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Long-non-coding RNA AK001058: A genetic biomarker in cisplatin sensitivity of non-small-cell lung carcinoma

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ABSTRACT

Previous evidences have shown that lncRNA AK001058 serves as an oncogene. This study aims to elucidate the expression characteristic of AK001058 in NSCLC samples, and its potential influence on the malignant progression and cisplatin resistance of NSCLC. Relative levels of AK001058 and IGF2 in NSCLC and non-tumoral tissues were detected by qRT-PCR. Proliferation inhibition rate and migratory rate in DDP-induced SPC-A1 and A549 cells were examined by CCK-8 and Transwell assay, respectively. Subsequently, DDP-resistant SPC-A1 and A549 cell lines were generated, and the role of AK001058 in affecting their cell phenotypes was determined. Using dual-luciferase reporter assay, the binding relationship between AK001058 and IGF2 was verified. Their co-regulation on DDP-resistant NSCLC cells was finally explored *via* rescue experiments. AK001058 was upregulated in NSCLC samples. The proliferative rate was dose-dependently and time-dependently declined in DDP-induced SPC-A1 and A549 cells. Cisplatin induction upregulated AK001058 in NSCLC cells, and attenuated migratory potential. Transfection of sh-AK001058 reduced proliferative and migratory rates in SPC-A1/DDP and A549/DDP cells. IGF2 was the downstream target binding AK001058, which was lowly expressed in NSCLC samples. AK001058 upregulation in NSCLC reduces cisplatin sensitivity and promotes malignant progression by negatively regulating IGF2, leading to cisplatin resistance.

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Introduction

Lung carcinoma is a highly lethal malignant tumor, ranking first place in both cancer incidence and mortality. Its 5-year survival is only 15.6%. Each year, 1.4 million people die of lung carcinoma, accounting for 18% of cancer deaths (1, 2). According to the histological classification, 80-85% of lung carcinoma cases belong to non-small-cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma (3, 4). NSCLC has been well concerned because of its extremely poor prognosis (5, 6). In China, NSCLC is the leading cause of cancer death. Nevertheless, effective health management for diagnosis of NSCLC in the early phase and its treatment is immature (7, 8).

Platinum drugs are widely applied chemotherapy agents after the development of alkylating agents (9, 10). Chemotherapy resistance has become the most difficult obstacle that restricts anti-cancer efficacy (11). It is of significance to overcome primary drug resistance or cross-resistance, thus enhancing the sensitivity of cancer cells to platinum drugs (12, 13). At present, long non-coding RNAs (lncRNAs) have made great achievements in preventing and reversing chemotherapy resistance (14-16). In the meantime, abnormally expressed lncRNAs are involved in the malignant phenotypes of cancer cells (14, 17). Previous studies have shown that lncRNA AK001058 participates in the tumor progression of colorectal carcinoma and gastric cancer (18, 19). Its potential role in NSCLC remains unclear.

Bioinformatic prediction revealed that IGF2 may be a potential target of AK001058. This study first detected differential levels of AK001058 and IGF2 in clinical samples of NSCLC. Their regulatory effects on malignant phenotypes of NSCLC and cisplatin resistance were mainly explored.

Materials and Methods

NSCLC patients and samples

Forty pairs of NSCLC and adjacent normal tissues were collected from surgery, puncture biopsy or bronchoscopy biopsy. Adjacent tissues were at least 5 cm away from tumor lesions. None of the recruited patients had preoperative chemotherapy or radiotherapy. Tumor node metastasis (TNM) staging of NSCLC was defined by Union for International Cancer Control (UICC) criteria. Follow-up through telephone and outpatient review was conducted after discharge, including physical conditions, clinical symptoms and signs, and imaging examinations. This study was approved by the Ethical Committee of Zhongshan People's Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

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Cell culture

NSCLC cell lines (A549, H1299, PC-9, H358 and SPC-A1) and the human bronchial epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) at 37°C with 5% CO_2.

