

Long non-coding RNA AK001796 contributes to cisplatin resistance of non-small cell lung cancer

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Abstract. Cisplatin (DDP)-based chemotherapy is the most widely used therapy for non-small cell lung cancer (NSCLC). However, the existence of chemoresistance has become a major limitation in its efficacy. Long non-coding RNAs (lncRNAs) have been shown to be involved in chemotherapy drug resistance. The aim of the present study was to investigate the biological role of lncRNA AK001796 in cisplatin-resistant NSCLC A549/DDP cells. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to monitor the differences in the expression of AK001796 in cisplatin-resistant (A549/DDP) cells and parental A549 cells. Cellular sensitivity to cisplatin and cell viability were examined using an MTT assay. Cell apoptosis and cell cycle distribution were measured using flow cytometry. The expression levels of cell cycle proteins cyclin C (CCNC), baculoviral IAP repeat containing 5 (BIRC5), cyclin-dependent kinase 1 (CDK1) and G2 and S phase-expressed 1 (GTSE1) were assessed using RT-qPCR and western blot analyses. It was found that the expression of AK001796 was increased in A549/DDP cells, compared with that in A549 cells. The knockdown of AK001796 by small interfering RNA reduced cellular cisplatin resistance and cell viability, and resulted in cell-cycle arrest, with a marked increase in the proportion of A549/DDP cells in the G0/G1 phase. By contrast, the knockdown of AK001796 increased the number of apoptotic cancer cells during cisplatin treatment. It was also shown that the knockdown of AK001796 positively induced the expression of cell apoptosis-associated factors, CCNC and BIRC5, and suppressed the expression of cell cycle-associated factors, CDK1 and GTSE5. Taken together, these findings indicated that lncRNA AK001796 increased the resistance of NSCLC

Correspondence to: Dr Yi-Jiang Chen, Department of Thoracic and Cardiovascular Surgery, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, Guangzhou 210029, P.R. China E-mail: yijiangchenkxy@126.com cells to cisplatin through regulating cell apoptosis and cell proliferation, and thus provides an attractive therapeutic target for NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated mortality in men and women worldwide (1). Non-small cell lung cancer (NSCLC) currently accounts for almost 85% of lung cancer cases (2). Patients with NSCLC are often diagnosed at an advanced stage and associated with a poor prognosis. Currently, platinum-based adjuvant chemotherapy, particularly cisplatin (DDP), is the first-line therapeutic strategy for the treatment of NSCLC. However, intrinsic and acquired chemoresistance to the drugs remains a major obstacle to the efficacy of chemotherapy and results in failure of chemotherapy (3). The mechanisms underlying this chemotherapy resistance remain to be fully elucidated. Thus, there is an urgent requirement to reveal the molecular mechanism underlying cisplatin resistance in NSCLC.

Long non-coding RNAs (lncRNAs) are eukaryote non-coding RNAs, which are >200 nucleotides in length and are without protein-coding capacity (4). Accumulating evidence has confirmed that lncRNAs are vital in regulating diverse biological processes, including cell proliferation, embryonic development, tissue differentiation and tumorigenesis (5-7). To date, lncRNAs have been functionally characterized in cancer chemoresistance. For example, the overexpression of lncRNA HOTAIR promotes platinum resistance in ovarian cancer (8). Similarly, IncRNA HOTTIP increases the gemcitabine resistance of pancreatic cancer through regulating homeobox A13 (9), and IncRNA UCA1 promotes the chemoresistance of bladder cancer cells via Wnt signaling (10). In addition, the IncRNA PVT1 has been shown to contribute to the development of multidrug resistance in gastric cancer (11). A previous study showed that a novel lncRNA, AK001796, was overexpressed in lung cancer tissues and cell lines, and was involved in resveratrol resistance (12). However, the role of AK001796 in cisplatin-resistant NSCLC remains to be fully elucidated.

In the present study, a cisplatin-resistant lung cancer cell was established from parental A549 cells. It was found that lncRNA AK001796 was markedly increased in the cisplatin-resistant A549/DDP cell line. As the role of lncRNA AK001796 in the resistance of NSCLC to cisplatin remains

Key words: non-small cell lung cancer, cisplatin, chemoresistance, long non-coding RNA, AK001796

to be fully elucidated, the present study focused on the function of lncRNA AK001796 on chemotherapy resistance in NSCLC through the knockdown of lncRNA AK001796 using small interfering (si) RNA in the A549/DDP cell line. It was found that the knockdown of lncRNA AK001796 significantly decreased the 50% inhibitory concentration (IC₅₀) of the A549/DDP cells, compared with that in the parental cells. It was also shown that lncRNA AK001796 mediated A549/DDP cell cisplatin resistance via modulating cell proliferation and apoptosis. The results also confirmed that the knockdown of lncRNA AK001796 induced cell apoptosis and proliferation through regulating the expression of cell cycle proteins cyclin C (CCNC), baculoviral IAP repeat containing 5 (BIRC5), cyclin-dependent kinase 1 (CDK1), and G2 and S phase-expressed 1 (GTSE1). Together, these findings demonstrated that lncRNA AK001796 offers potential as a therapeutic target to reverse cisplatin resistance in patients with NSCLC.

