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Effect of lncRNA-BLACAT1 on drug resistance of non-small cell lung cancer cells in DDP chemotherapy by regulating cyclin D1 expression

Z.-S. JU[1], B. SUN[2], D. BAO[3], X.-F. ZHANG[4]

[1]Department of Internal Medicine-Oncology, Affiliated Zhongshan Hospital of Dalian University, Dalian, China
[2]Department of Thoracic Surgery, Jining No. 1 People's Hospital, Jining, China
[3]Department of Obstetrics and Gynecology, PLA Rocket Force Characteristic Medical Center, Beijing, China
[4]Department of Thoracic Surgery, The Second Hospital of Dalian Medical University, Dalian, China

Zaishuang Ju and Bo Sun contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding ribonucleic acid (lncRNA)-bladder cancer associated transcript 1 (BLACAT1) on the drug resistance of non-small cell lung cancer (NSCLC) cells in cisplatin (DDP) chemotherapy by regulating the expression of Cyclin D1.

MATERIALS AND METHODS: The analysis of the lncRNA expression profiles in 483 cases of NSCLC tissues and 347 cases of cancer-adjacent tissues in Gene Expression Omnibus (GEO) database revealed that lncRNA-BLACAT1 was differentially expressed in NSCLC and related to prognosis. In order to further study its mechanism of action on DDP-resistant cells, the expression level of lncRNA-BLACAT1 in normal human lung bronchial epithelial cell line BEAS-2B, NSCLC cell line A549, and DDP-resistant cell line A549 (A549/DDP) was detected by quantitative Polymerase Chain Reaction (qPCR). LncRNA-BLACAT1 small interfering RNA (siRNA) (si-BLACAT1) and lncRNA-BLACAT1 negative control (si-NC) were transfected into A549/DPP cells. Then, qPCR was carried out to detect the changes in the expression of lncRNA-BLACAT1 before and after transfection. Thereafter, cell cycle and cell growth rate were detected by flow cytometry and the cell growth curve. Besides, the changes in cell migration, cell apoptosis, and Cyclin D1 were detected *via* wound healing assay, flow cytometry, and Western blotting (WB).

RESULTS: In GEO database, lncRNA-BLACAT1 was significantly overexpressed in NSCLC ($p<0.05$), and the prognosis of NSCLC in BLACAT1 low-expression group was better than that in the BLACAT1 high-expression group ($p<0.0001$). Compared with that in BEAS-2B cells, BLACAT messenger RNA (mRNA) was notably highly expressed in A549 cells ($p<0.05$), and compared with that in A549 cells, BLACAT1 mRNA in A549/DPP was significantly highly expressed in A549/DDP cells ($p<0.05$). Additionally, in comparison with that in the si-NC group, the content of lncRNA-BLACAT1 in si-BLACAT1 group was remarkably decreased ($p<0.01$). Moreover, flow cytometry detection of cell cycle revealed that compared with those in si-NC group, G0/G1 phase was markedly prolonged and S phase was shortened in si-BLACAT1 group. MTS assay manifested that the absorbance at 450 nm in si-BLACAT1 group was evidently decreased on the 3rd day compared with that in the si-NC group ($p<0.05$), and the difference between the two groups was the most significant on the 5th day ($p<0.001$). According to wound healing assay, compared with those in si-NC group, the distance between cells became larger, the cell migration ability was remarkably weakened ($p<0.05$), and cell apoptosis was prominently reduced in si-BLACAT1 group ($p<0.05$). WB results showed that compared with si-NC group, si-BLACAT1 group had significantly reduced Cyclin D1 ($p<0.05$)

CONCLUSIONS: LncRNA-BLACAT1 regulates the expression of Cyclin D1, reduces the malignant phenotype of drug-resistant cells, and increases the sensitivity of lung cancer cells to DDP.

Key Words:
LncRNA-BLACAT1, Cyclin D1, DDP, Non-small cell lung cancer, GEO database.