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in a 6-well plate and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 48, 72 and 96 h, the optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell assay

The cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS were respectively added on the top and bottom of a Transwell insert, and cultured for 48 h. Cells were migrated from the top to the bottom, which was induced with methanol for 15 min, 0.2% crystal violet for 20 min and captured using a microscope. Five random fields per sample were selected for capturing and counting cells.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Japan), followed by qRT-PCR using StepOne Plus Real-time PCR (Applied Biosystems, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. AK001058: Forward: 5'-GCTTGCTGTACAAGAGGGGA-3', Reverse: 5'-CTGCGATGGCCCTCTCTATT-3'; IGF2: Forward: 5'-TCTGCCCCGTCGCACATTC-3', Reverse: 5'-GATTCCCATTGGTGTCTGGAAGC-3'; GAPDH: Forward: 5'-CCTGGCACCCAGCACAAT-3', Reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Dual-luciferase reporter assay

Wild-type and mutant-type AK001058 vectors were synthesized based on the predicted binding sites, which were co-transfected to cells with pcDNA-IGF2 or pcDNA-NC, respectively. Luciferase activity (Promega, Madison, WI, USA) was measured at 48 h in a standard method.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. Potential influences of AK001058 and IGF2 on clinical features of NSCLC were analyzed by Chi-square test. Kaplan-Meier survival curves were depicted, followed by a log-rank test for comparing differences between curves. Pearson correlation test was conducted to assess the relationship between expressions of AK001058 and IGF2 in NSCLC tissues. $P<0.05$ was considered as statistically significant.

Results

AK001058 was highly expressed in NSCLC

In comparison to non-tumoral tissues, AK001058 was highly expressed in NSCLC tissues (Figure 1A). *In vitro,* the level of AK001058 was consistently higher in NSCLC cell lines than that in the bronchial epithelial cell line (Figure 1B). It is concluded that AK001058 was an oncogene involved in NSCLC progression.

AK001058 reduced cisplatin sensitivity of NSCLC

To clarify the cisplatin sensitivity of NSCLC cells, they were induced with DMSO (negative control), 2 μM, 4 μM, 8 μM and 16 μM cisplatin for 24 h. CCK-8 assay showed that viability dose-dependently decreased in DDP-induced SPC-A1 and A549 cells (Figures 2A and 2B). In particular, A549 and SPC-A1 cells presented the most pronounced

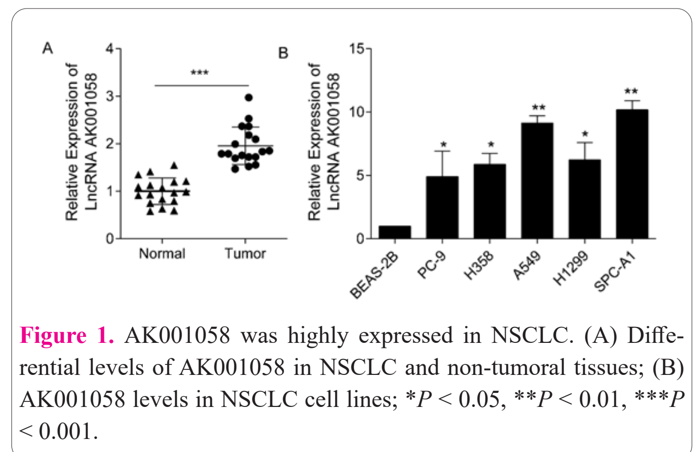

Figure 1. AK001058 was highly expressed in NSCLC. (A) Differential levels of AK001058 in NSCLC and non-tumoral tissues; (B) AK001058 levels in NSCLC cell lines; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

