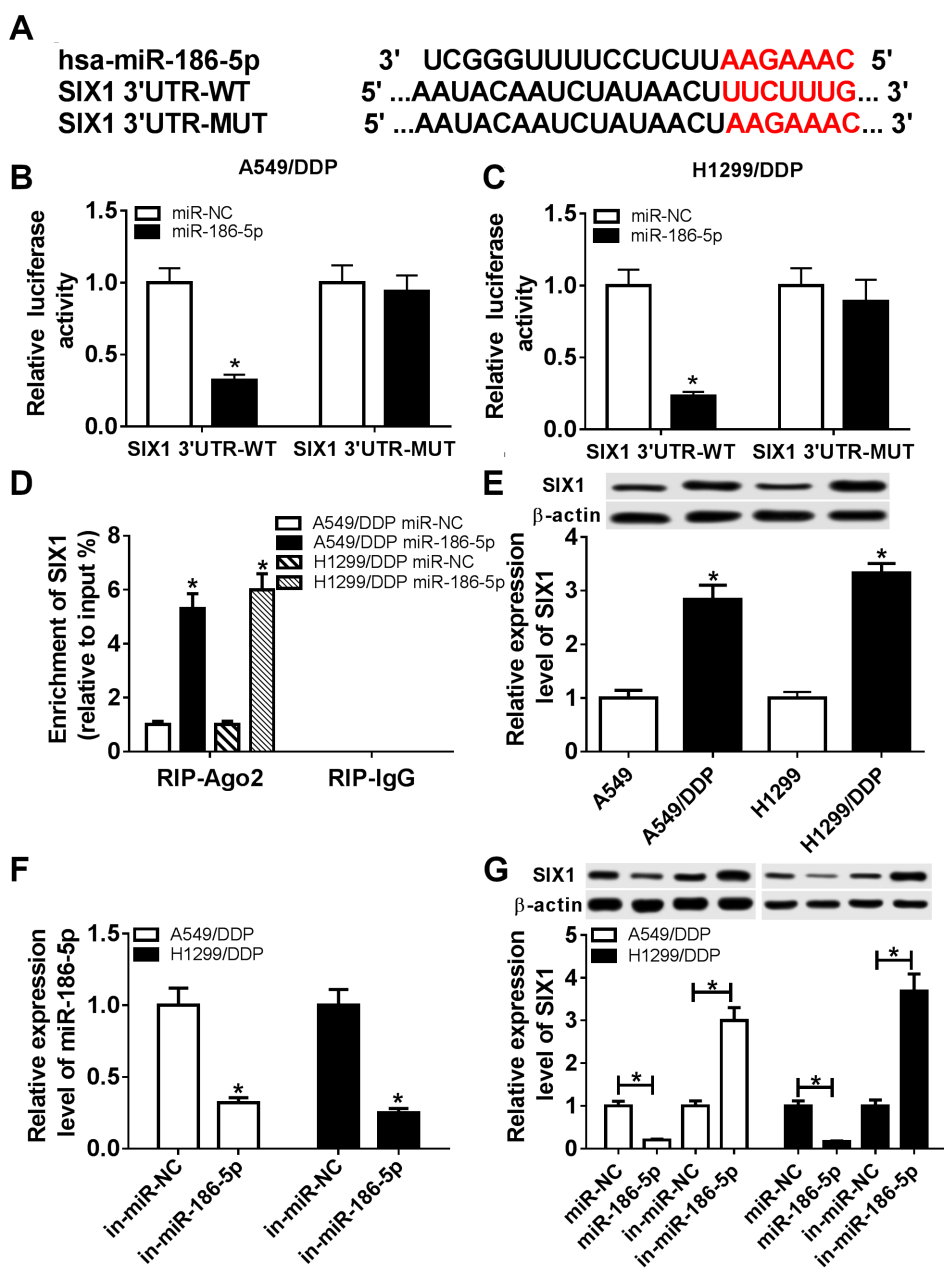
Figure 3. miR-186-5p negatively regulated sine oculis homeobox 1 (SIX1) expression by target binding. A) The potential binding site of miR-186-5p on SIX 3' UTR was shown. (B–D) A549/DDP and H1299/DDP cells were transfected with miR-186-5p or miR-NC. B and C) Relative luciferase of pGL4-SIX 3' UTR wild type and mutant after transfection. D) Expression levels of SIX1 mRNA in RNA immunoprecipitation from Ago2 after transfection. E) Levels of SIX1 protein were measured by western blot in A549/DDP and H1299/DDP cells, compared with the parental cells. F) Levels of SIX1 mRNA were measured by RT-qPCR in A549/DDP and H1299/DDP cells when transfected with miR-miR-186-5p/NC inhibitor (in-miR-miR-186-5p/NC). G) The regulatory effect of miR-186-5p on SIX1 expression was detected using western blot. Levels of SIX1 protein were measured in A549/DDP and H1299/DDP cells when transfected with miR-miR-186-5p/NC or in-miR-miR-186-5p/NC. All experiments were carried out in triplicate, and *p<0.05 versus miR-NC or in-miR-NC.

