Original Article

Suppression of ZNF205-AS1/EGR4 positive feedback loop attenuates cisplatin resistance of non-small cell lung cancer cells via targeting miR-138-5p/OCT4 pathway

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Background: Long non-coding RNAs (lncRNAs) are frequently reported to involve in the onset and development of non-small cell lung cancer (NSCLC). Cisplatin (DDP) resistance continues to pose a daunting challenge for improving the prognosis of NSCLC patients. The current study intends to elucidate the molecular mechanisms underlying the function of lncRNA ZNF205 AS1/early growth response 4 (EGR4) positive feedback loop in DDP resistance of NSCLC.

Methods: A series of assays, including real-time polymerase chain reaction (PCR), western blotting, flow cytometry, and dual-luciferase reporter, were performed to evaluate the effect of ZNF205-AS1/EGR4 loop in the established DDP-resistant A549 cell line and its progenitor A549 cell line. Immunohistochemistry (IHC) technique was conducted to investigate the expression pattern of EGR4 and octamer-binding protein 4 (OCT4) in NSCLC tissues. RNA pull-down assay was carried out to evaluate the interaction between miR-138-5p and EGR4 and OCT4. Transwell assay and wound healing assay was used to evaluate the invasive and migratory potential of cells subject to various treatment. The protein levels of Bcl2, Bax, Cl-caspase 3, Cl-PARP and OCT4 were measured in western blotting assay.

Results: The levels of ZNF205-AS1, EGR4 and OCT4 were notably upregulated in post-chemotherapy DDP-resistant lung specimens, as opposed to those pre-chemotherapy, and in A549/DDP cells than the progenitor DDP-sensitive A549 cells. In contrast, the level of miR-138-5p was significantly reduced in A549/DDP cells ($P<0.05$). Luciferase reporter assay confirmed the interaction between ZNF205-AS1 and miRNA-138-5p. Protein-RNA interaction was validated between miR-138-5p, EGR4 and OCT4. The higher chemosensitivity of DDP-resistant cells induced by the loss-of-function of ZNF205-AS1 could be diminished by a miR-138-5p inhibitor.

Conclusions: Our data demonstrated that miR-138-5p/OCT4 functions as a downstream effector of the ZNF205-AS1/EGR4 positive feedback loop and mediates resistance of NSCLC cells to DDP. Our work sheds light on the therapeutic strategies for NSCLC with DDP chemoresistance.

Keywords: Cisplatin resistance (DDP resistance); non-small cell lung cancer (NSCLC); ZNF205-AS1; miR-138-5p; early growth response 4 (EGR4)

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Introduction

Cancer is the leading cause of death for people younger than 70 years in 91 out of 172 surveyed countries (1). Lung cancer, as the most frequently diagnosed type of cancerous malignancy, ranks the first place for cancer-caused mortality on a worldwide scale. With more than 1.2 million cases of death, lung cancer has inflicted an overwhelmingly heavy social and economic burden. Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two histological groups of lung cancer, with the latter making up for over 80% of total cases (2). The deliberately lowered rate of smoking, technological development in clinical diagnosis, and the continuous emergence of new therapeutic options have jointly contributed to improved outcomes, the 5-year survival rate of patients suffering from NSCLC is still lower than 30%, which is considerably lower than that of other malignancies, such as thyroid cancer and prostate cancer (2,3). The efficacy of cisplatin (DDP), a first-line medication for the treatment of NSCLC, has been well acknowledged, yet chemoresistance to DDP has been frequently observed in NSCLC patients receiving chemotherapy and is a key indicator of dismal prognosis (4,5). Hence, it is essential to unravel the molecular mechanism of DDP-resistance in NSCLC and to uncover more therapeutic strategies for the management of NSCLC.

A growing line of evidence have indicated that long non-coding RNAs (lncRNAs) constitute a family of vital regulators in numerous cellular events in cancers, including proliferation, invasion, and apoptosis, as indicated by their aberrant expression level (6,7). Mechanistically, lncRNAs interact with a vast variety of proteins and mRNAs to mediate the downstream pathways, aside from sponging miRNAs (8,9). Lu *et al.* suggested that small nucleolar RNA host gene 1 (SNHG1) promotes the progression of NSCLC by repressing miR-145-5p expression, thereby upregulating the level of metadherin (MTDH) (10). Shi *et al.* revealed that lncRNA PCAT6, an oncogene, binds to EZH2 to repress LATS2 expression in non-small-cell lung cancer (11). Our previous work revealed that the level of ZNF205-AS1 was markedly up-regulated in NSCLC cell lines and tissue specimens, contributing to a dismal prognosis of patients with NSCLC. Moreover, our result confirmed the existence of the positive feedback loop between ZNF205-AS1 and early growth response 4 (EGR4), which functions to boost the proliferation of NSCLC cells and could be applied as a promising therapeutic method for NSCLC (12).

MicroRNA-138-5p, as a non-coding small RNA molecule, has been identified in a recent study as a cancer suppressor repressing the expression of proto-oncogenes post-transcriptionally (13). Song *et al.* revealed that miRNA-138-5p functions to thwart tumor growth and activate the immune system via suppressing checkpoint programmed cell death 1/programmed death-ligand 1 (PD-1/PD-L1) axis (14). Nevertheless, the involvement of miR-138-5p in mediating DDP resistance of NSCLC cells has not been elucidated. Located on chromosome 6 (6p21.31), octamer-binding protein 4 (OCT4) has multiple transcription start sites (TSS) and has a length of 16.40 kb (15). OCT4, as a member of Pit-Oct-Unc (POU) family of transcription factors, is mainly expressed in embryonic and germinal stem cells and is profoundly implicated in the maintenance of embryonic stem cells with multidirectional potential and self-renewal (16). Liu *et al.* reported the high expression of OCT4 expression is intimately associated with the onset and progression of NSCLC, and that the knockdown of OCT4 thwarts cellular proliferation, induces excessive apoptosis of DDP-resistant cells, suggesting that OCT4 maintains the stemness of cancer cells and contributes to chemoresistance (17).

In our pilot study, we applied bioinformatics prediction

Highlight box

Key findings

- The current study, for the first time, revealed the existence of ZNF205-AS1/early growth response 4 (EGR4) positive feedback loop and that miR-138-5p/octamer-binding protein 4 (OCT4) serves as a downstream effector of such a positive feedback loop to mediates chemo-resistance of non-small cell lung cancer (NSCLC) cells to cisplatin. The current study provides new insights into the therapeutic strategies for NSCLC with cisplatin chemoresistance.

What is known and what is new?

- Our previous work confirmed the existence of the positive feedback loop between ZNF205-AS1 and EGR4 and its implication in NSCLC. OCT4 maintains the stemness of cancer cells and contributes to chemoresistance.
- The current paper demonstrated that miR-138-5p/OCT4 functions as a downstream effector of the ZNF205-AS1/EGR4 positive feedback loop and mediates resistance of NSCLC cells to cisplatin, thus revealing potential therapeutic strategies for NSCLC with cisplatin chemoresistance.

What is the implication, and what should change now?