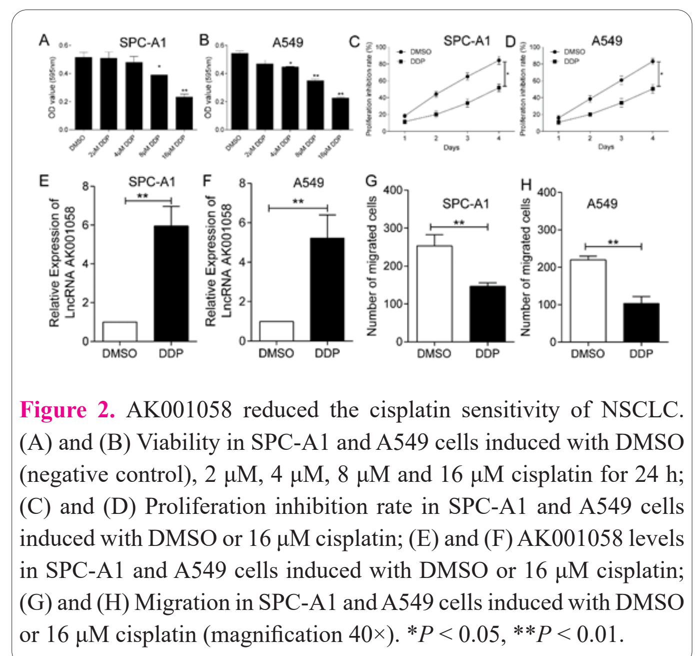

Figure 2. AK001058 reduced the cisplatin sensitivity of NSCLC. (A) and (B) Viability in SPC-A1 and A549 cells induced with DMSO (negative control), 2 μM, 4 μM, 8 μM and 16 μM cisplatin for 24 h; (C) and (D) Proliferation inhibition rate in SPC-A1 and A549 cells induced with DMSO or 16 μM cisplatin; (E) and (F) AK001058 levels in SPC-A1 and A549 cells induced with DMSO or 16 μM cisplatin; (G) and (H) Migration in SPC-A1 and A549 cells induced with DMSO or 16 μM cisplatin (magnification 40×). *$P < 0.05$, **$P < 0.01$.

resistance to the induction of 16 μM cisplatin. Besides, the proliferation inhibition rate was markedly inhibited in NSCLC cells with the prolongation of cisplatin induction (Figure 2C and 2D). Compared with DMSO-induced cells, the AK001058 level was higher and the migratory rate was much lower in DDP-induced NSCLC cells (Figure 2E-2H).

Knockdown of AK001058 inhibited proliferative and migratory potentials in NSCLC

DDP-resistant SPC-A1 and A549 cell lines were generated. Transfection of sh-AK001058 effectively downregulated AK001058 in SPC-A1/DDP and A549/DDP cells, indicating the effective transfection (Figure 3A and 3B). Knockdown of AK001058 reduced viability and migratory cell number in DDP-resistant NSCLC cells (Figure 3C- 3F). It is suggested that AK001058 stimulated proliferative and migratory potentials in DDP-resistant NSCLC cells.

IGF2 was a direct target of AK001058

In DDP-resistant SPC-A1 and A549 cells transfected with sh-AK001058, mRNA levels of IGF2 were upregulated (Figure 4A). IGF2 was lowly expressed in NSCLC tissues (Figure 4B). As expected, IGF2 was downregulated in NSCLC cell lines (Figure 4C).

Co-regulation of AK001058 and IGF2 on DDP-resistant NSCLC cells

Dual-luciferase reporter assay uncovered that overexpression of IGF2 could decline luciferase activity in the wild-type AK001058 vector. However, it had no impact on luciferase activity in the mutant-type one (Figure 5A-5C). The lower level of IGF2 was detected in SPC-A1/DDP and A549/DDP cells with co-knockdown of AK001058 and IGF2 than those with sole knockdown of AK001058 (Figure 5D). Moreover, co-knockdown of AK001058 and IGF2 enhanced viability and migratory cell number in DDP-resistant NSCLC cells with sole knockdown of AK001058 (Figure 5E-5H).