after transfection; SIX1 levels were declined by transfection of si-SIX1 alone and improved when transfected with si-SIX1 together with in-miR-186-5p (Figure 4A). IC50 of DDP in A549/DDP and H1299/DDP cells was decreased with SIX1 knockdown, and then rescued when co-expression of SIX1

and miR-186-5p were concurrently inhibited (Figure 4B). SIX1 downregulation alone could inhibit cell proliferation ability in A549/DDP and H1299/DDP cells under 60 μM of DDP treatment, which was abolished by low expression of miR-186-5p (Figures 4C and 4D). Abilities of cell migration
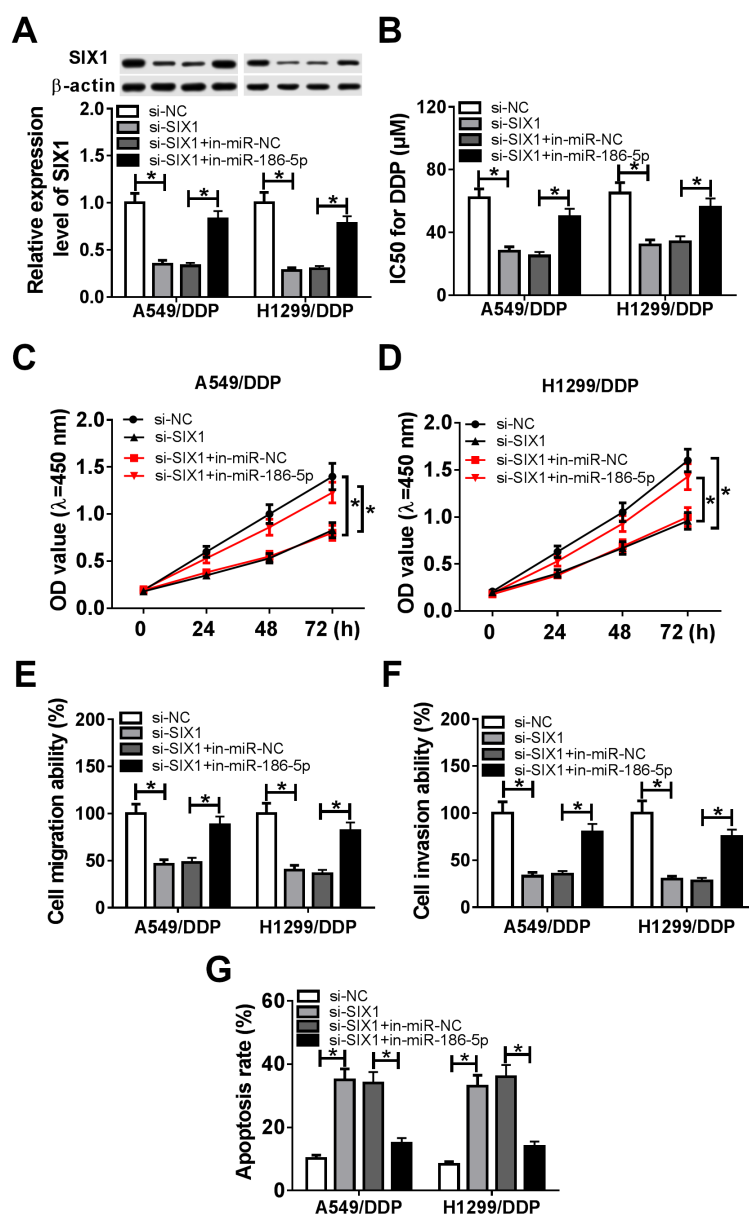
Figure 4. Knockdown of SIX1 suppressed DDP resistant NSCLC cell proliferation, migration and invasion depending on miR-186-5p upregulation. A549/DDP and H1299/DDP cells were transfected with siRNA SIX1/NC (si-SIX1/NC) or co-transfected with si-SIX1 and in-miR-186-5p/NC. A) Expression levels of SIX1 were measured by western blot after transfection. B) IC50 of DDP was measured with cell viability assay (with MTT) after transfection. C and D) Cell viability was measured after transfection followed with DDP treatment for 0, 24, 48 and 72 h. E and F) Ability of cell migration and invasion was examined with Transwell assay after transfection. G) Apoptosis rate was measured by flow cytometry. All experiments were carried out in triplicate, and *p<0.05 versus control cells transfected with si-NC or si-SIX1 and in-miR-NC.