- ZNF205-AS1/EGR4 positive feedback loop could be applied in pre-clinical study to mediate chemo-resistance of NSCLC cells to cisplatin. It provides a new perspective for the management of NSCLC.

programs, in combination with experimental validation, to reveal that EGR4 could directly bind to the promoter of lncRNA *ZNF205-AS1*, thereby increasing its promoter activity and activating its transcription. Of note, the ZNF205-AS1 transcript could directly interact with EGR4 mRNA to maintain the stability of EGR4 mRNA and to elevate the expression of EGR4 through RNA-RNA interaction, hence the formation of ZNF205-AS1/EGR4 positive feedback loop (12). Specifically, the elevated level of EGR4 in NSCLC was significantly and positively correlated with the expression level of ZNF205-AS1. Moreover, the results of loss-of-function and gain-of-function assays consistently revealed that both EGR4 and ZNF205-AS1 boosted growth and invasion of NSCLC cells *in vitro* and *in vivo*. Herein, we hypothesize that the lncRNA ZNF205-AS1/EGR4 positive feedback loop inhibits the expression level of miRNA-138-5p and thereby diminishing the suppressive effect of miRNA-138-5p on OCT4. To verify such a hypothesis, we combine cellular and molecular biology techniques to study the role of lncRNA ZNF205-AS1/EGR4 feedback loop and miR-138-5p/OCT4 pathway in regulating DDP resistance of NSCLC. We constructed DDP-resistant NSCLC cells (A549/DDP cells), and applied siRNA technique to mediate the level of lncRNA ZNF205-AS1 and miRNA-138-5p of cancer cells to observe their effect on phenotypic characteristics. Furthermore, we conducted RNA pulldown assay to examine the possible interaction between miR-138-5p and EGR4, and between miR-138-5p and OCT4. Luciferase reporter assay was conducted to examine the binding between miR-138-5p and lncRNA ZNF205-AS1.

The current study intended to provide novel combined intervention targets for the treatment of NSCLC using DDP by shedding light on new mechanisms of DDP resistance. To this end, we detected the expression patterns of ZNF205-AS1, EGR4, miRNA-138-5p and OCT4 in DDP-resistant A549 cells and non-resistant A549 cells to explore the correlation effects of the ZNF205-AS1/EGR4 positive feedback loop and miRNA-138-5p in chemo-resistant NSCLC cancer cells. We present this article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1171/rc).

Methods

Cell lines and tissue specimen

Human lung adenocarcinoma A549 cell line and human lung epithelial BEAS-2B cell line were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), and were cultured in DMEM (Sigma Aldrich, St. Louis, MO, USA), which was supplemented with 10% fetal bovine serum (FBS), and were maintained at the temperature of 37 ℃ in the 5% humidified CO_2.

Forty pairs of specimens, including NSCLC tissues and the respective adjacent noncancerous lung tissues, were collected from NSCLC patients receiving surgical resection at the Taizhou Hospital affiliated to Wenzhou Medical University (Linhai, China). The patients were notified about the aim of the project, and written consent was obtained from all the subjects. All the specimens, after being diagnosed through pathological examination, were preserved at the temperature of –80 ℃ for the following assay. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The design and protocol of the current study has been approved by the Ethics Committee of Taizhou Hospital (No. K20210705).

Establishment of DDP-resistant A549 cells

DPP-resistant cell line of A549 was constructed by subjecting the proliferating A549 cells to exposure to a gradually increasing concentration of DDP, which was finalized to 8 μM. A549 cells were cultured in the media supplemented with DDP for 10 weeks. The chemo-resistant cells belonging to the logarithmic phase were collected for the following analyses.

RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

Chemo-resistant A549/DDP cells were cultured in Carlsbad the 6-well plates and were then transfected with si-ZNF205-AS, si-scrambled, miRNA-138-5p mimic, miR-138-5p inhibitor, negative control (NC) mimic, respectively, by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The sequences of interference vectors are listed in Table S1. These DDP-resistant cells were applied for the subsequent analyses 48 hours after the transfection.

We extracted total RNAs from the said cell lines by using Trizol reagent (Invitrogen), which were reversely transcribed with 50 units of MultiScribe Reverse Transcriptase (cat#4311235; Thermo Fisher, Carlsbad, MA, USA) in a volume of 20 μL by using PrimeScriptTM RT-PCR Kit (Takara, Dalian, China). The experiment was

carried out according to the following conditions: 5-min denaturing at 95 ℃, subsequently 45 cycles of amplification were performed for 50 s at 90 ℃, and then 60 ℃ for 20 s and 75 ℃ for 40 s. The tests of every sample were repeated in triplicate on an ABI PRISM 7700 Sequence System (PerkinElmer, Waltham, MA, USA). The level of mRNA was measured using $2^{-\Delta\Delta Cq}$ method. GAPDH and U6 were used as endogenous control. The sequence primers used in the current study were as follows: ZNF205-AS1: forward, 5'-CCTCAGAATGGGACCTTATTG-3'; reverse, 5'-GCATGGGAAGAAGAGCGAAA-3'. GAPDH: forward 5'-CCATGACAACTTTGGTATCGTGGAA-3'; reverse, 5'-GGCCATCACGCCACAGTTTC-3'. hsa-mir-138-5p: forward, 5'-AGCTGGTGTTGTGAATCAGGCCG-3'; U6: forward, 5'-CGCTTCGGCAGCACATATACTAA-3'. Universal reverse primer (URP): 5'-AGTGCAGGGTCCGAGGTATT-3'.

Cell Counting Kit-8 (CCK8) assay

CCK-8 assay was conducted to explore the biological effect of ZNF205-AS1 knockdown and miRNA-138-5p overexpression on the viability of cells by using Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Briefly speaking, the cells were transferred into the 96-well plate at a density of 5×10^3 cells per well, followed by incubation with CCK8 solution for 2 h with 5% humidified CO_2 at 37 ℃. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to calculate the optical density of the cell mixture.

Western blotting assay

OCT4 (#ab19857), Cl-caspase 3 (#ab32042), Cleaved-PARP (#ab32064), Bcl2 (#ab182858) and Bax (#ab32503) primary antibodies were obtained from Abcam (Cambridge, MA, USA). The transfected cells were washed twice with New phosphate-buffered saline containing 0.1% Tween (PBST) before extracting the total protein at 4 ℃ by using RIPA lysis buffer with protease inhibitors. We then centrifuged the lysates for 25 min at 15,000 rpm to remove the cell debris. The concentration of protein was measured using protein quantification kit (Thermo Fisher, Carlsbad, CA, USA). Fifty μg extracts of protein were added into each lane and then were fractionated by using 12% SDS-PAGE gel, and were then transferred onto the 0.22 μm PVDF membrane (Merck Millipore, Billerica, MA, USA). Subsequently, the PVDF membrane was blocked for 2 hours at radiotherapy (RT) using 5% non-fat milk as the blocking reagent, and was then incubated with the primary antibodies for 2 hours at RT. GAPDH was used as housekeeper control. Two hours later, the membrane was washed twice with Tris buffered saline with Tween 20 (TBST), and was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours. Finally, the signal of the target proteins was detected with enhanced chemiluminescence (ECL, Thermo Fisher).

Immunohistochemistry (IHC) assay

Formalin-fixed paraffin embedded (FFPE) tissue specimens were sectioned sequentially at the thickness of 4 μm. After de-paraffinization and rehydration, the FFPE sections were treated with 0.3% H_2O_2, prior to being incubated along with 10% goat serum. The retrieval of antigen was performed using EDTA (pH =8.0) for 25 minutes at 100 ℃. The sections were washed, and then incubated with OCT-4 primary antibody and EGR-4 primary antibody over night at the temperature of 4 ℃. Subsequently, the FFPE sections were incubated at 37 ℃ for 40 minutes, and were then washed and incubated with secondary antibody at RT. One hour later, the FFPE sections were washed and incubated with diaminobenzidine tetrachloride (DAB) for 20 minutes, followed by haematoxylin and eosin (HE) counterstaining. The pathological diagnosis of these lesions was performed by two gynecologic pathologists who were blind to the clinic-pathologic characteristics of the subjects.