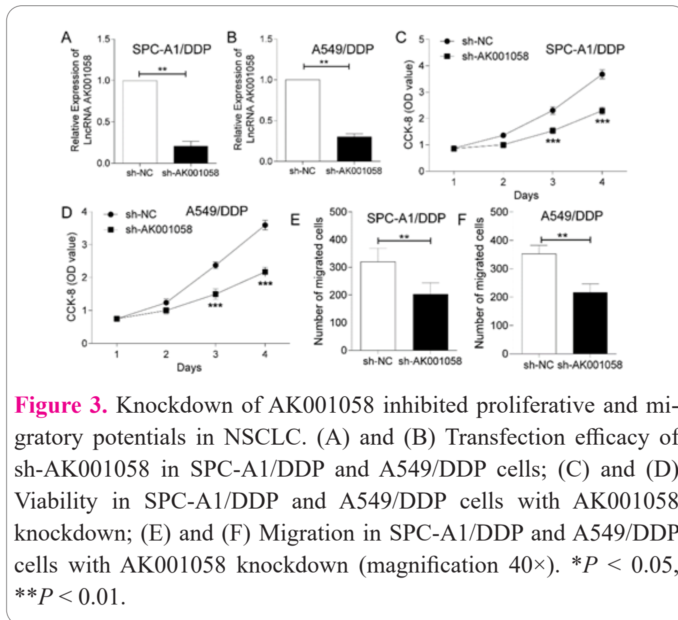

Figure 3. Knockdown of AK001058 inhibited proliferative and migratory potentials in NSCLC. (A) and (B) Transfection efficacy of sh-AK001058 in SPC-A1/DDP and A549/DDP cells; (C) and (D) Viability in SPC-A1/DDP and A549/DDP cells with AK001058 knockdown; (E) and (F) Migration in SPC-A1/DDP and A549/DDP cells with AK001058 knockdown (magnification 40×). *P < 0.05, **P < 0.01.

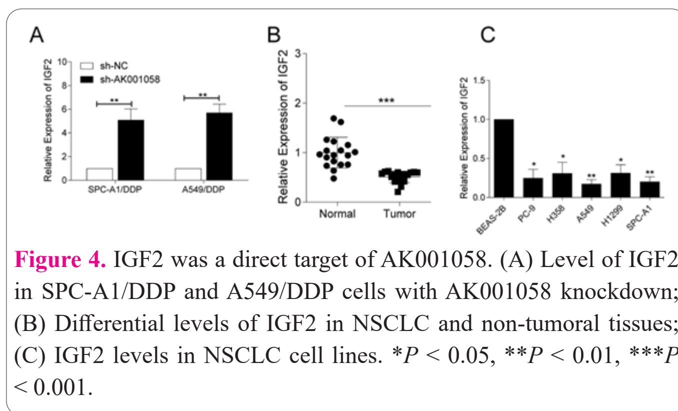

Figure 4. IGF2 was a direct target of AK001058. (A) Level of IGF2 in SPC-A1/DDP and A549/DDP cells with AK001058 knockdown; (B) Differential levels of IGF2 in NSCLC and non-tumoral tissues; (C) IGF2 levels in NSCLC cell lines. *P < 0.05, **P < 0.01, ***P < 0.001.