and invasion were higher when transfected with si-SIX1, and this effect was reversed when co-transfected with si-SIX1 and in-miR-186-5p (Figures 4E and 4F). Inversely, apoptosis rate was greatly elevated when SIX1 was downregulated, which was partially reversed by simultaneously reduced miR-186-5p expression (Figure 4G). These results indicated that the effects of SIX1 knockdown in DDP resistant NSCLC cells *in vitro* were abated by miR-186-5p downregulation.

Overexpression of miR-186-5p suppressed xenograft NSCLC tumor growth with DDP treatment. In present study, we concluded that miR-186-5p sensitized the DDP resistant NSCLC cells to DDP *in vitro*. Then, we wondered whether miR-186-5p overexpression could suppress cisplatin resistance *in vivo*. A549/DDP cells stably expressing miR-186-5p by lentivirus infection were subsequently xenogeneic transplanted into nude mice. As shown in Figure 5, xenograft

experiments were divided into three groups: Lenti-control (Lenti-miR-NC+PBS), Lenti-miR-NC (+DDP), Lenti-miR-186-5p (+DDP). A549/DDP cells-induced xenograft tumor growth was significantly inhibited by enforced expression of miR-186-5p comparing to Lenti-miR-NC group (Figures 5A and 5B); while, there was no difference between miR-NC tumors with and without DDP treatment. Furthermore, we dug out the expression of miR-186-5p and SIX1 in xenograft tumors. As expected, miR-186-5p was highly upregulated and SIX1 was extremely downregulated in Lenti-miR-186-5p group (Figures 5C and 5D). These data showed NSCLC xenograft tumors were retarded by miR-186-5p under DDP treatment, implying the suppressive role of miR-186-5p on cisplatin resistance in NSCLC *in vivo*.

Discussion

Drug resistance still remains a major obstacle to the efficacy of chemotherapy, even though great advances have been achieved in adjuvant therapies [18]. Cancer chemotherapy is limited by the development of drug resistance and the adverse effects of anti-tumor drugs [19]. Besides, 70–80% of patients are diagnosed at advanced stages due to