Introduction

Non-small cell lung cancer (NSCLC) is characterized by high morbidity and mortality rates[1]. Cisplatin (DDP) is a common component of

Corresponding Author: Xuefei Zhang, MD; e-mail: xuefei-zhang@mails.qust.edu.cn

postoperative first-line therapeutic drugs[2]. Platinum-based chemotherapy, especially DDP-based chemotherapy, has become a leading adjuvant therapy for NSCLC after surgical resection[3]. However, NSCLC has drug resistance to DDP-based therapies, leading to a remarkable decrease in the clinical efficacy of DDP[4]. Compared with untreated control group, the curative effect of 5-year DDP chemotherapy shows that it can reduce lung cancer-related deaths by 6.9%[5]. The exact anti-tumor mechanism of DDP is still not fully understood[6]. Vance et al[7] manifest that the cytotoxicity of DDP is mainly mediated by nuclear deoxyribonucleic acid (DNA) binding and leads to the cross-linking of DNA double chains, eventually resulting in cell cycle arrest and promoting cell apoptosis. However, since only about 1% of intracellular DDP binds to nuclear DNAs, the above standpoint may be too untenable. DDP is activated in cells into aquatic complexes that react with many membrane and cytoplasmic components, indicating that DDP may also exert cytotoxic effects through a mechanism independent of nuclear DNA binding[8].

Long non-coding ribonucleic acids (lncRNAs), originally considered as DNA garbage, are messenger RNAs (mRNAs) with no ability to code proteins, and are important factors for transcriptional regulation[7]. Schmitt et al[8] showed that lncRNAs can induce the occurrence and development of diseases and even exert a decisive effect in some diseases. It has been recently indicated that some lncRNAs can affect the chemoresistance of NSCLC, displaying their roles as potential therapeutic targets for DDP-resistant NSCLC therapy[9,10]. Sun et al[11] found that small nucleolar RNA host gene 1 (SNHG1) is an lncRNA abundant in nuclei, located on chromosome 11q12.3, and has been identified as an oncogene in many human cancers. Upregulation of SNHG1 is conducive to the progression of glioma and is associated with poor prognosis[12]. Besides, SNHG1 is upregulated in NSCLC tissues and cells, but its knockdown suppresses the proliferation of NSCLC cells[13]. In addition, the upregulation of SNHG1 promotes the development of NSCLC by sponging micro RNA (miR)-145-5p and regulating MTDH expression[14]. LncRNA BLACAT1 was previously reported to be involved in chemoresistance of NSCLC by regulating autophagy. Gene Expression Omnibus (GEO) database also showed that lncRNA-BLACAT1 was differentially expressed in NSCLC. As we know, DDP resistance is a major problem to be solved in the treatment of NSCLC[15]. This study mainly aims to investigate the mechanism of lncRNA-BLACAT1 in the drug resistance of NSCLC cells in DDP chemotherapy. A better understanding of the mechanism of DDP resistance in NSCLC will provide effective methods to reverse DDP resistance and improve the efficacy of NSCLC therapy.

Materials and Methods

Materials

A549, A549/DPP, and BEAS-2B [American Type Culture Collection (ATCC) Manassas, VA, USA], acrylamide (Amresco, Atlanta, GA, USA), bicinchoninic acid assay (BCA) kit (Beyotime, Shanghai, China), MTS kit (BD Biosciences, Franklin Lakes, NJ, USA), transwell invasion chamber (BD Biosciences, Franklin Lakes, NJ, USA), radioimmunoprecipitation assay (RIPA) buffer (APPLYGEN, Beijing, China), and BLACAT1 primer (Sigma-Aldrich, St. Louis, MO, USA).

Research Objects and Grouping

The research objects of this experiment were NSCLC cell line (A549/DPP) and normal lung epithelial cell line (BEAS-2B). After transfection, A549/DPP cells were divided into si-negative control (NC) group and si-BLACAT1 group.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction (qPCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) and chloroform were used to extract the total RNAs from cells. After quantification, RNAs were transcribed into complementary deoxyribonucleic acids (cDNAs). According to reverse transcription instructions, reagent 1 was added and preheated at 65°C for 10 min. Then, the remaining six reagents were added, respectively, and the concentration of cDNAs was quantified. After that, a 20 mL system was composed of 1 mL upstream primer and 1 mL downstream primer, 1 mL cDNA, SYBR, and enzyme-free water. Ct values were detected and recorded on machine. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference, and $2^{-\Delta\Delta Ct}$ was applied to calculate Ct difference statistics. Primer sequences: LncRNA-BLACAT1-F: GTCTCTGCCCTTTTGAGCCT; LncRNA-BLACAT1-R: GTGGCTGCAGTGTCATACCT. GAPDH-F: TGACTTCAACAGCGACACCCA; GAPDH-R: CACCCTGTTGCTGTAGCCAAA.