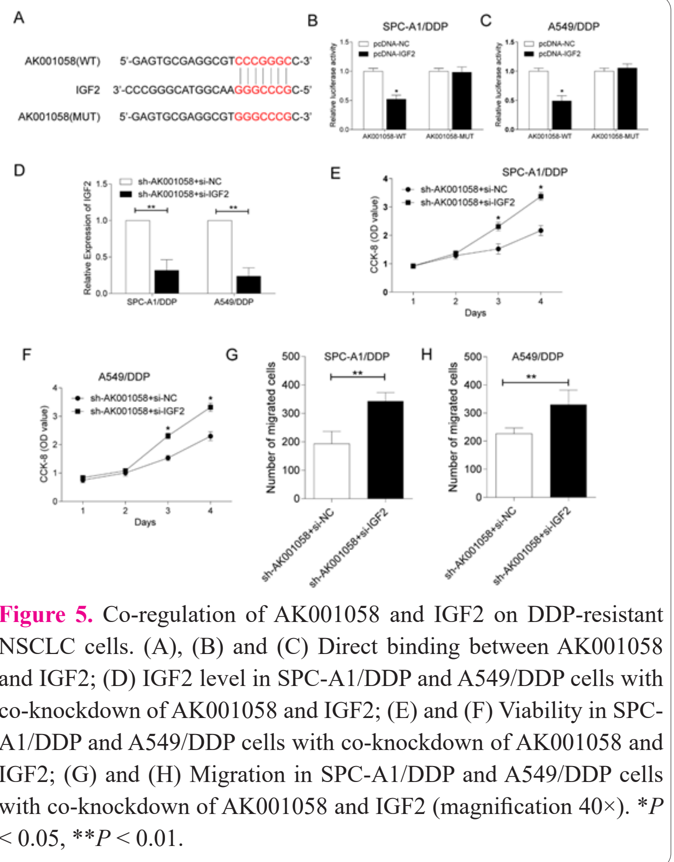

Figure 5. Co-regulation of AK001058 and IGF2 on DDP-resistant NSCLC cells. (A), (B) and (C) Direct binding between AK001058 and IGF2; (D) IGF2 level in SPC-A1/DDP and A549/DDP cells with co-knockdown of AK001058 and IGF2; (E) and (F) Viability in SPC-A1/DDP and A549/DDP cells with co-knockdown of AK001058 and IGF2; (G) and (H) Migration in SPC-A1/DDP and A549/DDP cells with co-knockdown of AK001058 and IGF2 (magnification 40×). *P < 0.05, **P < 0.01.

Discussion

Both incidence and mortality of NSCLC are the top of malignant tumors (1, 2). Radical resection is not suitable for most NSCLC patients because they have local progression or distant metastases at the first time of diagnosis (4, 5). At present, a combined chemotherapy treatment approach that includes cisplatin and another agent is the first-line strategy preferred to driver-negative advanced NSCLC (9, 10). Cisplatin has been used as the first-line anti-cancer therapy for decades. It not only brings survival benefits to advanced NSCLC patients but also contributes to improving the prognosis of surgical patients as an adjuvant therapy drug (11). Our results revealed that cell viabilities of A549 and SPC-A1 cells were dose-dependently and time-dependently reduced by cisplatin induction. In addition, cisplatin intervention markedly inhibited proliferative and migratory potentials in NSCLC.

It is well known that intrinsic or acquired resistance severely limits the clinical use of cisplatin, and effective strategies for reversing cisplatin resistance are lacking (12, 13). The latest evidences have shown that lncRNAs are able to regulate cisplatin sensitivity in NSCLC *via* regulating target gene expressions. We detected upregulated

AK001058 and downregulated IGF2 in NSCLC tissues, and they both had close relations to tumor staging, metastasis and prognosis of NSCLC. Notably, the knockdown of AK001058 reduced proliferative and migratory rates in SPC-A1/DDP and A549/DDP cells, suggesting that AK001058 decreased cisplatin sensitivity in NSCLC.

As bioinformatic analysis uncovered, an IGF2 binding site was predicted in AK001058 sequences. Furthermore, the AK001058 vector with mutant IGF2 binding site failed to enrich IGF2, thus confirming the direct binding between AK001058 and IGF2. Missense mutations of IGF2 have been detected in many kinds of malignant tumors, which are of significance in tumor growth, invasiveness and metastasis. Importantly, IGF2 was able to reverse the role of AK001058 in regulating malignant phenotypes of DDP-resistant NSCLC cells. Taken together, AK001058 stimulated NSCLC to proliferate and migrate as an oncogene, and it also reduced cisplatin sensitivity by negatively regulating IGF2.

Conclusions

AK001058 is upregulated in NSCLC cases, it stimulates the malignant progression of NSCLC and reduces cisplatin sensitivity through negatively regulating IGF2.

Conflict of Interest

The authors declared no conflict of interest.

Acknowledgments

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