European Review for Medical and Pharmacological Sciences 2020; 24: 3130-3142

# ELF1 activated long non-coding RNA CASC2 inhibits cisplatin resistance of non-small cell lung cancer via the miR-18a/IRF-2 signaling pathway

X.-H. XIAO[1], S.-Y. HE[2]

[1]Department of Respiratory, Gansu Provincial Hospital, Lanzhou, China
[2]Department of Respiratory Medicine, Lanzhou Second People's Hospital, Chengguan Dist., Lanzhou, China

**Abstract. – OBJECTIVE: Chemoresistance is the leading cause of recurrence in non-small cell lung cancer (NSCLC). The long non-coding RNA (lncRNA) cancer susceptibility candidate 2 (CASC2) inhibits the tumorigenesis of various cancers. However, the regulatory function of CASC2 on the chemoresistance of NSCLC remains unclear.**

**PATIENTS AND METHODS: The levels of CASC2 and miR-18a in cisplatin (DDP)-resistant NSCLC tissues and cell lines were evaluated by quantitative Polymerase Chain Reaction (qPCR). The role of low CASC2 levels on overall survival in patients with NSCLC was tested using the log-rank test. The Chi-squared test was used to assess the relation between CASC2 expression and clinicopathological features of NSCLC patients. Cell Counting Kit-8 (CCK-8) assays tested the cell proliferation of cisplatin-resistant NSCLC cells (H226/DDP and A549/DDP). The underlying regulatory mechanism between CASC2 and miR-18a or miR-18a and interferon regulatory factor 2 (IRF-2) was predicted by bioinformatics and verified by a Dual-Luciferase reporter assay, RNA transfection, qPCR, and Western blotting. Chromatin immunoprecipitation (ChIP) assay was done to exam the relation between E74 like factor 1 (ELF1) and CASC2 gene. Mice xenografts were applied to exam the function of CASC2 on chemosensitivity of cisplatin of NSCLC cells *in vivo*.**

**RESULTS: Low CASC2 expression is more likely to present in patients with advanced TNM stage (IV), cisplatin-resistance, and poor overall survival. The expression of CASC2 sharply decreased in cisplatin-resistant NSCLC tissues and cell lines (H226/DDP and A549/DDP). CASC2 overexpression strongly inhibited proliferation, migration, and invasion of cisplatin-resistant NSCLC cells (H226/DDP and A549/DDP) in vitro and inhibited tumor growth *in vivo*. Besides, CASC2 repressed miR-18a function by binding to the complementary sites of miR-18a as competing endogenous RNAs (ceRNAs). MiR-18a released by the declining expression of CASC2 reduced the protein concentration of IRF-2 in NSCLC cells. Furthermore, the transcription factor ELF1 was found to be promotor of CASC2 and increased its levels in cisplatin-resistant NSCLC cells.**

**CONCLUSIONS: IRF-2 expression mediated by the ELF1/CASC2/miR-18a axis is markedly associated with the proliferation, migration, and invasion of cisplatin-resistant NSCLC, resulting in inferior survival. These findings suggest that this regulatory axis may serve as a novel therapeutic target in NSCLC.**

*Key Words:*
Non-small cell lung cancer, E74 like factor 1, Cancer susceptibility candidate 2, Interferon regulatory factor 2, MiR-18a, Cisplatin resistance.

## Introduction

Lung cancer ranks first in both cancer incidence and the leading cause of cancer-related mortality globally[1]. Two histological subtypes, adenocarcinoma (51%) and squamous cell carcinoma (30%), mainly constitute non-small cell lung cancer (NSCLC), representing 85% of all lung cancers[2]. The 5-year survival rate for patients with NSCLC undergoing surgery or chemotherapy has reached 20%, and unfortunately, the recurrence rate has not significantly decreased over the past 20 years[1,3]. The chemoresistance of residual tumor cells is an essential cause of NSCLC recurrence after regular treatments[4,5]. The overexpression of filamin A, a biomarker of resistance to various chemotherapeutics [including cisplatin (DDP)], is associated with disease relapse and metastases status[6]. SIRT6 in NSCLC is involved in paclitaxel resistance and autophagy activation by boosting nuclear factor kappaB (NF-κB) and Beclin1 signaling, resulting in creating an environment in



*Corresponding Author:* Siyun He, MD; e-mail: hesiyunlz@126.com.

favor of tumor recurrence[7]. Currently, the first-line treatment strategy after surgical resection of NSCLC is still based on cisplatin chemotherapy[8]. Therefore, novel prevention for NSCLC chemoresistance, especially cisplatin-resistance, may be a promising strategy to intervene in the development and relapse of NSCLC.

Low survival rates and tumor stem cell characteristics are mediated by long non-coding RNAs (lncRNAs)[9,10]. LncRNAs are transcripts with more than 200 bases and cannot encode proteins[11] but inhibit microRNA (miRNA) function through interactions[12]. Generally, carcinogenic lncRNAs are abnormally expressed in malignant tumors, enhancing cell proliferation, metastasis, and chemoresistance[13]. The inhibition of these carcinogenic lncRNAs can impair cellular malignant biological behavior, thereby controlling cancer growth and improving therapeutic outcomes[14]. It has been reported that the dysregulation of lncRNA is linked to the proliferation, metastasis, and even resistance to chemotherapeutics of NSCLC cells[15,16]. LncRNA epidermal growth factor receptor antisense 1 (EGFR-AS1) is found to be related to the chemotherapy-resistant features[15]. LncRNA bladder cancer-associated transcript 1 (BLACAT1) not only augments the autophagy-related protein 7 (ATG7) signal by completely binding miR-17 but also boosts the autophagy-related effector, including autophagy-associated proteins light chain 3 and Beclin 1 to accelerates the resistance to cisplatin NSCLC cells[16]. LncRNA nicotinamide nucleotide transhydrogenase-antisense RNA1 (NNT-AS1) plays an essential role in the maintenance of chemoresistance by mitogen activated protein kinase/slug pathway[13]. Therefore, studying the critical role of lncRNA in tumorigenesis, especially chemoresistance, may have far-reaching value for identifying novel molecular targets in the development of NSCLC.

Although increasing lncRNAs are found to be involved in resistance properties of NSCLC, the ones exerting tumor-suppressing functions are still few. In our investigation, we found that lncRNA cancer susceptibility candidate 2 (CASC2) was upregulated in adjacent tissue rather than NSCLC cancerous ones. The data of our study identified that CASC2 restrained the proliferation and chemoresistance of NSCLC, being activating by E74 like factor 1 (ELF1). All these data indicated that the ELF1/CASC2 pathway could be a helpful molecular target for improving the prognosis of NSCLC patients.

## Patients and Methods

### Patients, Chemotherapy, Therapy Responses, and Follow-up

The Ethics Committee of Gansu Provincial Hospital approved the study. All patients gave their written informed consent before this study. After a transbronchial lung biopsy, a total of 85 NSCLC patients were collected from December 2013 to September 2014. All patients were diagnosed by the biopsy-histology and received chemotherapy. Any distant clinical metastasis (M1 stage, the 7$^{th}$ AJCC TNM staging system) is confirmed by MRI (magnetic resonance imaging) or bone scanning. Tissue specimens were frozen in -80°C for further experiments.

The enrollment criteria of qualified patients were as follows: NSCLC verified by biopsy-histology, no neoadjuvant therapy and surgery, arranged chemotherapy treatment finished, KPS (Karnofsky Performance Status) scores > 70. Patients were defined as unqualified if they met the following criteria: absence of measurable disease, second malignancies, incomplete chemotherapy, incomplete follow-up data, and other conditions that required medical treatment.