Cell invasion assay

The cells, subject to various treatment and grouping, were seeded onto the culture plate (density =6×10^5 cells per well), were trypsinized and washed twice with phosphate buffered saline (PBS) and were re-suspended in RPMI-1640 culture medium. The Transwell unit was coated by using Matrigel (cat#354234; Corning, New York, NY, USA) which was diluted in cold serum-free media to the concentration at 1 mg/mL. The lower chambers were filled with serum-free medium, whereas the upper chambers were loaded with 100 μL of 3×10^5/mL cell suspension from each group in triplicate. The cells that could infiltrate through the Matrigel-coated filter membrane were fixed and stained with 0.1% Giemsa stain and were counted for invasion. We calculated the number of the invading cells in the chose field of each group.

Early apoptosis assay

Following the protocols, we used Annexin V/FITC and PI detection kit (Miltenyi, Bergisch Gladbach, Germany) for the detection of early apoptosis on a flow cytometer (BD, San Jose, CA, USA). The cells were collected and were then gently resuspended in the binding buffer, before being incubated with Annexin V-FITC and PI for 20 minutes in the dark, following the instruction of the manufacturer.

In vitro wound healing assay

The collective Migration capacity of the cells was assessed using by performing the cell scratch assay. A total of 6×10^5 cells belonging to the logarithmic phase were collected and seeded into the 96-well culture plates. After reaching a 90% confluence, linear wounds were made in each dish by using a Micro Scratch Tester (CSM Instruments, Switzerland). After an additional 24 h-culture, the representative images of the scratched areas from each dish of each group at 0 and 24 hours post-wound were photographed and quantified on a microscope for the analysis.

Luciferase assay

The wild type-fragments of ZNF205-AS1 that might contain putative binding sites for mutant-type fragments and miRNA-138-5p were cloned into pmirGLO vector between NheI and SalI restriction enzyme sites. The miRNA-138-5p mimic and the constructed plasmids were co-transfected into chemo-resistant A549/DDP cells for 36 hours by using Lipo 3000. We measured the Firefly and Renilla luciferase activity of each group by applying the dual luciferase assay system (Promega, Madison, WA, USA). The values were normalized to that of Renilla luciferase activity.

RNA pulldown assay

ZNF205-AS1 was transcribed *in vitro* from pSPT19-ZNF205-AS1 and was then labelled by biotin using T7 RNA polymerase and biotin RNA Labelling Mix. The *in vitro* transcribed RNAs were treated with DNase I (Takara, China) and were purified by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). After that, the purified biotin-labelled RNAs (5 μg) were incubated along with SPC-A1 whole-cell lysates (1 mg) for 1 hour. We then used streptavidin agarose beads (Invitrogen, Life, Carlsbad, CA, USA) to isolate the complexes. Real-time qPCR was performed to detect the enriched RNAs from the pulldown material.

Statistical analysis

Quantitative data obtained from triplicated independent experiments were presented as mean ± standard deviation (SD) in the current study. SPSS 21.0 (IBM, Chicago, IL, USA) was applied for statistical analysis. Inter-group difference was analyzed using one-way analysis of variance (ANOVA), and Newman-Keuls test was used as *post hoc* analysis. The level of statistical significance was set at $P<0.05$.

Results

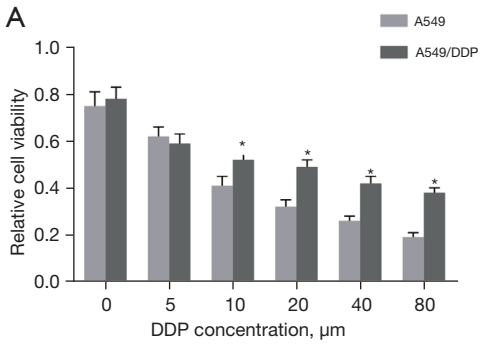
Expression level of lncRNA ZNF205-AS1 and miRNA-138-5p level in DDP-resistant NSCLC cells and non-resistant cells

We constructed DDP-resistant A549 cell line (A549/DDP) via exposing the cells to a gradient increasing concentration of DDP. To investigate the effect of ZNF205-AS1/EGR4 positive feedback loop on DDP-resistance in NSCLC, the established chemo-resistant cells were cultured in the medium with DDP at a concentration of 8 μM for 12 weeks. The half-maximal inhibitory concentration (IC_{50}) values of non-resistant A549 cells and DDP-resistant A549 cells were evaluated in performing CCK8 assay. As illustrated in *Figure 1A*, the IC_{50} value of A549 cell line being significantly lower than that of A549/DDP cell line (10.28±0.92 *vs.* 38.13±5.72 μM; $P<0.001$) indicated that the construction of DDP-resistant cell line was effective.

As seen in *Figure 1B*,*1C*, the results of real-time PCR found that the levels of ZNF205-AS1, EGR4, and OCT4 were significantly elevated in DDP-resistant cells than those in A549 cells ($P<0.05$), and were lowest in BEAS-2B cells. In contrast, the levels of miR-138-5p were significantly down-regulated in A549 cells ($P<0.05$).

These results tentatively indicated the high probability of ZNF205-AS1/EGR4 feedback loop and miR-138-5p implicating in mediating chemoresistance of NSCLC cells. Furthermore, the inverse correlation between ZNF205-AS1 and miRNA-138-5p, which predicts potential binding in DDP-resistant A549 cells, will be clarified in the subsequent assay.

Subsequently, we performed real-time PCR assay and IHC assay to examine expression pattern of EGR4



Cells	IC_{50}, μm	P value
A549	10.28±0.92	
A549/DDP	38.13±5.72	<0.001

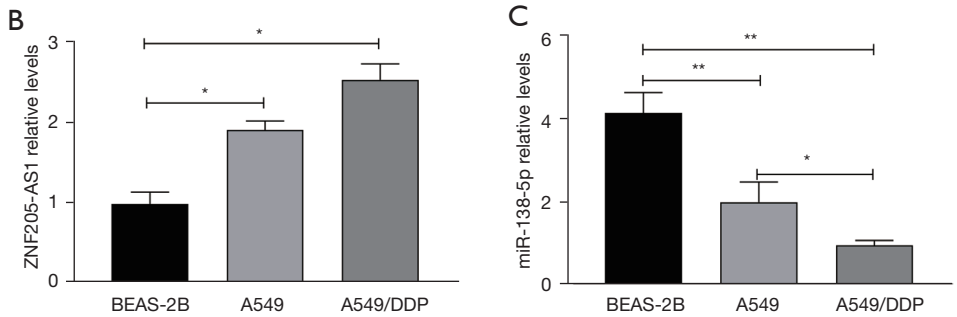


Figure 1 The levels of ZNF205-AS1 and miRNA-138-5p in non-resistant NSCLC cells and DDP-resistant cells (*, P<0.05 and **, P<0.01). (A) The biological effect of various concentrations of DDP on the viability of A549 cells and A549/DDP cells was measured in CCK8 assay, which showed that the IC_{50} value of A549 cell line was significantly lower than that of A549/DDP (10.28±0.92 *vs.* 38.13±5.72 μM; P<0.001). (B) qPCR was conducted to evaluate the expression of ZNF205-AS1 in DDP-sensitive A549 cells and A549/DDP cells (GAPDH as endogenous control). (C) qPCR was employed to evaluate the expression of miRNA-138-5p in DDP sensitive A549 cells and A549/DDP cells (U6 as endogenous control). DDP, cisplatin; IC_{50}, half-maximal inhibitory concentration; NSCLC, non-small cell lung cancer; CCK8, Cell Counting Kit-8; qPCR, quantitative polymerase chain reaction.