Materials and methods

Cell culture. The A549 human lung cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The cisplatin-resistant A549/DDP cell line was developed from parental A549 cells, which were subjected to exposure to increasing concentrations of cisplatin, and then cultured in medium containing 1.0 μ g/ml cisplatin. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C and 5% CO₂.

Cell transfection. Three specific siRNAs for lncRNA AK001796 (siRNA-1, siRNA-2 and siRNA-3) and a scramble non-specific siRNA (si-NS) were synthesized. The sequences were as follows: siRNA-1, forward 5'-GGUCACUACUGC UUUAUAATT-3' and reverse 5'-UUAUAAAGCAGUAGU GACCTT-3', siRNA-2, forward 5'-GGUGGCCUGUACCUA UAAUTT-3' and reverse 5'-AUUAUAGGUACAGGCCAC CTT-3', siRNA-3, forward 5'-GCCCAGAUUUAAGGG CUAUTT-3' and reverse 5'-AUAGCCCUUAAAUCUGGG CTT-3', si-NS, forward 5'-UUCUCCGAACGUGUCACG UTT-3' and reverse 5'-ACGUGACACGUUCGGAGAATT-3'. The A549 cells $(5x10^5)$ were seeded in 6-well plates with antibiotic-free medium and cultured for 24 h, and were then transfected with siRNA-1, siRNA-2, siRNA-3 and si-NS using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The efficiency of knockdown was detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis following transfection for 48 h.

RT-qPCR analysis. Total RNA was extracted from the cultured cells using TRIzol reagent (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μ g RNA using MMLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The total reaction system (final reaction volume, 25 μ l) for RT-qPCR included 2 μ l of the cDNA

template, 0.5 μ l of the upstream primer, 0.5 μ l of the downstream primer, 12.5 μ l of the SYBR-Green super mix (cat. no. RR036A; Takara Biotechnology, Co., Ltd., Dalian, China), 0.5 μ l of Takara Ex Taq HS (5 U/ μ l; cat. no. RR036A; Takara Biotechnology, Co., Ltd.), 0.5 µl of PrimeScript RT Enzyme Mix II (cat. no. RR036A; Takara Biotechnology, Co., Ltd.) and 8.5 μ l of RNase Free dH₂O. The qPCR procedure was performed under the following conditions: 95°C for 10 min and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The primers used were as follows: AK001796, sense 5'-GCCCAG AUUUAAGGGCUAUTT-3' and antisense 5'-AUAGCCCUU AAAUCUGGGCTT-3'; CCNC, sense 5'-GATTGCTGCTGC TACTTCTG-3' and antisense 5'-CATGTCCTGCACATA CTG-3'; BIRC5, sense 5'-TCATAGAGCTGCAGGGTGGAT TGT-3' and antisense 5'-AGTAGGGTCCACAGCAGTGTT TGA-3'; CDK1, sense 5'-TCCTAGTACTGCAATTCGGGA AA-3' and antisense 5'-CAGATCCATGGAAAGAAACTC AAAG-3'; GTSE1, sense 5'-AGAAGCACACGTGGCTGT AGGA-3' and antisense 5'-CAGGGCTCAGCTGGATCA GA-3'; GAPDH, sense 5'-TCCTAGTACTGCAATTCGGGA AA-3' and antisense 5'-CAGATCCATGGAAAGAAACTC AAAG-3'. All mRNA levels were analyzed using the comparative Cq method (13) and normalized to GAPDH.