At least two cycles of the first-line platinum-based chemotherapy were administrated to all these patients. An enhanced computed tomography before the chemotherapy and another one after 2-3 cycles of chemotherapy were applied to evaluate the response to treatment. According to the RECIST1.1 criteria about tumor response (complete response, CR; partial response, PR; stable disease, SD; progressive disease, PD), all those patients were divided into chemosensitive group (n=52, including CR and PR) and chemoresistant group (n=33, including SD and PD)[17]. The overall survival of patients was followed up, defined as the time from the first-time chemotherapy to death or the last follow-up.

### Total RNA Extraction and Quantitative Analysis

The total RNA of tissue and cell line was extracted using RNAiso Plus (TaKaRa, Beijing, China) according to the instruction. The extracted RNA was synthesized to cDNA by the PrimeScript™ RT reagent Kit (TaKaRa, Beijing, China). Quantitative Polymerase Chain Reaction (PCR) was done using SYBR® Green Realtime PCR Master Mix (TOYOBO, Shanghai, China) on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The quantitation of the target RNA expression was assessed using the endogenous control by

the $2^{-\Delta\Delta Ct}$ method [glyceraldehyde-phosphate dehydrogenase (GAPDH)]. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quality of the prepared RNA, and cDNA was also measured.

### Culture of Cell Lines

The cell lines H226, H520, SK-MES-1, A549, H1975, H1299, and normal human bronchial epithelium cell line BEAS-2B were purchased from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). The cell line A549/DDP, which exhibits stable cisplatin-resistant features, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). A continuous exposure with an escalating dose of cisplatin was applied to establish DDP-resistant H226 cell lines (H226/DDP). Briefly, DDP (Sigma-Aldrich, St Louis, MO, USA) with an initial concentration of 0.5 μg/mL was administrated to H226 cells in the exponential phase. DDP dosage was increased twofold when H226 exhibited resistance to the current DDP concentration until DDP was increased to 8 μg/mL. The induced cell line was defined as cisplatin-resistant when it can maintain a normal morphology and activity in the highest concentration of DDP (8 μg/mL) for eight weeks. The cell lines H226, H520, SK-MES-1, A549, H1975, H1299, and BEAS-2B were cultured in 10% fetal bovine serum-supplementing Roswell Park Memorial Institute-1640 (RPMI-1640; Thermo Fisher Scientific, Waltham, MA, USA). Moreover, H226/DDP and A549/DDP were cultured in the medium mentioned above, plus 2 μg/mL DDP. All of them were in a 37°C atmosphere with 5% $CO_2$.

### Determination of Half Maximal Inhibitory Concentration

The NSCLC cells ($1\times10^5$ cells/ml) were seeded into a 96-well plate for overnight culture. Then, different wells were treated with various concentrations (0, 2, 4, 6, 8 μg/mL) of DDP for 48 h. The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was used to measure cell viability based on the protocol. The cell viability of each well was analyzed at optical density (OD) 450 nm to compute the toxicity curves of DDP.

### Gene Overexpression and Cells Transfection

For gene overexpression, lentiviral vectors (pcD-ciR vector, Geenseed Biotech, Guangzhou, China) were used to construct CASC2 expressing particles (oe-CASC2, CASC2 sequence was shown in **Supplemental Table I**). CASC2-cDNA or Vector-cDNA (MOI = 20) with polybrene was

**Table I.** Relation between CASC2 expression and clinicopathological features in NSCLC (n = 85).

| Variables | Total No. | CASC2 expression | | *p*-value |
|---|---|---|---|---|
| | | Low = 42 | High = 43 | |
| ***Gender*** | | | | 0.149 |
| Male | 55 | 24 | 31 | |
| Female | 30 | 18 | 12 | |
| ***Age*** | | | | 0.425 |
| ≤60 | 34 | 15 | 19 | |
| >60 | 51 | 27 | 24 | |
| ***Pathology*** | | | | 0.756 |
| Squamous carcinoma | 50 | 24 | 26 | |
| Adenocarcinoma | 35 | 18 | 17 | |
| ***T stage*** | | | | 0.088 |
| T1-2 | 32 | 12 | 20 | |
| T3-4 | 53 | 30 | 23 | |
| ***N stage*** | | | | 0.221 |
| N0-1 | 36 | 15 | 21 | |
| N2-3 | 49 | 27 | 22 | |
| ***TNM stage*** | | | | 0.040* |
| II+III | 44 | 17 | 27 | |
| IV | 41 | 25 | 16 | |
| ***Chemotherapy response*** | | | | 0.037* |
| Sensitive | 52 | 21 | 31 | |
| Resistant | 33 | 21 | 12 | |

Notes: *$p<0.05$ represents statistical difference.

transfected into H226/DDP and A549/DDP. 24 h after transfection, a new medium replacement was done to the culture medium. Stably transfected cells were selected by puromycin (1 μg/ml). The puromycin (1 μg/ml, 2-3 times) selection was made until green fluorescence was shown in all cancer cells *via* the fluorescence microscope (Olympus IX71, Tokyo, Japan).

### Proliferation Ability of Tumor Cells

NSCLC cells were cultured in 96-well plates ($5\times 10^3$ cells/well) for five days. Based on the protocol, CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was used to measure cell viability every 24 h. The analyzed O.D. 450 nm at each test was accumulated to compute the proliferation curves.

### Migration and Invasion Ability of Tumor Cells

NSCLC cells transfected with CASC2 overexpressing cDNA or vector control were incubated for 24 hours. According to the manufacturer's instruction of invasion (Matrigel) assay, $5 \times 10^5$ transfected cells in 200 μL of medium [fetal bovine serum (FBS) free] were seeded in the upper chamber, and 600 μL of medium with 10% FBS was added into the lower chamber using BioCoat Matrigel chambers (Corning, New York, NY, USA). After 24 hours of incubation, the remaining cells were removed from the polycarbonate membrane of the upper chamber. Moreover, the cells in the lower chamber were fixed, stained, and photographed by a microscope (Olympus, Tokyo, Japan). For the migration assay, the chamber was not pre-coated with Matrigel.

### Tumor Growth In Vivo and Mice Experiment

All the procedures of animal experiments were performed in strict accordance with the guiding principles of the Institutional Animal Ethics Committee and approved by the Institutional Animal Care and Use Committee of Gansu Provincial Hospital. BALB/c-nude mice (4-week-old) were purchased from the Laboratory Animal Center of Lanzhou Veterinary Research Institute. All mice were fed in a specific pathogen-free atmosphere with a 12 h light/dark cycle. Tumor cells transfected with an expressing vector (oe-CASC2) or control (oe-Vec) were respectively incubated subcutaneously into mice ($2 \times 10^5$ cells in 100 μL for each mouse, each group n = 5). The tumor sizes of every mouse were evaluated every week. Tumor weight was measured when the mice were sacrificed (the fifth week).

### Dual-Luciferase Reporter Assay

The wildtype lncRNA CASC2 sequences containing miR18a-binding sites or mutant sites were cloned to psiCHECK-2 plasmid (Promega, Madison, WI, USA). Similarly, the wildtype fragments of interferon regulatory factor 2 (IRF-2) 3'UTR or the one containing a mutant miR18a-binding area were cloned into the reporter vector with a Luciferase gene (Promega, Madison, WI, USA). Then, following the manufacturer's instruction, the Renilla Luciferase reporter vector was transfected into DPP-resistant NSCLC cells along with the synthetic vectors, miR-18a, anti-miR-18a, or relative controls. The above transfected NSCLC cells were seeded for incubation 48 hours. The Luciferase activities of the target gene were tested by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

### Western Blotting Assay

Total proteins of NSCLC cells were extracted using RIPA buffer added with 1% protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). According to the manufacturer's protocol, the concentration of the protein samples was tested by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). After denatured at 96°C for 10 minutes, the proteins were divided by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a transfer to a nitrocellulose polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After an incubation with 5% non-fat milk for blockade of non-specific signals, PVDF membranes were incubated with primary antibodies against MRP1 (1:1000), P-gp (1:2000), IRF-2 (1:3000), ELF1 (1:2000), and GAPDH (1:4000) (Abcam, San Francisco, CA, USA) overnight at 4°C. Then, the PVDF membrane was incubated with the secondary antibody with horseradish peroxidase (HRP) conjugated secondary antibody (1:5000; Cell Signaling Technology, Danvers, MA, USA). The protein blots were photographed using a chemiluminescence detection system (ECLTM, Pierce, Waltham, MA, USA). The density of bands was quantified by Image J software (Bio-Rad, Hercules, CA, USA).