and OCT4 in NSCLC cell and tissues. The results of qPCR found that the miRNA levels of EGR4 and OCT4 were the highest in A549/DDP cells, being significantly higher than that in BEAS-2B cells (P<0.05), although the difference in EGR4 level between A549 cells and A549/DDP cells did not reach the level of significance (*Figure 2A*,*2B*). Consistently, the results of IHC assay found that the protein levels of EGR4 and OCT4 were notably higher in DDP-resistant NSCLC tissue collected from patients post-chemotherapy, as opposed to the NSCLC tissue from patients pre-chemotherapy (*Figure 2C*,*2D*).

ZNF205-AS1 is involved in mediating the expression level of miR-138-5p

The correlation between ZNF205-AS1 and miRNA-138-5p was explored by transfecting DDP-resistant A549 cell lines with three designed ZNF205-AS1 small interfering RNA fragments (si-ZNF205-AS1) to effectively knockdown the level of ZNF205-AS1. The effectiveness of knockdown was measured in qPCR assay. As shown in *Figure 3A*, the relative level of ZNF205-AS1 was significantly down-regulated in three paralleled knockdown group than that in blank control group and NC group (P<0.001). Notably, ZNF205-AS1 loss-of-function led to significantly higher expression level of miR-138-5p was detected in three paralleled ZNF205-AS1 loss-of-function group, as opposed to NC group (P<0.05) (*Figure 3B*). The result of bioinformatics analysis predicted the binding of ZNF205-AS1 with miRNA-138-5p, as shown in *Figure 3C*. Of note, we performed dual-luciferase assay to convincingly reveal that ZNF205-AS1 could directly bind with miRNA-138-5p (*Figure 3D*).

MiRNA-138-5p mediates chemosensitivity to DDP in NSCLC cancer

qPCR was carried out to examine the effect of miR-138-

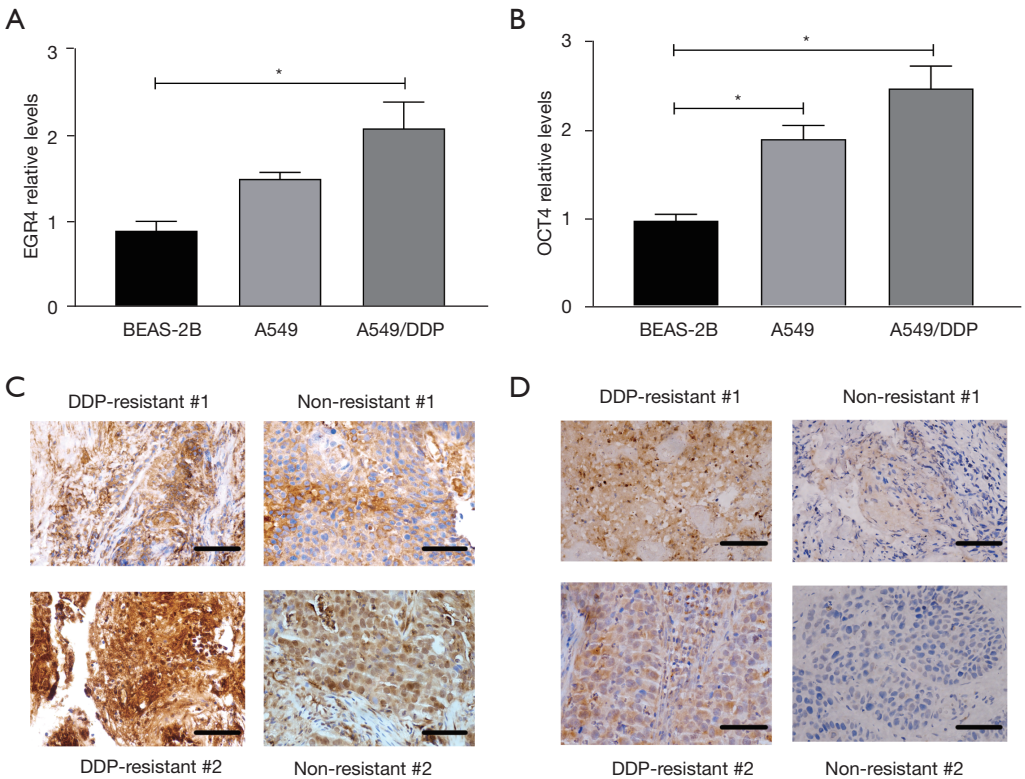


Figure 2 The expression levels of EGR4 and OCT4 in chemo-resistant cell lines and tissues from patients before and after DDP-chemotherapy (*, P<0.05). (A,B) Real-time PCR confirmed that markedly up-regulation of EGR4 and OCT4, respectively, in A549/DDP cells, as opposed to that in A549 cells and BEAS-2B cells. (C,D) IHC results found that protein expression levels of EGR4 and OCT4, respectively, were notably higher in DDP-resistant tissues (the scale bars in IHC represent 50 μm) (left: post-DDP therapy; right: pre-DDP therapy). DDP, cisplatin; PCR, polymerase chain reaction; IHC, immunohistochemistry.

5p mimic transfection on A549/DDP cells. As seen in *Figure 4A*, the level of miRNA-138-5p was significantly higher in cells transfected with miRNA-138-5p mimic than those with NC (P<0.05). Intriguingly, the invasion capacity of cells with miRNA-138-5p gain-of-function was significantly suppressed, as seen in the representative images of *Figure 4B*. The influence of miRNA-138-5p up-regulation on the viability of A549/DDP cells exposed to DDP was measured in CCK8 assay. The results found that the overexpression of miRNA-138-5p significantly suppressed cell viability, in comparison with those without miRNA-138-5p over-expression. The IC_{50} value of A549/DDP cell line with miRNA-138-5p over-expression was significantly lower than that transfected with NC (49.19±7.89 *vs.* 17.58±1.29 μM, P<0.001, *Figure 4C*).