Western blot analysis. The cells were washed with ice-cold PBS and lysed in lysis buffer containing 50 mM Tris (pH 8.0), 0.5% deoxycholic acid, 1% NP-40, 150 mM NaCl and 0.1% SDS. Protein concentrations were measured using a Takara Bicinchoninic Acid Protein Assay kit (cat. no. T9300A; Takara Biotechnology, Co., Ltd.), according to the manufacturer's instructions. Equal quantities $(20 \,\mu g)$ of the denatured proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with primary antibodies against CCNC (cat. no. AB38997; AbSci, Nanjing, China), BIRC5 (cat. no. MAB2365; Abnova, Taipei City, Taiwan), CDK1 (cat. no. PAB18458; Abnova), GTSE1 (cat. no. H00051512-A01; Abnova) in a dilution of 1:1,000 and with β -actin (cat. no. A1978; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a dilution of 1:10,000 at 4°C overnight. They were subsequently incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG (H+L)-HRP; cat. no. BS13278; Biogot Technology Co., Ltd, Nanjing, China) in a dilution of 1:10,000 for 1 h at room temperature. The signals were visualized using the ECL Western Blotting kit (BioVision, Inc., Milpitas, CA, USA).

MTT assay. To monitor the IC₅₀, an MTT assay was performed. The cells were seeded in 96-well plates $(5x10^3/\text{well})$, incubated at 37°C for 24 h, and then treated with different concentrations $(0, 1, 5, 10, 15, 20, 25, 30, 35 \text{ and } 40 \ \mu g/\text{ml})$ of cisplatin for 48 h in a humidified atmosphere of 5% CO₂ at 37°C. At the end of the experiment, 0.5 mg/ml MTT was added. Following incubation for 4 h, the culture medium was replaced with 150 μ l DMSO and vortexed for 8 min. An automatic microplate reader was used to measure the absorbance at 490 nm. The cell proliferation rate was determined as follows: Cell proliferation=absorbance of drug treatment panel/absorbance of control panel x 100%. Cell viability was detected on days 0, 1, 2, 3 and 4 with or without cisplatin treatment.





Figure 1. Expression level of AK001796 in non-small cell lung cancer cells. (A) RT-qPCR analysis of expression levels of AK001796 in A549 and A549/DDP cells. ***P<0.001, vs. A549 cells. (B) MTT assay of the IC₅₀ values of A549 and A549/DDP cells to cisplatin. ***P<0.001, vs. A549 cells. (C) RT-qPCR analysis of the expression levels of AK001796 following knockdown of AK001796 in A549/DDP cells. *P<0.05 and *P<0.01, vs. Con. (D) MTT assay of the IC₅₀ values of si-AK001796 A549/DDP cells to cisplatin. *P<0.05 and *P<0.01, vs. Con. (D) MTT assay of the IC₅₀ values of si-AK001796 A549/DDP cells to cisplatin. *P<0.05 and *P<0.01, vs. Con. (D) MTT assay of the IC₅₀ values of si-AK001796 A549/DDP cells to cisplatin. *P<0.05, vs. si-NS groups. Data are presented as the mean ± standard deviation. A549/DDP, cisplatin-resistant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; si-NS, non-specific siRNA; IC₅₀, 50% inhibitory concentration; Con, control.

Cell apoptosis analysis. The cells were cultured in 6-well plates with a final concentration of 1×10^5 /ml, and then treated with 0.25% trypsin and fixed in 70% ice-cold ethanol. A cell suspension was prepared using DMEM with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and washed twice with PBS. The cells were then double stained with 5 μ l Annexin V-FITC and 1 μ l propidium iodide (PI; 50 μ g/ml). Following incubation in the dark for 15 min, the cells were analyzed using a flow cytometer equipped with CellQuest Pro 5.1 software (BD Bioscience, San Jose, CA, USA).

Cell cycle analysis. The cells for cell cycle analysis were prepared with a final concentration of $2x10^5$ /ml, treated with RNase A and fixed with 70% ice-cold ethanol overnight. The cells were then washed twice in PBS and stained with 50 mg/ml PI, 100 μ g/ml RNase A and 0.2% Triton X-100 for 30 min at 4°C in the dark. The percentages of the populations of cells in the G0/G1, S and G2/M phases were calculated using a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis. Data are shown as the mean ± standard deviation. Student's t-test was performed using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

IncRNA AK001796 mediates cisplatin-resistace in A549/DDP cells. To investigate the potential role of lncRNA AK001796 in the resistance of human lung cancer cells to cisplatin, RT-qPCR analysis was performed to quantify the expression of lncRNA AK001796 in the cisplatin-resistant A549/DDP cell line and parental A549 cell line. The mRNA level of lncRNA AK001796 was almost 3-fold higher in the A549/DDP cells, compared with that in the A549 cells (P<0.001; Fig. 1A). Subsequently, the IC_{50} of A549/DDP cells and A549 cells were determined. As shown in Fig. 1B, the IC_{50} values of the A549/DDP and A549 cells were 30.2 and 7.4 μ g/ml, respectively (P<0.001). The A549/DDP cells were then transfected with lncRNA AK001796 siRNA (siRNA-1, siRNA-2 and si-RNA-3, respectively) and the negative control si-NS. Following transfection for 48 h, the RT-qPCR analysis revealed that the expression of IncRNA AK001796 was differentially decreased in the three siRNA-transfected in A549/DDP cells (P<0.05, P<0.01 and P<0.01, respectively; Fig. 1C). siRNA-3 was most efficient at suppressing the expression of lncRNA AK001796, therefore, the siRNA-3 A549/DDP cells were selected for subsequent experiments. It was also found that the knockdown of lncRNA AK001796 significantly reduced the IC_{50} of the A549/DDP cells (P<0.05; Fig. 1D). These data suggested that lncRNA AK001796 may be associated with cisplatin resistance in lung cancer cell.