### Chromatin Immunoprecipitation (ChIP) Assay

According to the instruction, the ChIP Assay Kit (Beyotime, Shanghai, China) was used to investigate the relation between ELF1 and CASC2. Briefly, 1% formaldehyde was used to cross-link cells. After a sonication on ice for producing 200-500 bp

DNA fragments, chromatin was stained and incubated with primary antibodies against ELF1 or IgG (isotype control; Abcam, San Francisco, CA, USA) overnight at 4°C. The target chromatin DNA was precipitated by the antibody. After the DNA was recovered, a qPCR assay was applied to evaluate it.

### Statistical Analysis

All data were presented as the mean ± standard deviation (SD). The student's *t*-test made a comparison between the means from the two groups. Survival analysis was described using the Kaplan-Meier method, followed by comparison using the Log-rank test. Each experiment was done independently three times. $p < 0.05$ was considered as the statistical significance. All statistical analyses were carried out by GraphPad Prism 6 software (San Diego, CA, USA).

## Results

### CASC2 Expression is Highly Related to Cisplatin-Sensitive and Superior Survival in NSCLC

By analyzing 85 pairs of cancer tissues and corresponding adjacent tissues, we found declined mostly in NSCLC tissues relative to adjacent normal tissues (Figure 1A). Then, we observed that the ex-

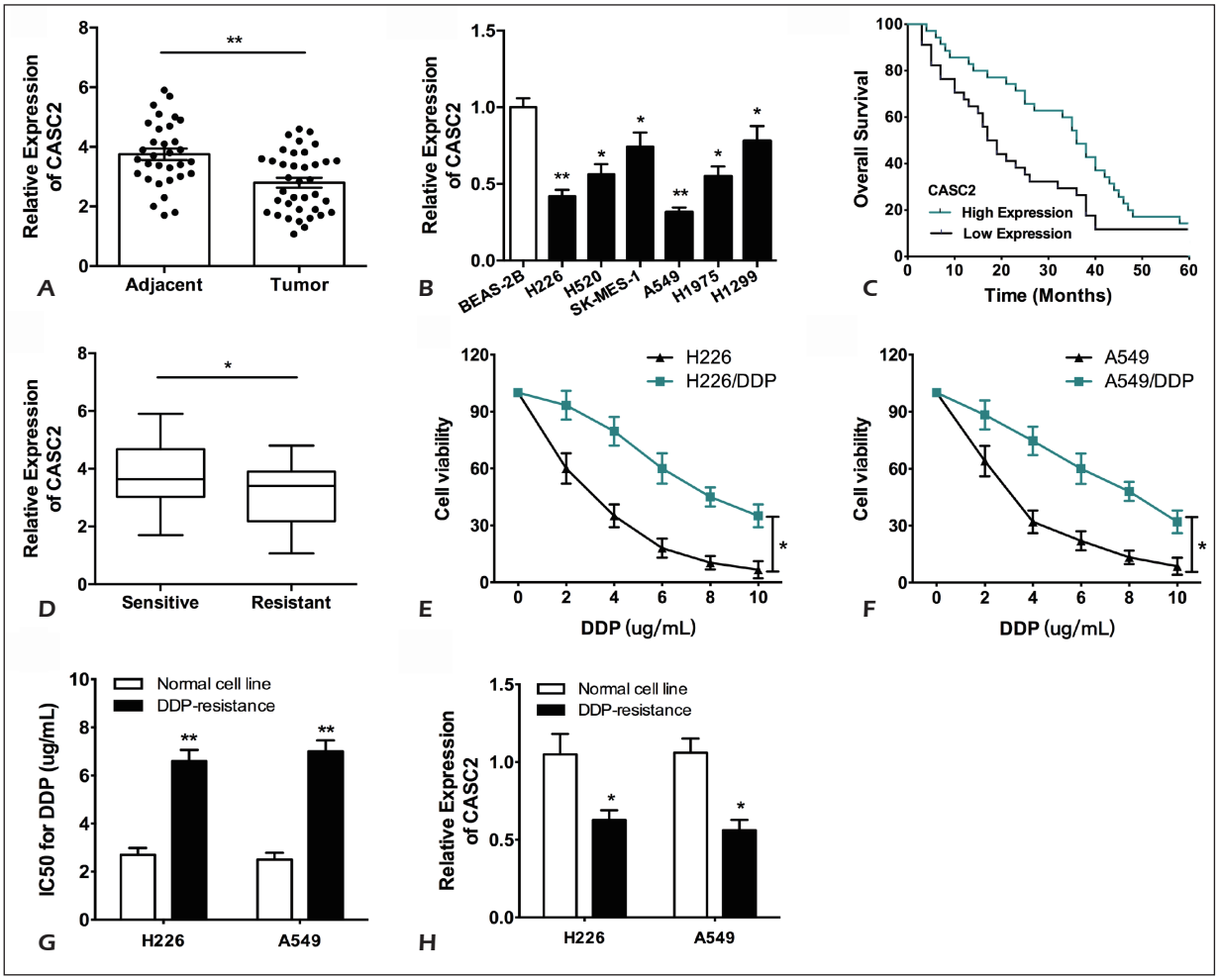


**Figure 1.** CASC2 expression is highly related to cisplatin-sensitive and superior survival in NSCLC. **A**, Expression of CASC2 in NSCLC tissues and adjacent normal tissues shown by qPCR. **B**, CASC2 levels in NSCLC cells (H226, H520, SK-MES-1, A549, H1975, and H1299) and BEAS-2B cells. **C**, Overall survival of NSCLC patients with high and low CASC2 expression. **D**, Expression of CASC2 in NSCLC tissues with different responses to cisplatin (Sensitive, n = 52; Resistant n = 33). **E**, Cell viability of H226 and H226/DDP by CCK8 assay. **F**, Cell viability of A549 and A549/DDP by CCK8 assay. **G**, IC50 of cisplatin -sensitive and -resistant NSCLC by CCK-8 assays. **H**, Expression of CASC2 in NSCLC cells (sensitive: H226 and A549) with different responses to cisplatin (Resistant: H226/DDP and A549/DDP). $^{**}p<0.01$, $^{*}p<0.05$.

pression of CASC2 was lower in NSCLC cell lines (H226, H520, SK-MES-1, A549, H1975, and H1299) than normal human bronchial epithelial cells (BEAS-2B) (Figure 1B). Based on Kaplan-Meier analysis, a lower expression of CASC2 was associated with inferior overall survival (Figure 1C, $p < 0.05$). Moreover, NSCLC patients with advanced TNM stages (IV) or chemotherapy resistance exhibited notably decreased CASC2 expression than NSCLC tissues with less advanced TNM stages (II/III, Table I, $p = 0.040$) or chemotherapy sensitivity (Table I, $p = 0.040$; Figure 1D, $p < 0.05$), respectively. Thus, we adopted cisplatin-resistant NSCLC cell lines H226/DDP and A549/DDP to investigate the association between chemotherapeutics response and CASC2. Under increasing DDP pressure (0-10 μg/mL), A549/DDP and H226/DDP cells have higher viability (Figure 1E and F, $p < 0.05$) and IC50 (Figure 1G, $p < 0.05$), compared to A549 and H226 cells. Last but not least, quantitative analysis showed that CASC2 expression in A549/DDP and H226/DDP cells were lower than their counterparts (Figure 1H, $p < 0.05$). Altogether, our results indicated that CASC2 is downregulated in NSCLC tissues, and it demonstrates a tumor-suppressive role in NSCLC by inducing cisplatin response.