To further validate whether miRNA-138-5p could potently facilitate apoptosis of DDP-resistant NSCLC cells, A549/DDP cells transfected with miRNA-138-5p mimic were exposed to DDP at a concentration of10 μM. The results of flow cytometry found that miR-138-5p overexpression led to significantly higher rate of apoptosis, as opposed to those transfected with NC (*Figure 5A*; P<0.05). The significantly down-regulation of Bcl-2 and OCT4 in DDP-treated A549/DDP cells with miR-138-5p gain-of-function was found in western blotting, along with the significant elevation of Cleaved-caspase 3, Cleaved-PARP and Bax (*Figure 5B*; P<0.05). We further performed RNA pulldown to examine the possible interaction between miR-138-5p and EGR4 and OCT4, the retrieved proteins are detected in immunoblotting assay. Intriguingly, RNA pulldown assay confirmed that miR-138-5p directly binds to the 3'-UTR of EGR4 and OCT4 (*Figure 5C*). These

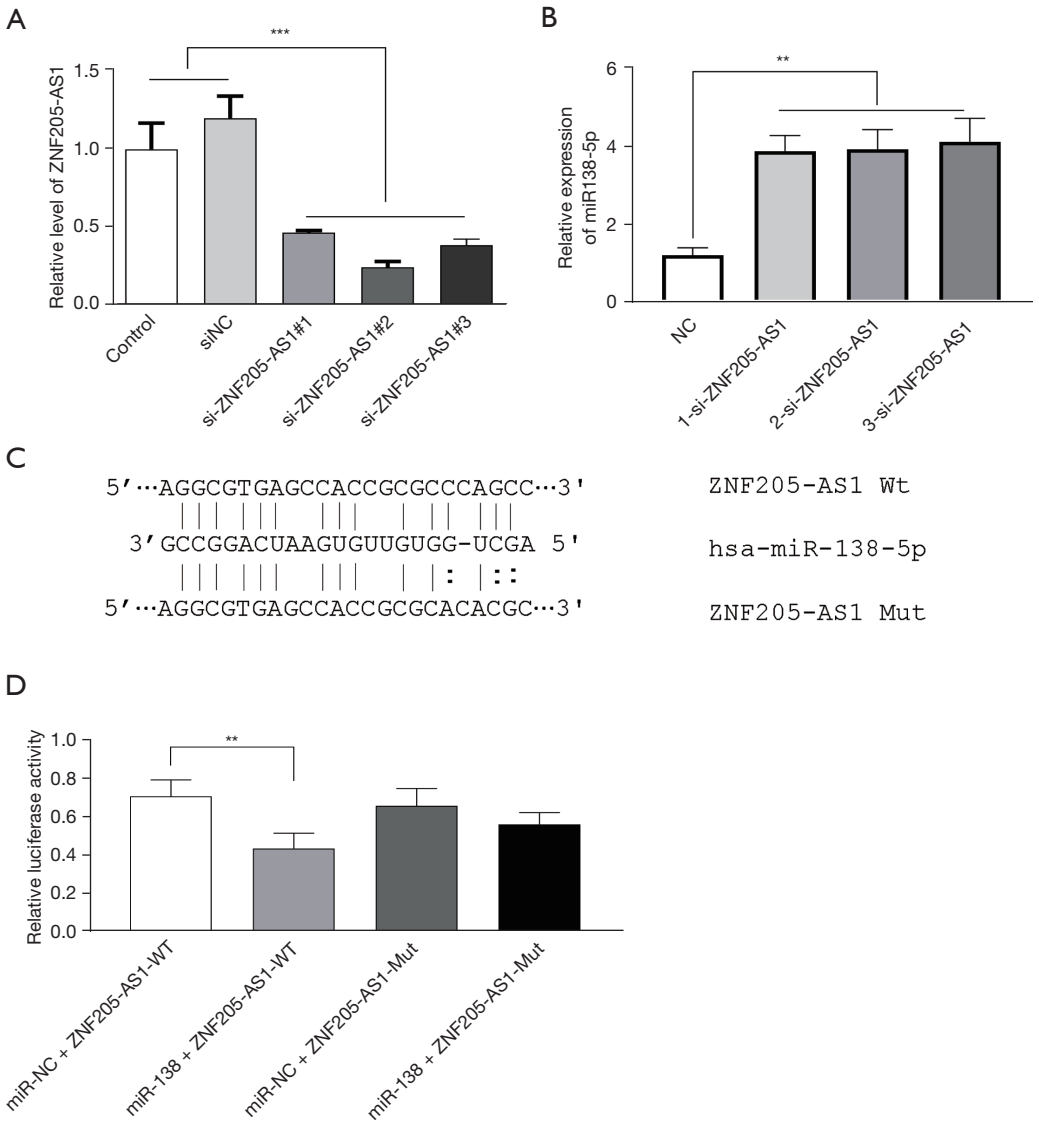
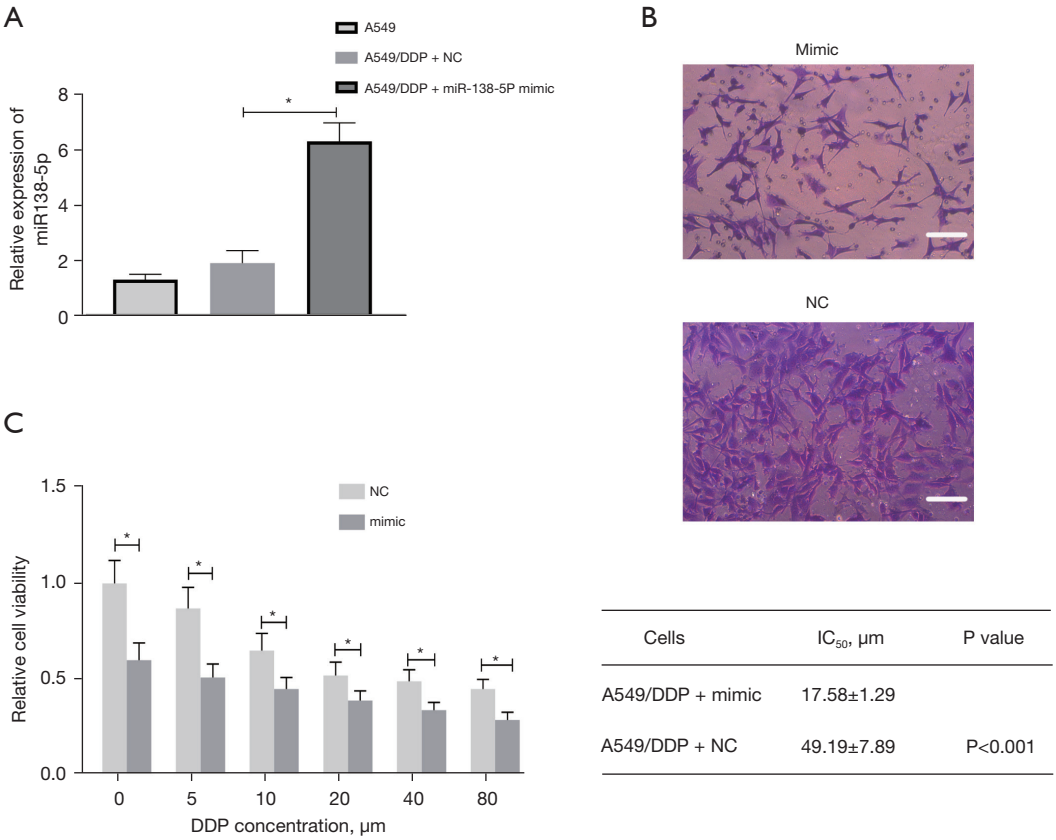


Figure 3 Knockdown of ZNF205-AS1 led to elevated miRNA-138-5p levels in A549/DDP cells. (A) The down-regulation of ZNF205-AS1 was realized by transfecting three designed small interfering RNAs. ZNF205-AS1 level was significantly higher in blank control group and NC group than the three paralleled knockdown group (***, P<0.001). (B) The levels of miR-138-5p in A549/DDP cells with ZNF205-AS1 loss-of-function were assessed in qPCR. ZNF205-AS1 loss-of-function led to significantly up-regulation of miR-138-5p as opposed to NC group (**, P<0.01) (GAPDH and U6 were used as endogenous control for detection of ZNF205-AS1 and miRNA-138-5p, respectively). (C) The binding site of lncRNA ZNF205-AS1 with miRNA-138-5p was predicted in bioinformatics analysis. (D) The significantly inhibited relative luciferase activity of cells co-transfected with wild-type ZNF205-AS1 and miRNA-138-5p mimic, as opposed to that transfected with wild-type ZNF205-AS1 and miR-NC was detected (**, P<0.01). NC, negative control; WT, wide type; Mut, mutant; DDP, cisplatin; qPCR, quantitative polymerase chain reaction.