Figure 2. Knockdown of AK001796 promotes A549/DDP cell apoptosis. Flow cytometry of apoptosis in si-AK001796 A549/DDP cells during treatment of cisplatin (0, 1 and $2 \mu g/ml$). **P<0.01 and ***P<0.001. vs. si-NS groups. Data are presented as the mean \pm standard deviation. A549/DDP, cisplatin-resistant; siRNA, small interfering RNA; si-NS, non-specific siRNA.



Figure 3. Knockdown of AK001796 reduces A549/DDP cell proliferation. (A) MTT assay of the proliferation of si-NS- and siRNA-transfected AK001796 A549/DDP cells. (B) Flow cytometric analysis of cell cycle distribution in si-NS- and siRNA-transfected AK001796 A549/DDP cells. *P<0.05, **P<0.01 and ***P<0.001. vs. si-NS groups. Data are presented as the mean \pm standard deviation. A549/DDP, cisplatin-resistant; siRNA, small interfering RNA; si-NS, non-specific siRNA; OD, optical density.

lncRNA AK001796 knockdown promotes A549/DDP cell apoptosis. As the knockdown of lncRNA AK001796 appeared to downregulate the chemosensitivity of A549/DDP cells to cisplatin, the present study examined the effects of lncRNA AK001796 on the apoptosis of A549/DDP cells. The flow cytometric analysis showed that the apoptotic rate of A549/DDP cells transfected with siRNA-3 was significantly increased, compared with the control cells transfected with si-NS, when the cells were treated with increasing concentrations of cisplatin (0, 1 and 2 μ g/ml), as shown in Fig. 2A and B. These results demonstrated that lncRNA AK001796 promoted the resistance of A549/DDP cells to cisplatin via regulating cell apoptosis.

lncRNA AK001796 knockdown affects A549/DDP cell proliferation and cell cycle. To further investigate the biological role and mechanism of lncRNA AK001796 on the cisplatin resistance of A549/DDP cells, the present study examined the effects of lncRNA AK001796 on the proliferation of A549/DDP cells. As shown in the MTT assay, cell viability was markedly reduced in the A549/DDP cells transfected with siRNA-3, compared with the control cells (Fig. 3A). Cell cycle progression in the A549/DDP cells was then monitored using a flow cytometric assay. It was found that the percentage of siRNA-3-transfected A549/DDP cells in the G0/G1 phase was significantly increased (38.21 to 58.13%; P<0.01) and in the S phase it was significantly decreased (37.72 to 23.40%; P<0.05), when compared with the control cells (Fig. 3B). These results demonstrated that lncRNA AK001796 may enhance the cisplatin resistance of A549/DDP cells via regulating cell proliferation.

lncRNA AK001796 regulates the expression of CCNC, BIRC5, CDK1 and GTSE1. To better understand the role of lncRNA AK001796 in mediating the resistance of A549/DDP cells to cisplatin through regulating cell apoptosis and cell proliferation, the expression levels of several apoptosis-associated proteins (BIRC5 and GTSE1) and proliferation-associated proteins





Figure 4. Knockdown of AK001796 regulates the expression of CCNC, BIRC5, CDK1 and GTSE1. (A) Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression levels of CCNC, BIRC5, CDK1 and GTSE1 in si-AK001796 A549/DDP cells. (B) Western blot analysis of protein expression levels of CCNC, BIRC5, CDK1 and GTSE1 in si-AK001796 A549/DDP cells. (B) Western blot analysis of protein expression levels of CCNC, BIRC5, CDK1 and GTSE1 in si-AK001796 A549/DDP cells. (B) Western blot analysis of protein expression levels of CCNC, BIRC5, CDK1 and GTSE1 in si-AK001796 A549/DDP cells. **P<0.01 and ***P<0.001, vs. si-NS groups. Data are presented as the mean ± standard deviation. A549/DDP, cisplatin-resistant; siRNA, small interfering RNA; si-NS, non-specific siRNA; CCNC, cell cycle proteins cyclin C; BIRC5, baculoviral IAP repeat containing 5; CDK1, cyclin-dependent kinase 1; GTSE1, G2 and S phase-expressed 1.8.