Western Blotting

The cells were placed on ice. Then, protein lysis mixture was added to the cell precipitation, and the cells were crushed for three times at 100 W and incubated for 30 min to measure the protein concentration. After that, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was utilized to separate 60 mg of protein samples, and the samples on the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) and covered with the mixture of blocking reagent A and liquid B (1:1) under the constant current of 300 mA. Subsequently, primary antibody was added after 1 h, secondary antibody was added after 16 h in a refrigerator, and the membrane was scanned. In the above process, Phosphate-Buffered Saline and Tween 20 (PBST) was employed to wash the membrane for 3 times with 6 min each time.

Detection of Cell Cycle Via Flow Cytometry

Cells were paved into a 6-well plate at 2.0×10^5 cells per well and incubated overnight at normal oxygen concentration. After 24 h, the pipette was used to gently suck the supernatant into the centrifuge tube, and the liquid and supernatant washed by phosphate-buffered saline (PBS) were collected in the same tube. Subsequently, the cells were centrifuged at 200 × g for 5 min and fixed with 79% ethanol at 4°C for 24 h after the supernatant was discarded. After fixation, the cells were centrifuged at 1400 g for 5 min, washed with PBS, and incubated with 100 μg/mL RNaseA for 30 min at room temperature. Next, the cells were dyed with a flow dye and detected using a flow cytometer.

Detection of Apoptosis Via Flow Cytometry

Cells were paved into the 6-well plate at 3.0×10^5 cells/well and normally cultured without any treatment. When the cell fusion rate reached over 80%, the cells were treated with trypsin for 3 min, and culture medium was added to terminate digestion. Next, centrifugation was carried out at 1500×g for 4 min to precipitate cell suspension, and flow binding buffer was added to resuspend the precipitate. Finally, propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) dyes were added to the cell suspension. After pre-cooling for 15 min, the flow cytometer was used for testing on machine.

Cell Proliferation Assay

A total of 3,000 target cells were inoculated to a 96-well plate and the time was recorded. At 1, 2, 3, 4, and 5 days after cell growth, the absorbance at 490 nm of the cells was detected, as follows: when the cell grew for a specific time, the supernatant was discarded, MTS reagent was added in the dark, and the cells were detected on machine after incubation for other 2 h. It should be ensured that three duplicate wells were set for each well.

Cell Transfection

Preparation of transfection experiment: on the day before the experiment, si-BLACAT1 and si-NC were first diluted with opti-MEM and Lipofectamine 2000, the diluted Lipofectamine 2000 and si-BLACAT1/NC were mixed evenly, and 1×10^5 target cells were inoculated into the 6-well plate. When the cell fusion reached over 70%, the transfection experiment started, and the final mixed solution of Lipofectamine 2000 and si-BLACAT1/NC was added into cells after the supernatant of the culture medium was discarded. After transfection for 6 h, the original medium was replaced with normal culture medium.

Cell Wound Healing Assay

Cells were inoculated into the 6-well plate at the same density (3×10^5 cells/well), and a 200 μL measuring range pipette was selected to make a sign of the cross on the bottom of the wells. In order to avoid pollution, the culture medium was replaced after cleaning, and the cells were continuously incubated in an incubator for 24 h. Finally, a high-pixel camera was employed to photograph changes in intercellular distance.

Statistical Analysis

The obtained experimental results were statistically processed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA), and *t*-test and univariate analysis were adopted. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression and Prognosis of LncRNA-BLACAT1 in GEO Database

A total of 483 cases of NSCLC tissues and 347 cases of cancer-adjacent tissues were analyzed in GEO database, and it was found that lncRNA-BLACAT1 was significantly highly ex-