Cells	IC_{50}, μm	P value
A549/DDP + mimic	17.58±1.29	
A549/DDP + NC	49.19±7.89	P<0.001

Figure 4 miRNA-138-5p gain-of-function enhanced the sensitivity of A549/DDP cells to DDP. (A) Overexpression of miRNA-138-5p was verified in qPCR assay to find that miRNA-138-5p level was significantly higher in miRNA-138-5p mimic group than NC group (*, P<0.05) (U6 as endogenous control). (B) Up-regulation of miR-138-5p promoted the invasion of A549/DDP cells. The cells were stained with 0.1% Giemsa stain (the scale bars represent 25 μm). (C) The results of CCK8 assay showed that the viability of cells exposed to gradient concentrations of DDP was significantly inhibited in miRNA-138-5p mimic group, compared with that of NC group (*, P<0.05). DDP, cisplatin; NC, negative control; IC_{50}, half-maximal inhibitory concentration; qPCR, quantitative polymerase chain reaction; CCK8, Cell Counting Kit-8.

finding consistently revealed that the overexpression of miR-138-5p could increase the chemosensitivity of A549/DDP cells to DDP.

Downregulation of ZNF205-AS1 contributes to a higher sensitivity to DDP in NSCLC cells by upregulating miR-138-5p expression

To examine the plausible inverse correlation between ZNF205-AS1 and miRNA-138-5p, we transfected ZNF205-AS-loss-of-function A549/DDP cells with miRNA-138-5p inhibitor and miRNA-138-5p NC respectively. Cell viability, apoptosis rate and invasion capacity were measured in each group of cells treated with 10 μM of DDP. The expression levels of Bax, Bcl-2, Cl-caspase-3, Cl-PARP and OCT4 were measured by performing western blotting assay.

Our data found that miRNA-138-5p-knockdown improved cell viability (*Figure 6A*), led to decreased protein levels of OCT4 and Bcl-2, along with notably increased expression of Cl-caspase-3, Cl-PARP and Bax (P<0.05) (*Figure 6B*). Significantly lowered apoptosis rate of A549/DDP cells was observed in si-ZNF205-AS1 + miRNA-138-5p group and in NC group, as opposed to si-ZNF205-AS1 group (P<0.05) (*Figure 6C*). The number of invasive cells was significantly higher in si-ZNF205-AS1 + miRNA-138-5p group and in NC group than si-ZNF205-AS1 group (P<0.05) (*Figure 6D*). Consistently, the results of wound healing assay prove the migratory ability of chemo-resistant cell was bolstered after miRNA-138-5p-knockdown (P<0.01), as shown in *Figure 6E*. Therefore,

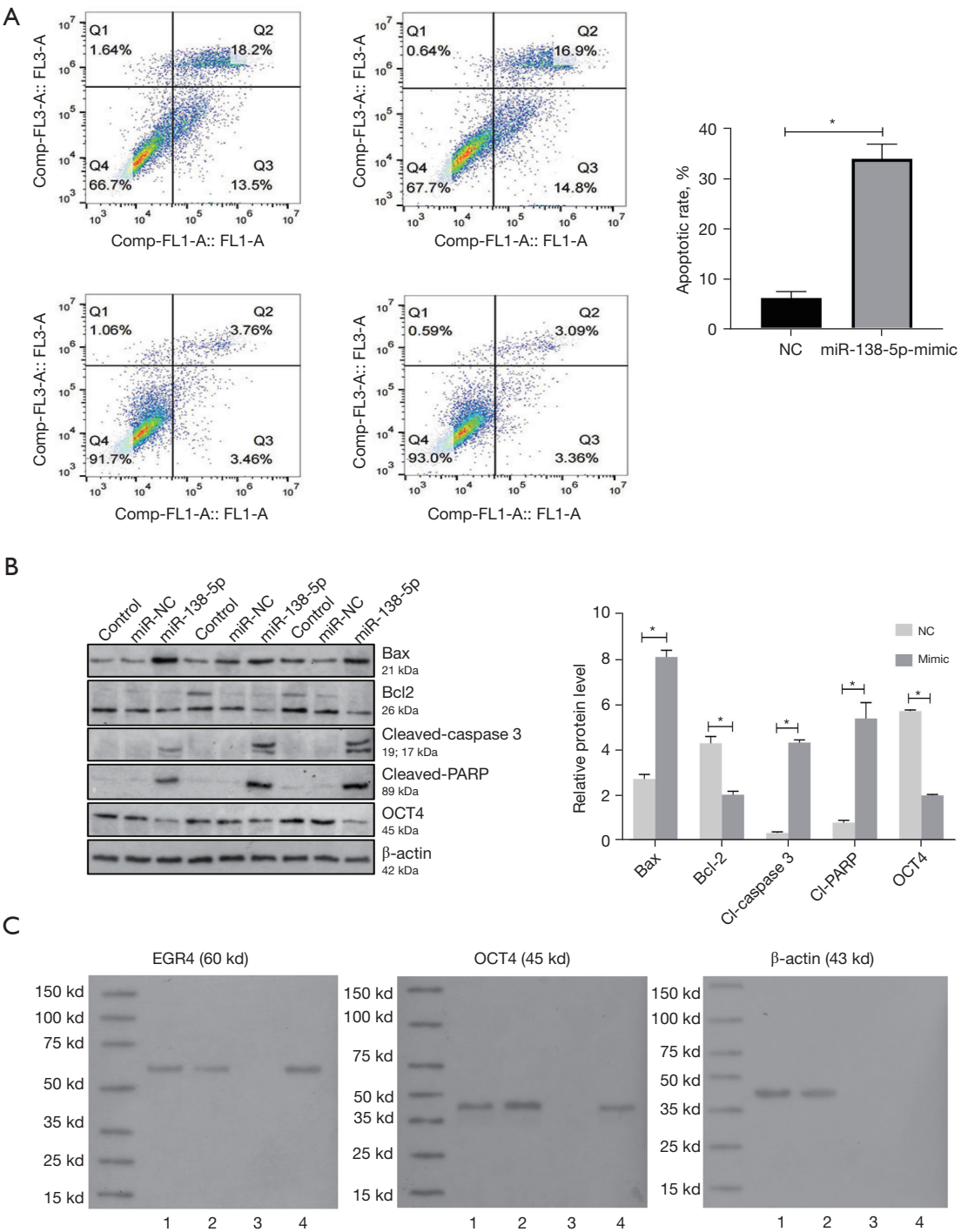


Figure 5 miR-138-5p up-regulation facilitated DDP-induced apoptosis of A549/DDP cells. (A) The cell apoptosis rate of DDP-treated A549/DDP cells with miR-138-5p overexpression was significantly increased than those without miR-138-5p overexpression (*, P<0.05). (B) The protein levels of Cleaved-caspase 3, Cleaved-PARP, Bax, Bcl2, and OCT4 were measured in western blotting (*, P<0.05). (C) RNA pulldown assay was performed to verify the potential binding of miR-138-5p to OCT4, and EGR4, respectively (lane 1: miR-NC input; lane 2: miR-138-input; lane 3: miR-NC pulldown; lane 4: miR-138-5p pulldown). NC, negative control; OCT4, octamer-binding protein 4; EGR4, early growth response 4; DDP, cisplatin.