(CCNC and CDK1) were examined using RT-qPCR analysis. The mRNA levels of CCNC and BIRC5 in A549/DDP cells transfected with si lncRNA AK001796 were significantly higher, compared with those in the control (P<0.01 and P<0.001, respectively; Fig. 4A). By contrast, the mRNA levels of CDK-1 and GTSE1 were significantly decreased in A549/DDP cells transfected with si lncRNA AK001796, compared with the control (P<0.01; Fig. 4A). The results of the western blot analysis revealed that the protein levels of CCNC and BIRC5 were increased in the si lncRNA-transfected AK001796 A549/DDP cells, compared with the control cells, whereas the protein levels of CDK-1 and GTSE1 were decreased in these A549/DDP cells (Fig. 4B). These data indicated that lncRNA AK001796 contributed to the resistance of A549/DDP cells to cisplatin via regulating the expression of CCNC, BIRC5, CDK1 and GTSE1.

Discussion

Human lung cancer is one of the most life-threatening forms of cancer in humans worldwide (14). The majority of cases of lung cancer are NSCLC, for which chemotherapy is one of the most effective treatments (15). Cisplatin, which is the most commonly used chemotherapeutic drug in NSCLC, can induce cell apoptosis by activating the DNA damage response (16-18). However, acquired cisplatin resistance is the primary obstacle to clinical chemotherapy (19,20). Therefore, it is imperative to investigate the molecular mechanism involved in chemotherapy resistance.

Accumulating evidence has demonstrated that the misregulation of lncRNA results in tumorigenesis in several types of cancer, including gastric cancer and human lung adenocarcinoma (16,21,22). In addition, lncRNAs have been shown to be important in the regulation of cancer chemoresistance (23-25). lncRNA AK001796 has been shown to be significant in the regulation of lung cancer carcinogenesis and is involved in the anticancer effects of resceratrol (12). The present study offers the first insight, to the best of our knowledge, into the function of lncRNA AK001796 on cisplatin resistance. It was found that the expression level of lncRNA AK001796 was increased in cisplatin-resistant A549/DDP cells, compared with parental A549 cells. The decreased expression of lncRNA AK001796 appeared to reverse the chemoresistance of A549/DDP cells to cisplatin. In addition, the results of the MTT assay and flow cytometry revealed that lncRNA AK001796 knockdown significantly decreased the survival rate and enhanced the percentage of apoptotic cells in the A549/DDP cell line. Additionally, lncRNA AK001796 knockdown contributed to G0/G1 stage arrest of the A549/DDP cells. Further investigations are required to detect these effects of lncRNA AK001796 *in vivo*.

CCNC and BIRC5 have been reported to be associated with the anti-apoptotic response and upregulated by cisplatin-treated cancer cells (26,27). CDK1 and GTSE1, as cell cycle regulators, are downregulated in cisplatin-treated cancer cells (28). In addition, microarray results reported in a previous study indicated that CCNC and BIRC5 were increased following lncRNA AK001796 knockdown in lung cancer cells, whereas CDK1 and GTSE1 were found to be downregulated in lung cancer cells with lncRNA AK001796 knockdown (12). Consistent with this previous study, the present study confirmed that knockdown of lncRNA AK001796 in the A549/DDP cells increased the mRNA and protein levels of CCNC and BIRC5. By contrast, knockdown of lncRNA AK001796 in the A549/DDP cells resulted in decreased mRNA and protein levels of CDK1 and GTSE1. Therefore, it was hypothesized that lncRNA AK001796 elevated cisplatin resistance by targeting CCNC, BIRC5, CDK1 and GTSE1, further modulating cell apoptosis and cell proliferation.

In conclusion, the results of the present study demonstrated that lncRNA AK001796, which was significantly overexpressed in A549/DDP cell lines, was vital in NSCLC cisplatin chemoresistance. The results also showed that lncRNA AK001796 exerted its effects in NSCLC, at least in part, as a cell apoptosis and cell proliferation regulator by mediating the expression of cell apoptosis-associated proteins (CCNC and BIRC5) and cell cycle-associated proteins (CDK1 and GTSE1). Therefore, lncRNA AK001796 is required for the maintenance of cisplatin resistance in lung cancer cells, and provides a potential therapeutic target for patients with lung cancer who are unresponsive to cisplatin.

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