### CASC2 Overexpression Enhanced Drug Response of Cisplatin-Resistant NSCLC Cells In Vitro

A synthesized lentiviral CASC2-overexpressing (oe-CASC2) vector was transfected into A549/DDP and H226/DDP cells to augment CASC2 expression (Figure 2A). Then, we applied molecular experiments *in vitro* to verify the regulatory function of CASC2 on drug response in NSCLC cells tolerant to cisplatin. CCK-8 assays revealed that the proliferation of H226/DDP (Figure 2B, $p < 0.05$) and A549/DDP (Figure 2C, $p < 0.05$) cells decreased notably after CASC2 overexpression. Moreover, compared to H226 and A549 cells, CASC2 overexpression strongly suppressed migration (Figure

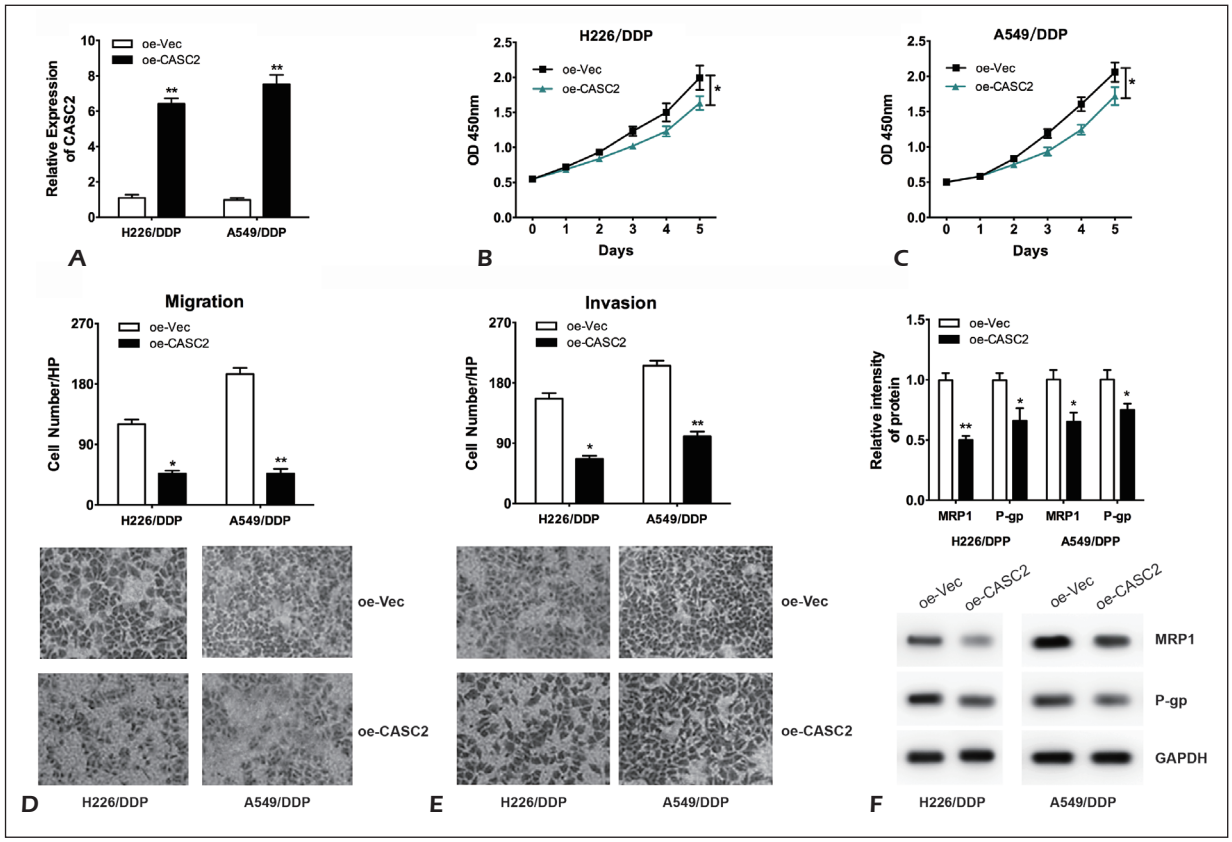


**Figure 2.** CASC2 overexpression enhanced drug response of cisplatin-resistant NSCLC cells *in vitro*. **A**, CASC2 expression was increased by lentiviral vectors in H226/DDP and A549/DDP cells. **B**-**C**, Proliferative ability of H226/DDP and A549/DDP after CASC2 overexpression. **D**, Migration ability of H226/DDP and A549/DDP transfected with overexpressing vector or control (×200). **E**, Invasion ability of H226/DDP and A549/DDP after CASC2 overexpression (×200). F, Western blot assay showed the MRP1 and P-gp protein expression in H226/DDP and A549/DDP after CASC2 overexpression. $^{**}p<0.01$, $^{*}p<0.05$.

2D, $p < 0.05$) and invasion behaviors (Figure 2E, $p < 0.05$) of H226/DDP and A549/DDP cells, showed by transwell assays. Western blot assay suggested that several intracellular biomarkers for drug tolerance (Multidrug Resistance-associated Protein 1, MRP1 and P-glycoprotein, P-gp) were significantly decreased in CASC2-overexpressing H226/DDP and A549/DDP cells compared to vector control (Figure 2F, $p < 0.05$). Therefore, these data suggest that CASC2 can mediate the destructive properties (proliferation, migration, and invasion), especially cisplatin response of NSCLC cells *in vitro*.

### CASC2 Boosts NSCLC Tumor Limitation and Cisplatin Response In Vivo

A xenograft tumor model inoculated with CASC2-overexpressing H226/DDP and A549/DDP cells were induced to investigate the regulatory function of CASC2 on NSCLC *in vivo*. The results revealed that H226/DDP and A549/DDP cells exhibited smaller tumor size (Figure 3A and B, $p < 0.05$) and lower tumor weight (Figure 3C and D, $p < 0.05$) after CASC2 increase. Together with the macroscopic comparison of tumor nodes (Figure 3E and F), the data suggest that CASC2 dysregulation is involved in NSCLC tumor formation and cisplatin tolerance *in vivo*.

### CASC Modulates Cisplatin Response in NSCLC by Targeting MiR-18a

Bioinformatic analysis by miRTarBase predicted the complementary binding potential between CASC2 and miR18a (Figure 4A). The assays validated that the Luciferase activity of miR-18a was significantly repressed by the wildtype CASC2 rather than the mutant form in NSCLC cells (Figure 4B, C, $p<0.05$). Compared to the oe-Vec treated cells, the expression of miR-18a declined both in NSCLC cells and cisplatin-resistant NSCLC cells transfected with oe-CASC2 (Figure 4D and E, $p<0.05$). Further verifying that CASC2 could