the limitation of early diagnostic techniques and the lack of early specific clinical manifestations [20]. Numerous studies have demonstrated that miRNAs play important roles in mediating DDP sensitivity of NSCLC cells [21]. On one hand, dysregulation of some miRNAs contributes to the acquisition of chemoresistance in NSCLC. For example, the inhibition of miR-155 could enhance the sensitivity of A549 cells to DDP treatment by modulation of cellular apoptosis and DNA damage through an Apaf-1-mediated pathway [22]. Wang et al. [23] indicated that miR-224 targeting p21 promoted the chemoresistance of human lung adenocarcinoma cells to DDP via regulating G1/S transition and apoptosis. On the other hand, altered expression of many miRNAs suppresses chemoresistance in NSCLC. For instance, garcinol sensitized NSCLC cells to erlotinib and cisplatin by upregulating epithelial-to-mesenchymal transition (EMT)-modulating miRNAs such as miR-200c and let-7c [24]. Sun et al. [25] highlighted increased miR-202 expression could expand apoptosis signaling induced by DDP in NCI-H441 and A549 NSCLC cells through targeting KRas and inhibiting Ras/MAPK pathway. In our study, miR-186-5p was identified as tumor suppressor in NSCLC and miR-186-5p upregulation inhibited cell proliferation,
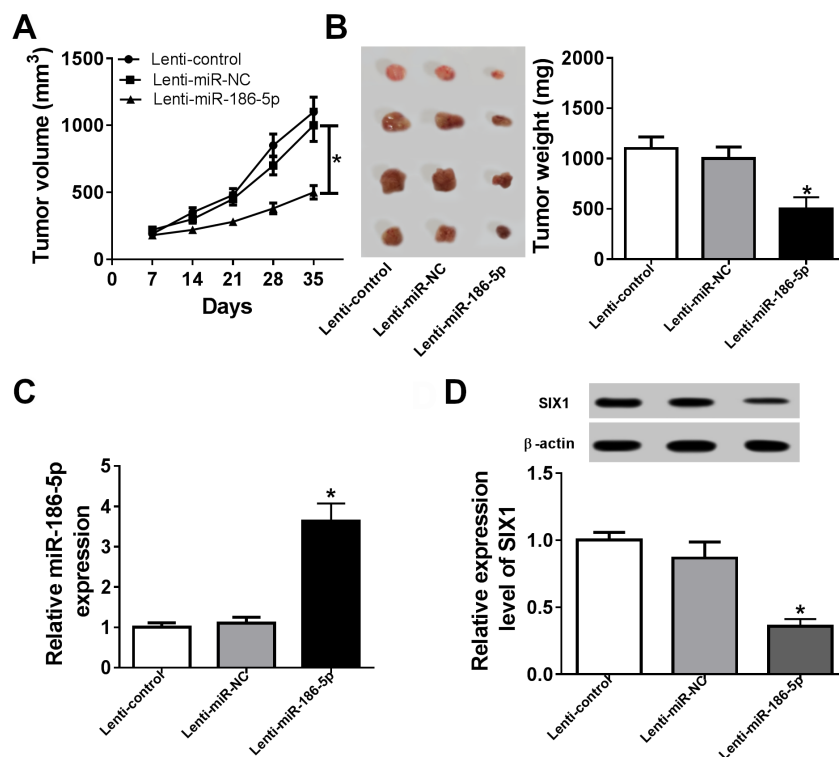

Figure 5. Effect of miR-186-5p overexpression on NSCLC tumor growth under DDP treatment. A549/DDP and H1299/DDP cells were infected with lentivirus particles carrying miR-186-5p/NC (Lenti-miR-186-5p/NC), followed by xenograft. After 7 days, xenograft experiments were divided into three groups: Lenti-control (Lenti-miR-NC+PBS), Lenti-miR-NC (+DDP), Lenti-miR-186-5p (+DDP). A) Tumor size was measured per 7 days and the growth curve was drawn. B) The representative images of tumors were presented and tumor weight was recorded on day 35. C and D) Expression levels of miR-186-5p and SIX1 were detected by RT-qPCR and western blot, respectively. All experiments were carried out in triplicate, and *p<0.05 versus Lenti-miR-NC.

migration and invasion, and promoted cell apoptosis rate in DDP resistant A549 and H1299 cells by downregulating its target SIX1 expression. Taken these data together, the molecular mechanism of cisplatin resistance may include regulation of miRNAs to decrease the cell proliferation and metastasis and to increase apoptosis.

miR-186-5p plays universal role in NSCLC tumorigenesis. Cai et al. [10] firstly claimed a close correlation between miR-186-5p expression and NSCLC patient survival. In that study, enforced overexpression of miR-186-5p in A549 and SK-MES-1 NSCLC cells inhibited the proliferation by inducing G1/S checkpoint arrest, and silencing its expression played the opposite effect. Then, overexpression of miR-186-5p was reported to inhibit invasion activity of NSCLC cells toward the SCID mice lung in research from Li et al. [26]. Moreover, a markedly decelerated proliferation of the tumor cells was caused by miR-186-5p upregulation [9, 27, 28]. The mechanisms underlying the anti-tumor role of miR-186-5p were recently uncovered in A549 and HCC827 cells through targeting Rho-associated protein kinase 1 [28], mitogen-activated protein kinase kinase kinase 2 [27] and Yin Yang 1 [9]. In this study, we focused on the role of this miRNA on DDP-resistant NSCLC cells. As a result, ectopic expression of miR-186-5p resulted in the inhibition of IC50 values of DDP, cell proliferation, migration and invasion, and enhancement of apoptosis rate in A549/DDP and H1299/DDP cells, as well as xenograft tumor growth. Notably, consistent downregulation of miR-186-5p was recognized in NSCLC tissues and cells, we further declared the lower expression level of miR-186 in DDP resistant NSCLC tissues and cells.