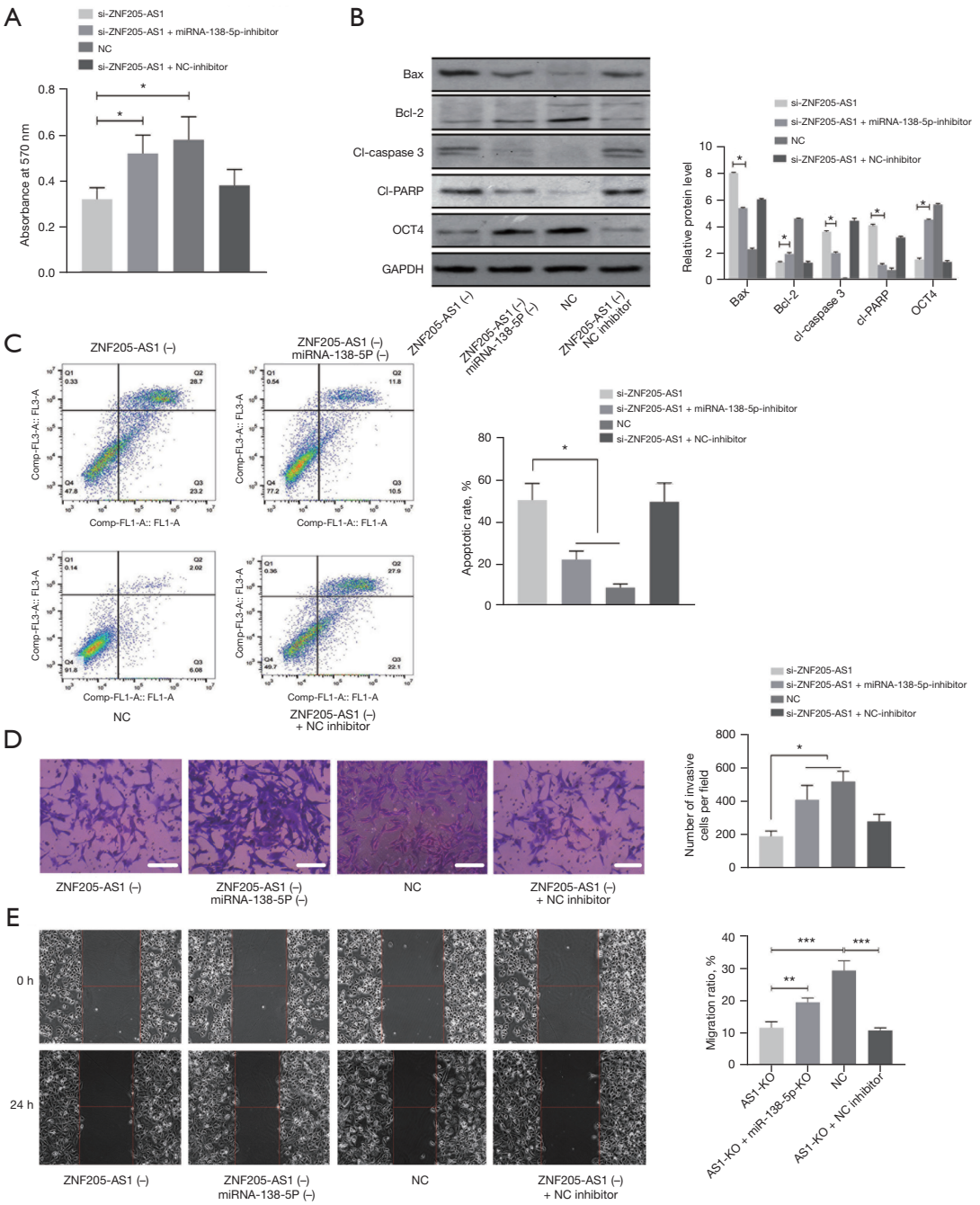


Figure 6 miRNA-138-5p loss-of-function diminished the pro-apoptosis activity induced by ZNF205-AS1-silencing in A549/DDP cells. (A) CCK8 assay confirmed that the cell viability of si-ZNF205-AS1 group was significantly lower than that with miR-138-5p knockdown and NC group (*, P<0.05). (B) Significantly higher protein levels of OCT4 and Bcl-2, as well as significantly lower protein levels of Cl-caspase-3, Cl-PARP and Bax were detected in si-ZNF205-AS1 with loss-of-function of miR-138-5p than that without (*, P<0.05). (C) The apoptosis rate of si-ZNF205-AS1 group was significantly higher than that of si-ZNF205-AS1 + miRNA-138-5p group and NC group (*, P<0.05). (D) The number of invasive cells was significantly higher in si-ZNF205-AS1 + miRNA-138-5p group and in NC group, in comparison with si-ZNF205-AS1 group (*, P<0.05). The cells were stained with 0.1% Giemsa stain (magnification 200×). (E) The migration ability of si-ZNF205-AS1 group being significantly lower than si-ZNF205-AS1 + miRNA-138-5p group (**, P<0.01) and NC group was validated in wound healing assay (the scratched areas from each group at 0 and 24 h post-wound were photographed and quantified on a microscope) (***, P<0.001). NC, negative control; DDP, cisplatin; CCK8, Cell Counting Kit-8.

miRNA-138-5p-loss-of-function facilitated the invasive potential and migratory capability of cells. Of note, the silencing of miRNA-138-5p could considerably reverse the effect of ZNF205-AS1 loss-of-function on increasing chemosensitivity of A549/DDP cells to DDP. Overall, these data suggested that ZNF205-AS1/EGR4 positive feedback loop regulates chemosensitivity of NSCLC cells to DDP by targeting miRNA-138-5p.

Discussion

The cases of NSCLC make up to over 80% of all lung cancer events and is a notoriously lethal cancer type and a leading cause of mortality on a global scale (18). DDP-based adjuvant chemotherapy in combination with postoperative radiotherapy is the most extensively applied approach for NSCLC (19); DDP functions to induce excessive cell apoptosis and cell cycle arrest via triggering DNA damage response (20,21). Unfortunately, the acquired chemo-resistance to DDP in clinical practice has impeded the effect of clinical therapy and resulted in weakened therapeutic efficiency, thus severely restraining the scope of available therapeutic regimens, especially in relapsed cases of NSCLC (21,22). Therefore, efforts should be made to elucidate the molecular mechanism underlying DDP-based chemotherapy resistance to improve the prognosis of patients with NSCLC.