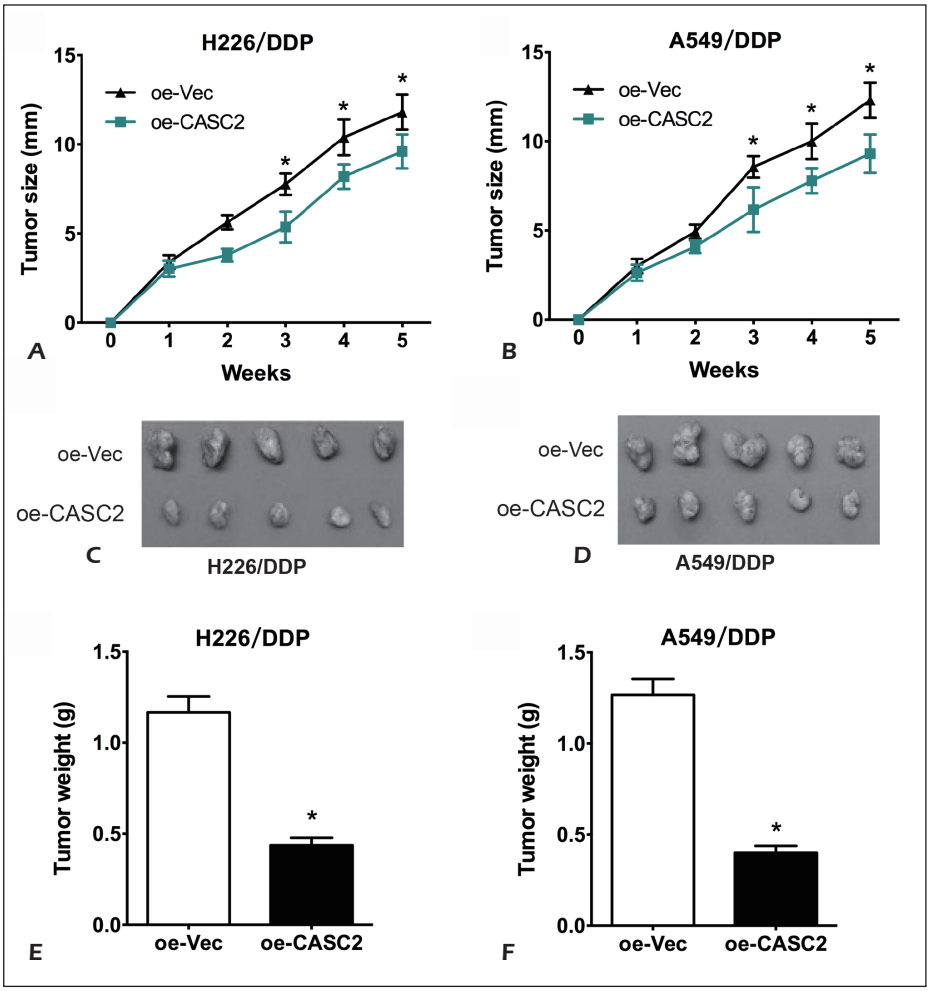


**Figure 3.** CASC2 boosts NSCLC tumor limitation and cisplatin response *in vivo*. **A-B**, Tumor size in CASC2 overexpression mice and the vector control ones. **C-D**, The macroscopic observation of tumor after sacrifice. **E-F**, Tumor weight after overexpression of CASC2. **$p<0.01$, *$p<0.05$.

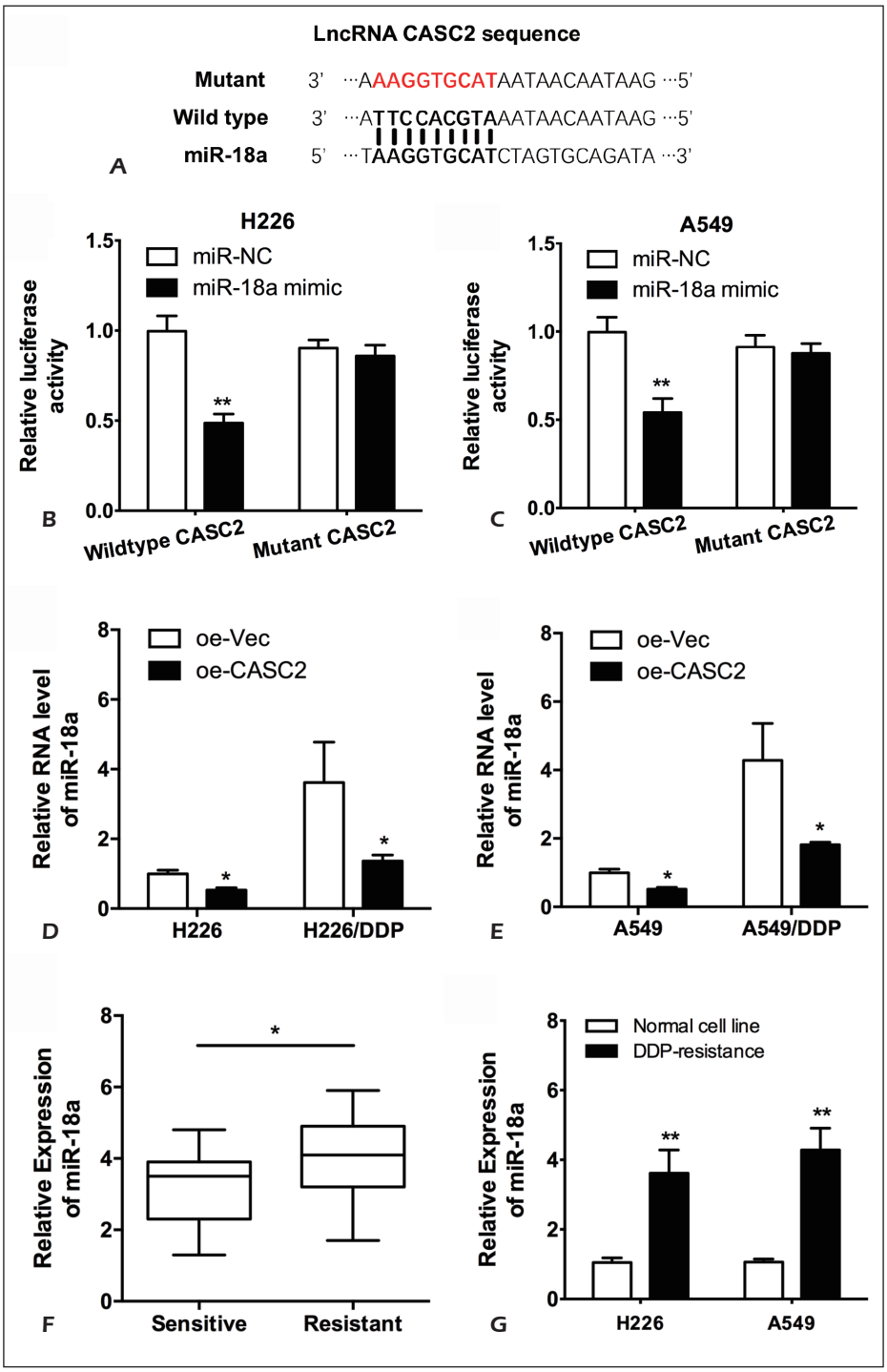


**Figure 4.** CASC modulates cisplatin response in NSCLC by targeting miR-18a. **A**, Bioinformatics tools reveal the complementary binding sites within CASC2 and miR-18a. B, C, Luciferase reporter assay confirmed the molecular binding between CASC2 and miR-18a in H226 (**B**) and A549 (**C**). **D**-**E**, qPCR showed the miR-18a expression in NSCLC cells transfected with CASC2-overexpressing vector or control in H226/DDP (**D**) and A549/DDP (**E**) cells. **F**, Expression of miR-18a in NSCLC tissues with different responses to cisplatin (Sensitive, n = 52; Resistant n = 33). **G**, MiR-18a expression in cisplatin -sensitive and -resistant NSCLC cells. $^{**}p<0.01$, $^{*}p<0.05$.

target miR18a, the expression of miR18a in cisplatin-resistant NSCLC tissues (Figure 4F, $p<0.05$) and cells (Figure 4G, $p<0.05$) was markedly higher than cisplatin-sensitive ones, showed by qPCR. Collectively, our data suggested that CASC2 interacted with miR-18a as a competing endogenous RNA to modulate cisplatin response in NSCLC.