miR-186-5p was involved in the occurrence of chemotherapeutic drug-resistance in NSCLC. It was observed that downregulated miR-186-5p occurred in NSCLC patients who were paclitaxel resistant, and this downregulation was associated with poor survival [11]. In NSCLC, there was no published data indicating the involvement between cisplatin resistance and miR-186-5p expression, even though miR-186-3p was early uncovered to be downregulated in the anti-cancer effects of curcumin in A549/DDP cells [19]. Here, we firstly developed the DDP resistant NSCLC cells in A549 and H1299 cells, namely A549/DDP and H1299/DDP. Expression of miR-186-5p was downregulated in DDP resistant NSCLC patients and A549/DDP and H1299/DDP cells. Overexpression of miR-186-5p distinctively attenuated DDP resistant phenotypes in A549/DDP and H1299/DDP cells, including IC50 value of DDP, proliferation, apoptosis, migration and invasion. A549/DDP cells-induced xenograft tumor growth was significantly inhibited by enforced expression of miR-186-5p; while, there was no difference between miR-NC tumors with and without DDP treatment. SIX1 knockdown may underlay the suppressive role of miR-186-5p on cisplatin resistance in NSCLC *in vitro* and *in vivo*.

SIX1 belongs to the sine oculis homeobox family, which is a homologue of the *Drosophila sine oculis* in vertebrates [29].

SIX family genes play an important role in organogenesis and disease [30]. Among them, SIX1 is the most heavily implicated SIX protein in cancers [31]. Over past decades, SIX1 has been related to "hallmarks of cancer", such as maintained cell proliferation, activated migration and invasion, resisted cell death, and etc. In lung cancer, upregulated SIX1 and Notch2 were investigated to be associated with invasive lung ADC, a mainly subtype of NSCLC, by inducing EMT and nuclear atypia, thus promoting advanced malignant phenotypes [32]. Roles of miRNAs in SIX1-mediated functions had gained much attention in several cancers including NSCLC [33]. Moreover, SIX1 was closely correlated with drug resistance and poor prognosis in some human cancers, such as breast cancer [34] and ovarian cancer [35, 36]. However, to the best of our knowledge, the contribution of SIX1 to cisplatin resistance in many other cancers remains unclear, including lung cancer. In this present study, we figured out the influence of SIX1 in miR-186-5p-induced DDP sensitivity in NSCLC. As a result, SIX1 downregulation by siRNA could reduce IC50 values of DDP in A549/DDP and H1299/DDP cells through inhibiting cell proliferation, migration and invasion and facilitating apoptosis. Furthermore, this effect was reversed by knockdown of miR-186-5p. Therefore, we concluded that miR-186-5p and SIX1 could be a new therapeutic target for DDP resistant NSCLC, even though the role and mechanism of SIX1 underlying cisplatin resistance has not been well explained.

Downregulation of miR-186-5p was well documented in NSCLC tumor tissues and cell lines, as well as its anti-tumor role in NSCLC cells both *in vitro* and *in vivo*. However, very recently, Feng et al. [37] published a conflicting announcement that miR-186-5p was upregulated in lung ADC tumor tissues and cell lines (H1299 and NCI-H1975) as compared with tumor-adjacent tissues and normal human lung epithelial cells, respectively. Notably, their finding was in consistent with data from Starbase v3.0 and they further observed that this upregulation accelerated lung ADC cell proliferation, migration and invasion through targeting PTEN. Unfortunately, they did not discuss the causes of this controversial outcome comparing to previous studies, as well as the results in this study. It remains a big question that should be concerned and settled in the further.

In conclusion, we showed that miR-186-5p was downregulated in DDP resistant NSCLC tissues and cells. Ectopic expression of miR-186a-5p sensitized DDP resistant NSCLC cells to DDP *in vitro* and *in vivo* through targeting SIX1. Our findings supported miR-186/SIX1 axis might be a promising biomarker and target for the treatment and prognosis of DDP resistant NSCLC.

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