A growing body of evidence in recent studies have revealed that lncRNAs are profoundly involved in a number of malignancy-related events (such as metastasis and chemoresistance) via various molecular mechanisms, including transcriptional regulation and epigenetic regulation (23,24). A variety of lncRNAs are applied as the biomarker for the diagnosis of malignancies, owing to aberrant expression levels. The validation of key lncRNAs and the advanced understanding their functional and molecular mechanism are of huge significance for the prognosis and treatment of various cancers. We have previously demonstrated that the positive feedback loop between EGR4 and ZNF205-AS1 promotes the proliferation of NSCLC, illuminating the prospect of such an intriguing feedback loop as a promising therapeutic approach for the management of NSCLC (12). Nevertheless, the mechanism of lncRNA-mediated DDP-resistance in NSCLC cases is yet to be clarified. We have identified the aberrantly elevated level of lncRNA ZNF205-AS1 in DDP-resistant cells and tissues contributes to the phenotype of DDP-resistance. We then studied the effect of the ZNF205-AS1/EGR4 positive feedback loop on the acquired resistance of NSCLC cells to DDP to reveal more therapeutic targets and new molecular mechanisms. The present study aims to provide a novel strategy for the clinical management of NSCLS by establishing the negative correlation of ZNF205-AS1/EGR4 positive feedback loop with miR-138-5p in mediating DDP resistance of NSCLC.

The aberrantly high expression level of lncRNA ZNF205-AS1 (NCBI refseq: NR_024167.1) in NSCLC tissues has been acknowledged to be associated with an unfavorable prognosis of NSCLC patients. The simultaneous knockdown of ZNF205-AS1 and EGR4 was found to potently thwart the proliferation of NSCLC cells *in vivo* (12). Mechanistically, EGR4 acts to activate the transcription of ZNF205-AS1 by interacting with ZNF205-AS1 promoter; ZNF205-AS1 maintains the stability of EGR4 and promotes the elevation of EGR4 translation level by interacting with EGR4-mRNA, hence the formation the lncRNA ZNF205 AS1/EGR4 feedback loop could exhibit the pro-progression effect on NSCLC. Nevertheless, the plausible implication of lncRNA ZNF205-AS1/EGR4 feedback loop in DDP resistance of NSCLC warrants exceptional research. Moreover, the targeting of OCT4 by miR-138-5p to mediate affects tumor cell stemness as a downstream mechanism of lncRNA ZNF205-AS1/EGR4 feedback loop to regulate DDP resistance in NSCLC should be clarified.

Evidence suggested that miRNA-138-5p acts as a tumor-suppressor in NSCLC, and the down-regulation of miRNA-138-5p promotes the proliferation, metastasis and invasive ability of NSCLC cancer cells. The level of miR-138-5p being significantly and negatively correlated with NSCLC DDP resistance has been established in previous study, and that the forced overexpression of miR-138-5p caused the restoration of higher susceptibility to DDP of chemo-resistant NSCLC cells (25). Moreover, it was proved that the drastic up-regulation of ITGA2 through competitive binding of miR-138-5p to drives metastasis of gastric cancer (26). Xu *et al.* revealed the role of miR-138-5p to modulate migration and chemoresistance of tumor cells by acting upon the NFIB-Snail1 axis (27). OCT4 plays a key role of maintaining the characteristics of embryonic stem cells with multi-directional potential and self-renewal (16). Liu *et al.* reported the aberrantly higher expression of OCT4 in gefitinib-resistant NSCLC cells to exhibit potent epithelial-mesenchymal transition (EMT) phenotype and to indicate multi-drug resistance (MDR) properties (28). OCT4-SOX2-Nanog interaction

mediates upregulation of miR-302 expression, thereby promoting clone formation and self-renewal ability of DDP-resistant stem cells in different cancers (29,30). Furthermore, a recent study has demonstrated that a high OCT4 expression is intricately correlated with the progression and chemoresistance of NSCLC and that the knockdown of OCT4 reduces the proliferative capacity of NSCLC cells and induces excessive apoptosis of A549/DDP cells (17). To reveal the underlying mechanism, we performed RNA pulldown to identify that EGR4 and OCT4 are the interactive proteins of miR-138-5p.

Notably, we found that the upregulation of ZNF205-AS, EGR4 and OCT4 in DDP-resistant A549 cells contributed to chemoresistance, and illustrated the pivotal function of the miR138-5p/OCT4 pathway in regulating DDP resistance in NSCLC. Consistently, a previous study reported the aberrantly higher expression of OCT4 in NSCLC specimens, and especially in DDP-resistant A549 cells and tumor specimens collected from EGFR-mutant NSCLC patients, thus concluding OCT4 conferred potent drug resistance to several chemotherapeutic agents (31).

We found increased levels of ZNF205-AS1 and decreased expression level of miR-138-5p in successfully constructed DDP-resistant NSCLC cells. The knockdown of ZNF205-AS1 led to elevated expression of miRNA-138-5p, thereby enhancing the sensitivity of A549/DDP cells to DDP. Furthermore, our data indicated the inverse regulatory between miRNA-138-5p and OCT4, which mediate the acquired DDP resistance of NSCLC. Interestingly, the loss-of-function of miRNA-138-5p markedly reversed ZNF205-AS1 silencing-mediated pro-apoptotic pattern and chemosensitivity to DDP. We reported the interaction between ZNF205-AS and miR-138-5p, validated OCT4 and EGR4 as the direct target of miRNA-138-5p, highlighting their contributing roles in the acquired chemoresistance to DDP.

The regulation levels of Bcl-2, Bax, cleaved PARP and cleaved caspase 3 proteins are indicative of induction of apoptosis in cancer cells (32). The application of PARP inhibitors for the treatment of homologous recombination-deficient tumor is under clinical investigation and has yielded promising response rates in spite of the rate of resistance (33). Caspase-3 is regarded as a primary target for the management of cancer using both synthetic and natural compounds (34). The results of western blotting were consistent with that of flow cytometry: both indicate that the forced upregulation of miRNA-138-5p led to the greater extent of cellular apoptosis.

The potential limitations of the current study should be noted. Firstly, while the vital role of ZNF205-AS1/miRNA-138-5p axis in regulating DDP resistance was well-established in the study, chemo-resistance is a multifactorial phenotype that involves both internal cellular processes and the microenvironment. Individual cases of NSCLC may present distinct single cell heterogeneity and plasticity in the respective tumor microenvironment (35). Secondly, *in vivo* assay, such as nude mouse xenograft model, could be performed to substantiate the findings of the study in a more comprehensive manner.

Conclusions

To conclude, this study elucidates, for the first time, the mechanism of the lncRNA ZNF205-AS1/EGR4 feedback loop in regulating DDP resistance of NSCLC and the exact function of the downstream effector of miR-138-5p/OCT4. In addition, we defined a new theoretical basis for DDP resistance in NSCLC and combined targets for DDP therapy of NSCLC, thus bridging the gap in the application of translational medicines from pre-clinical study to clinical medicine.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1171/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1171/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1171/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Taizhou Hospital (No. K20210705), with informed consent taken from all the participants.

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Supplementary

Table S1 Sequences used in the interference vectors

siRNA name	Sequence
1-si-ZNF205-AS1	F: 5'-GAUUGAUGCUAUUGUCAAUUUTT-3'
	R: 5'- AAAUUGACAAUAGCAUCAAUCTT-3'
2-si-ZNF205-AS1	F: 5'-CACUGACCUCAGCAGUGUAGATT-3'
	R: 5'- UCUACACUGCUGAGGUCAGUGTT-3'
3-si-ZNF205-AS1	F: 5'-GGAUGUCCACGAUCUGGAACCTT-3'
	R: 5'-GGUUCCAGAUCGUGGACAUCCTT-3'
si-NC	F: 5'-UUCUCCGAACGUGUCACGUTT-3'
	R: 5'-ACGUGACACGUUCGGAGAATT-3'

NC, negative control.

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