### *MiR-18a Directly Targets IRF-2 in NSCLC Development*

Bioinformatic analysis by TargetScan predicted the complementary binding potential between miR18a and IRF-2 3'UTR (Figure 5A). Confirmed by a Luciferase reporter assay using wildtype or mutant IRF-2 3'UTR, the inhibition

of miR18a increased the Luciferase activity of IRF-2 wildtype reporter in NSCLC cells (Figure 5B and C). Furthermore, IRF-2 expression was strongly induced in both NSCLC and cisplatin-resistant NSCLC cells transfected with oe-CASC2, compared to those with oe-vec controls (Figure 5D and E, $p$ <0.05). Therefore, these results indicate that IRF-2 is an effector protein targeted by the CASC2/miR-18a axis in the cisplatin response of NSCLC.

### *EFL1 Activates CASC2 Expression in NSCLC Cells*

To further investigate the upstream regulatory mechanism of CASC2 in NSCLC, we predicted the transcription factors which may target to the promoter of CASC2 using the JASPAR (https://jaspar.genereg.net/) databases. The transcription factor ELF1, is recommended by JASPAR **(Supplemental Table II)**. The binding sites in the CASC2 sequence are shown in Figure 6A. ChIP assays ex-

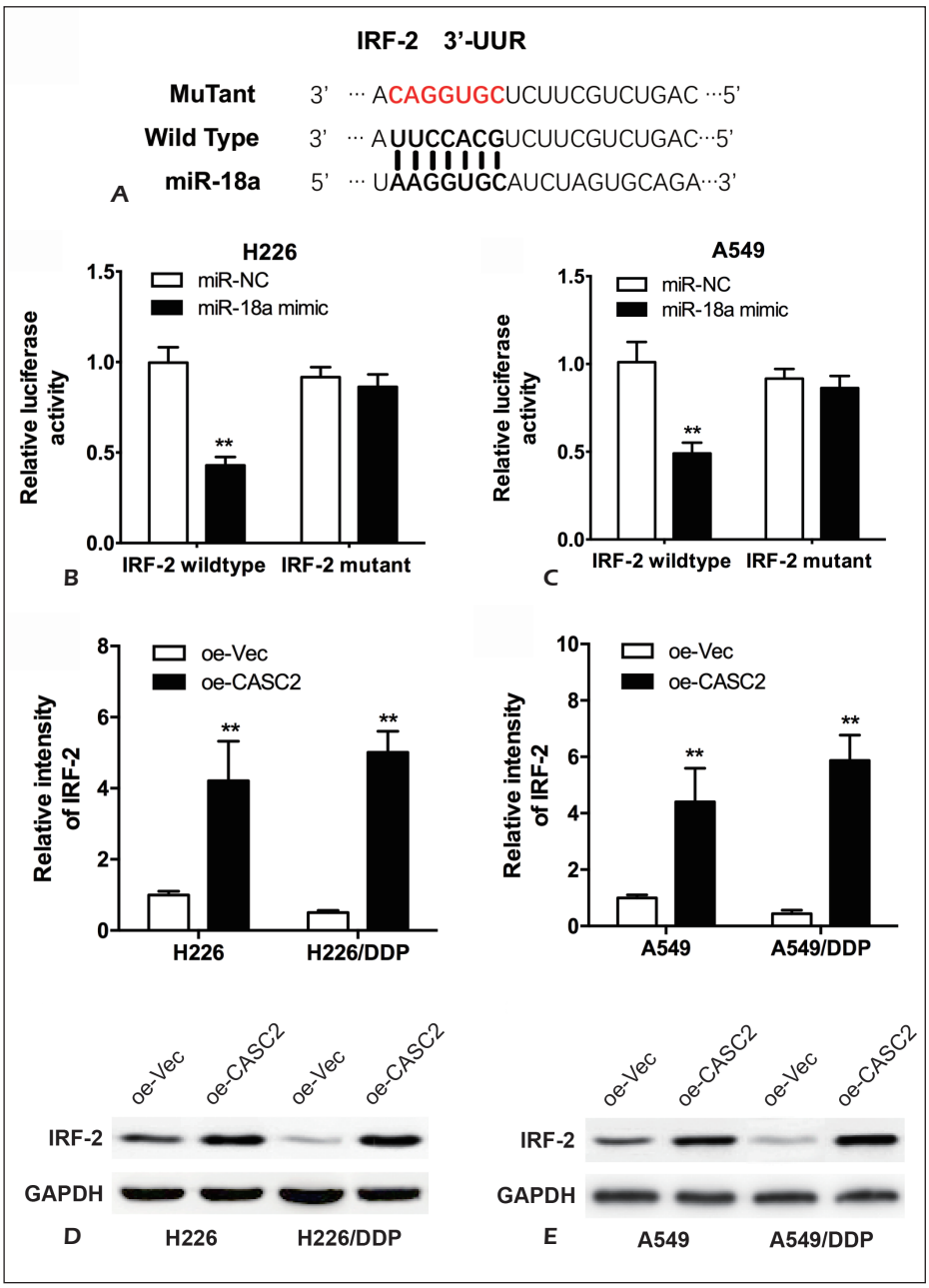


**Figure 5.** MiR-18a directly targets IRF-2 in NSCLC development. **A**, The schematic diagram presents the complementary binding sites within miR-18a and IRF-2 3'-UTR. **B-C**, Luciferase reporter assay validated the molecular binding between miR-18a and IRF-2 3'-UTR in H226 (**B**) and A549 (**C**). **D-E**, Western blot assay showed the IRF-2 protein expression in cisplatin -sensitive and -resistant NSCLC cells transfected with CASC2 overexpressing vector or control. **$p$<0.01, *$p$<0.05.

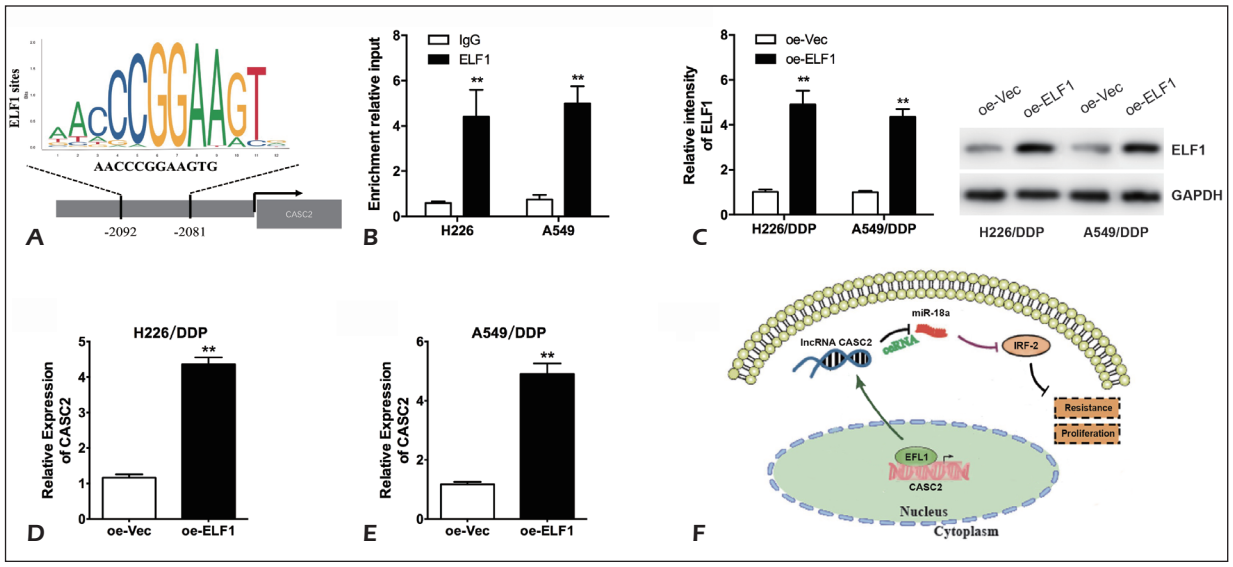


**Figure 6.** EFL1 activates CASC2 expression in NSCLC cells. **A**, JASPAR database reveals the complementary binding sites within the promoter region of CASC2. **B**, The targeting ability of ELF1 to the CASC2 promoter validated by ChIP assay and qPCR. **C**, Western blot assay showed the ELF1 protein expression after overexpression of ELF1. **D**-**E**, CASC2 expression was increased by lentiviral vectors of ELF1 in H226/DDP (**D**) and A549/DDP (**E**) cells. **F**, The regulatory network of ELF1 and CASC2 in NSCLC. $^{**}p<0.01$, $^{*}p<0.05$.

hibited that the CASC2 promoter was significantly pulled down by the antibody against ELF1 rather than the isotype antibody (Figure 6B). A synthesized lentiviral ELF1-overexpressing (oe-ELF1) vector was transfected into A549/DDP and H226/DDP cells to augment the ELF1 signal (Figure 6 C), leading to a notable increase of CASC2 levels (Figure 6D and E). Furthermore, the overexpression of ELF1 strongly induced CASC2 expression in A549/DDP and H226/DDP cells (Figure 6F). Therefore, these results indicate that ELF1 acts as a promotor for CASC2 in NSCLC.

## Discussion

Despite extensive research on the molecular mechanisms of drug resistance, there is still a lack of an effective intervention strategy. Studying the significant genetic characteristics of the chemoresistant phenotype in cancerous cells may lead to the discovery of more effective therapeutic targets. We investigated the molecular mechanism of CASC2/miR-18a regulatory axis in the maintenance of chemoresistance in NSCLC cells and explored potential new interventions in tumor progression. CASC2 acted as a tumor suppressor for various cancers[18-20], boosting the sensitivity to chemotherapeutics. Downregulated CASC2 was found to be significantly correlated with cisplatin-resistance in hepatocellular carcinoma[18]. Besides, miR-19a inhibition by CASC2 could overcome cisplatin resistance in gastric cancer[19]. Moreover, CASC2 accelerates the expression of mTOR, thus restraining protective autophagy and temozolomide resistance properties of glioma in a competitively binding way[20]. Although CASC2 has been shown to promote apoptosis and necrosis in a variety of cancer chemotherapy, little is known about the chemotherapy sensitizing mediated by CASC2 and its upstream transcription factor in NSCLC. In this study, we found that the aberrant decrease of CASC2 is strongly related to chemoresistance characteristics and may be an independent predictor of inferior overall survival of NSCLC (Figure 1). The overexpression of CASC2 in NSCLC cells inhibits the proliferation of *in vitro* cisplatin-resistant cell lines (Figure 2) and the growth of tumors *in vivo* (Figure 4). CASC2 can alter the biological behavior of tumor cells through interferon regulatory factor 2 (IRF2) *via* miRNA regulation. Moreover, ELF1 has been predicted and verified as the upstream regulatory factor of CASC2. Altogether, these results indicate that ELF1-activated CASC2 is involved in chemosensitivity maintaining and superior prognosis in NSCLC patients.

The transcription factor ELF1 can act as a tumor suppressor for various cancers, significantly inhibiting cell proliferation *in vitro* and tumor xenograft growth *in vivo*[21]. This repressing function is achieved by binding to sites in the regulatory regions of cell

cycle-control gene and apoptosis-related genes[21]. Investigators found that ELF1 can not only interfere with the oncogenic function of the transcription factors ETS Proto-Oncogene-1/Activator protein-1 but also target the genes that boost senescence in prostate cancer[22]; they increase the docetaxel sensitivity of prostate cancer[22]. However, few reports have exhibited the role of ELF1 in NSCLC chemoresistance and its underlying mechanism. Our data represent a pioneering research showing that ELF1 functions as a tumor-suppressing gene targeting to the promoter sites of CASC2, triggering the tumor response to cisplatin (Figure 6).

As competing endogenous RNAs (ceRNAs), sponging target miRNAs for post-transcriptional regulation is a functional property of lncRNAs[23]. In our study, we applied bioinformatics analysis to predict the interaction between the lncRNA CASC2 and miR-18a (Figure 4A). The Luciferase activity of the miR-18a was inhibited by wildtype lncRNA CASC2 but was not inhibited by the mutant form, thus demonstrating the direct-binding between them (Figure 4B, C). Furthermore, our results showed that lncRNA CASC2 overexpression suppressed the expression of miR-18a in tumor cells (Figure 5D). Therefore, since lncRNA CASC2 displayed a tumor-suppressive role and competitive repression of miR-18a, it is clear that miR-18a acts as a significant oncogene for tumor development. MiR-18a was proved to suppress Dicer function and enhance paclitaxel resistance in triple-negative breast cancer[24]. The negative regulation of CASC2 can mediate the boosting function of miR-18a on paclitaxel resistance of breast cancer cells[25]. In NSCLC, upregulated miR-18a was detected in peripheral blood samples of patients, being relative to shorter disease-free survival and overall survival[26]. Xiao et al[27] found that miR-18a could counteract phosphate and tension homology deleted on chromsome ten (PTEN) protein activity, leading to enhance cisplatin sensitivity of NSCLC. Our results represent a novel investigation of the critical role of the lncRNA CASC2-miR-18a-interaction in the chemoresistance of NSCLC.

IRF-2, as a transcription factor, can competitively repress the IRF-1 function[28]. Interferon β expression was decreased by IRF-2 *via* downregulating IRF-1[29]. Activated IRF-2 often inhibits malignant properties, especially chemoresistance, in various human cancers. The suppression of Fas-associated phosphatase 1 by IRF-2 accelerates apoptosis of colorectal cancer cells, promoting the therapeutic effect of oxaliplatin on targeted cells[30]. By targeting pegylated interferon-α2b (pIFN-α), IRF-2 is involved in the senescence of endothelial cells and angiogenesis inhibition, resulting in sensitive response to IFN-α combined with chemotherapy of melanoma cells[31]. In NSCLC, the activation of IRF-2 in cancer cells facilitates the epithelial-mesenchymal transition by strengthening N-cadherin expression[32]. MiR-18a can promote autophagy of NSCLC by inhibiting cell apoptosis through degradation of IRF-2[33]. IRF-2 is also the regulatory target of miR-450 in NSCLC[34]. Although IRF-2 is believed to be involved in tumor suppression and superior overall survival of patients with NSCLC, few studies have investigated the regulatory lncRNA upstream of IRF-2, particularly in chemotherapeutics response of NSCLC. Our study has shown that the lncRNA CASC2-miR-18a axis decreases the intracellular level of IRF-2 in NSCLC cells (Figure 5). Collectively, our results suggest that IRF-2 acts as a modulator of the malignant properties of NSCLC cells in the lncRNA-miRNA-interaction pathway.

## Conclusions

We have demonstrated that a low level of lncRNA CASC2 expression was associated with the advanced TMN stage, chemotherapy resistance, and inferior prognosis of NSCLC. LncRNA CASC2 can strongly inhibit the proliferation and growth of cisplatin-resistant NSCLC *in vitro* and *in vivo*. Furthermore, CASC2 was activated by ELF1 and mediated IRF2 expression *via* complementary binding to miR-18a in NSCLC chemoresistance (Figure 6F). These data suggest that the ELF1/CASC2/miR-18a/IRF2 axis may be a promising therapeutic target for NSCLC.

### Conflict of Interests

The authors declare that there are no conflicts of interest.

## References

1) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

2) Perez-Moreno P, Brambilla E, Thomas R, Soria JC. Squamous cell carcinoma of the lung: molecular subtypes and therapeutic opportunities. Clin Cancer Res 2012; 18: 2443-2451.

3) Torre LA, Siegel RL, Jemal A. Lung cancer statistics. Adv Exp Med Biol 2016; 893: 1-19.

4) Crino L, Cappuzzo F. Present and future treatment of advanced non-small cell lung cancer. Semin Oncol 2002; 29: 9-16.

5) Balata H, Fong KM, Hendriks LE, Lam S, Ostroff JS, Peled N, Wu N, Aggarwal C. Prevention and early detection for NSCLC: advances in thoracic oncology 2018. J Thorac Oncol 2019; 14: 1513-1527.

6) Gachechiladze M, Skarda J, Janikova M, Mgebrishvili G, Kharaishvili G, Kolek V, Grygarkova I, Klein J, Poprachova A, Arabuli M, Kolar Z. Overexpression of filamin-A protein is associated with aggressive phenotype and poor survival outcomes in NSCLC patients treated with platinum-based combination chemotherapy. Neoplasma 2016; 63: 274-281.

7) Azuma Y, Yokobori T, Mogi A, Altan B, Yajima T, Kosaka T, Onozato R, Yamaki E, Asao T, Nishiyama M, Kuwano H. SIRT6 expression is associated with poor prognosis and chemosensitivity in patients with non-small cell lung cancer. J Surg Oncol 2015; 112: 231-237.

8) Pirker R. Adjuvant chemotherapy in patients with completely resected non-small cell lung cancer. Transl Lung Cancer Res 2014; 3: 305-310.

9) Xie SY, Li G, Han C, Yu Y.Y., Li N. RKIP reduction enhances radioresistance by activating the Shh signaling pathway in non-small-cell lung cancer. Onco Targets Ther 2017; 10: 5605-5619.

10) Chen M, Liu P, Chen Y, Chen Z, Shen M, Liu X, Li X, Li A, Lin Y, Yang R, Ni W, Zhou X, Zhang L, Tian Y, Li J, Chen J. Long noncoding RNA FAM201A mediates the radiosensitivity of esophageal squamous cell cancer by regulating ATM and mTOR expression via miR-101. Front Genet 2018; 9: 611.

11) Mattick JS, Rinn JL. Discovery and annotation of long non-coding RNAs. Nat Struct Mol Biol 2015; 22: 5-7.

12) Akhade VS, Pal D, Kanduri C. Long noncoding RNA: genome organization and mechanism of action. Adv Exp Med Biol 2017; 1008: 47-74.

13) Cai Y, Dong ZY, Wang JY. LncRNA NNT-AS1 is a major mediator of cisplatin chemoresistance in non-small cell lung cancer through MAPK/Slug pathway. Eur Rev Med Pharmacol Sci 2018; 22: 4879-4887.

14) Gu J, Wang Y, Wang X, Zhou D, Shao C, Zhou M, He Z. Downregulation of lncRNA GAS5 confers tamoxifen resistance by activating miR-222 in breast cancer. Cancer Lett 2018; 434: 1-10.

15) Xu YH, Tu JR, Zhao TT, Xie SG, Tang SB. Overexpression of lncRNA EGFRAS1 is associated with a poor prognosis and promotes chemotherapy resistance in non-small cell lung cancer. Int J Oncol 2019; 54: 295-305.

16) Huang FX, Chen HJ, Zheng FX, Gao ZY, Sun PF, Peng Q, Liu Y, Deng X, Huang YH, Zhao C, Miao LJ. LncRNA BLACAT1 is involved in chemoresistance of non-small cell lung cancer cells by regulating autophagy. Int J Oncol 2019; 54: 339-347.

17) Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009; 45: 228-247.

18) Liu Z, Dang C, Xing E, Zhao M, Shi L, Sun J. Overexpression of CASC2 improves cisplatin sensitivity in hepatocellular carcinoma through sponging miR-222. DNA Cell Biol 2019; 38: 1366-1373.

19) Li Y, Lv S, Ning H, Li K, Zhou X, Xv H, Wen H. Down-regulation of CASC2 contributes to cisplatin resistance in gastric cancer by sponging miR-19a. Biomed Pharmacother 2018; 108: 1775-1782.

20) Jiang C, Shen F, Du J, Fang X, Li X, Su J, Wang X, Huang X, Liu Z. Upregulation of CASC2 sensitized glioma to temozolomide cytotoxicity through autophagy inhibition by sponging miR-193a-5p and regulating mTOR expression. Biomed Pharmacother 2018; 97: 844-850.

21) Ando M, Kawazu M, Ueno T, Koinuma D, Ando K, Koya J, Kataoka K, Yasuda T, Yamaguchi H, Fukumura K, Yamato A, Soda M, Sai E, Yamashita Y, Asakage T, Miyazaki Y, Kurokawa M, Miyazono K, Nimer SD, Yamasoba T, Mano H. Mutational landscape and antiproliferative functions of ELF transcription factors in human cancer. Cancer Res 2016; 76: 1814-1824.

22) Budka JA, Ferris MW, Capone MJ, Hollenhorst PC. Common ELF1 deletion in prostate cancer bolsters oncogenic ETS function, inhibits senescence and promotes docetaxel resistance. Genes Cancer 2018; 9: 198-214.

23) Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature 2013; 495: 384-388.

24) Sha LY, Zhang Y, Wang W, Sui X, Liu SK, Wang T, Zhang H. MiR-18a upregulation decreases Dicer expression and confers paclitaxel resistance in triple negative breast cancer. Eur Rev Med Pharmacol Sci 2016; 20: 2201-2208.

25) Zheng P, Dong L, Zhang B, Dai J, Zhang Y, Wang Y, Qin S. Long non-coding RNA CASC2 promotes paclitaxel resistance in breast cancer through regulation of miR-18a-5p/CDK19. Histochem Cell Biol 2019; 152: 281-291.

26) Xu X, Zhu S, Tao Z, Ye S. High circulating miR-18a, miR-20a, and miR-92a expression correlates with poor prognosis in patients with non-small cell lung cancer. Cancer Med 2018; 7: 21-31.

27) Xiao H, Liu Y, Liang P, Wang B, Tan H, Zhang Y, Gao X, Gao J. TP53TG1 enhances cisplatin sensitivity of non-small cell lung cancer cells through regulating miR-18a/PTEN axis. Cell Biosci 2018; 8: 23.

28) Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 1989; 58: 729-739.

29) Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y, Miyata T, Taniguchi T. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 1988; 54: 903-913.

30) Huang W, Bei L, Eklund EA. Inhibition of Fas associated phosphatase 1 (Fap1) facilitates apoptosis of colon cancer stem cells and enhances the effects of oxaliplatin. Oncotarget 2018; 9: 25891-25902.

31) Upreti M, Koonce NA, Hennings L, Chambers TC, Griffin RJ. Pegylated IFN-alpha sensitizes melanoma cells to chemotherapy and causes premature senescence in endothelial cells by IRF-1 mediated signaling. Cell Death Dis 2010; 1: e67.

32) Jin JJ, Liu YH, Si JM, Ni R, Wang J. Overexpression of miR-1290 contributes to cell proliferation and invasion of non small cell lung cancer by targeting interferon regulatory factor 2. Int J Biochem Cell Biol 2018; 95: 113-120.

33) Liang C, Zhang X, Wang HM, Liu XM, Zhang XJ, Zheng B, Qian GR, Ma ZL. MicroRNA-18a-5p functions as an oncogene by directly targeting IRF2 in lung cancer. Cell Death Dis 2017; 8: e2764.

34) Liu F, Yu X, Huang H, Chen X, Wang J, Zhang X, Lin Q. Upregulation of microRNA-450 inhibits the progression of lung cancer in vitro and in vivo by targeting interferon regulatory factor 2. Int J Mol Med 2016; 38: 283-290.