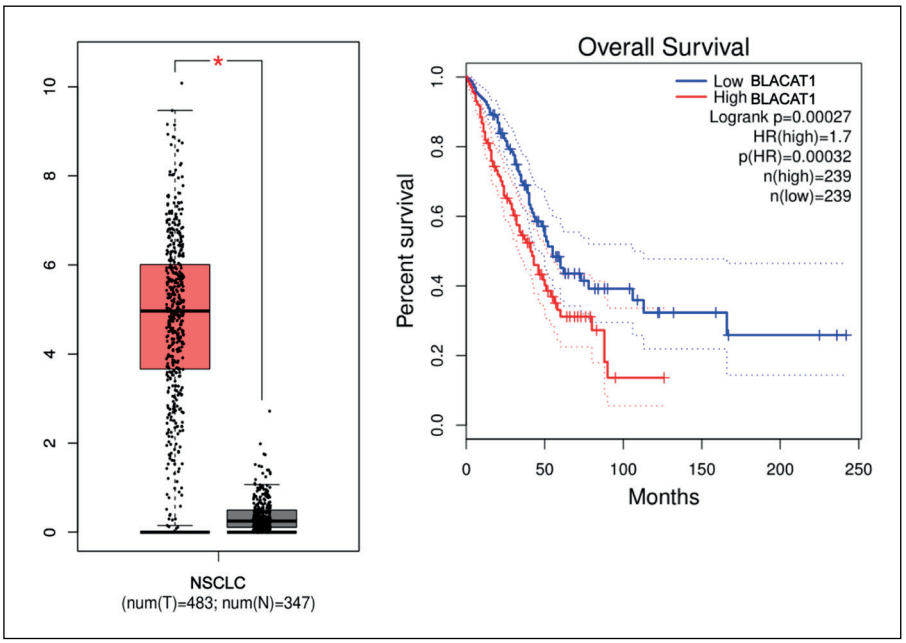

Figure 1. Expression and prognosis of lncRNA-BLACAT1 in GEO database (*left:* expression level of lncRNA-BLACAT1 in NSCLC tissues, *right:* survival curves of NSCLC patients) (*p<0.05, **p<0.01, ***p<0.001).

pressed in NSCLC, and the prognosis was poor in patients with highly expressed lncRNA-BLACAT1 (p<0.05) (Figure 1).

Verification of the LncRNA-BLACAT1 Expression in NSCLC Cells

In order to verify the expression of lncRNA-BLACAT1 in NSCLC cells, qRT-PCR detection showed that the lncRNA-BLACAT1 mRNA in A549 cells was markedly higher than that in BEAS-2B cells (p<0.05). Compared with A549 cells, A549/DPP cells exhibited notably highly expressed lncRNA-BLACAT1 (p<0.05). The above results indicate that lncRNA-BLACAT1 is highly expressed in NSCLC cells, which is consistent with the database results (Figure 2).

Effect of LncRNA-BLACAT1 on the Proliferation of A549/DPP Cells

In comparison with that in the si-NC group, the expression of lncRNA-BLACAT1 in si-BLACAT1 group was remarkably decreased (p<0.05). Flow cytometry analysis revealed that G0/G1 phase was prolonged and S phase was shortened in the si-BLACAT1 group (p<0.05). In addition, according to MTS results, there was a significant difference in the absorbance at 450 nm on the 3rd day between si-NC group and si-BLACAT1 group (p<0.05), and the difference was the most significant on the 5th day (p<0.001). It can be concluded that inhibiting the lncRNA-BLACAT1 expression can suppress the growth and proliferation of A549/DPP cells (Figure 3).

Influence of LncRNA-BLACAT1 on the Apoptosis of A549/DPP Cells

Compared with that in si-NC group, the proportion of apoptotic cells in the si-BLACAT1 group was remarkably decreased (p<0.05), indicating that inhibiting the expression of lncRNA-BLACAT1 can suppress A549/DPP cell apoptosis (Figure 4).

Effect of LncRNA-BLACAT1 on the Migration of A549/DPP Cells

The results of cell wound healing assay manifested that compared with those in the si-NC

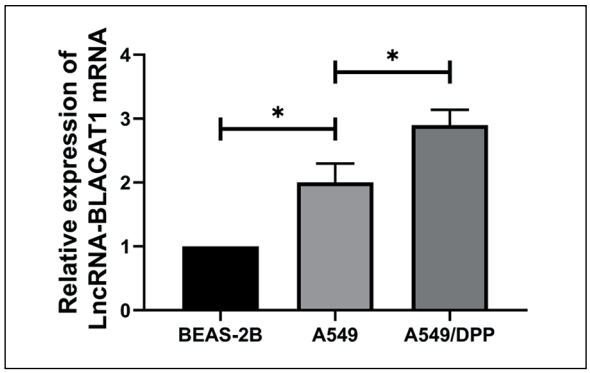

Figure 2. Expression level of lncRNA-BLACAT1 in NSCLC cells (*p<0.05, **p<0.01, ***p<0.001).

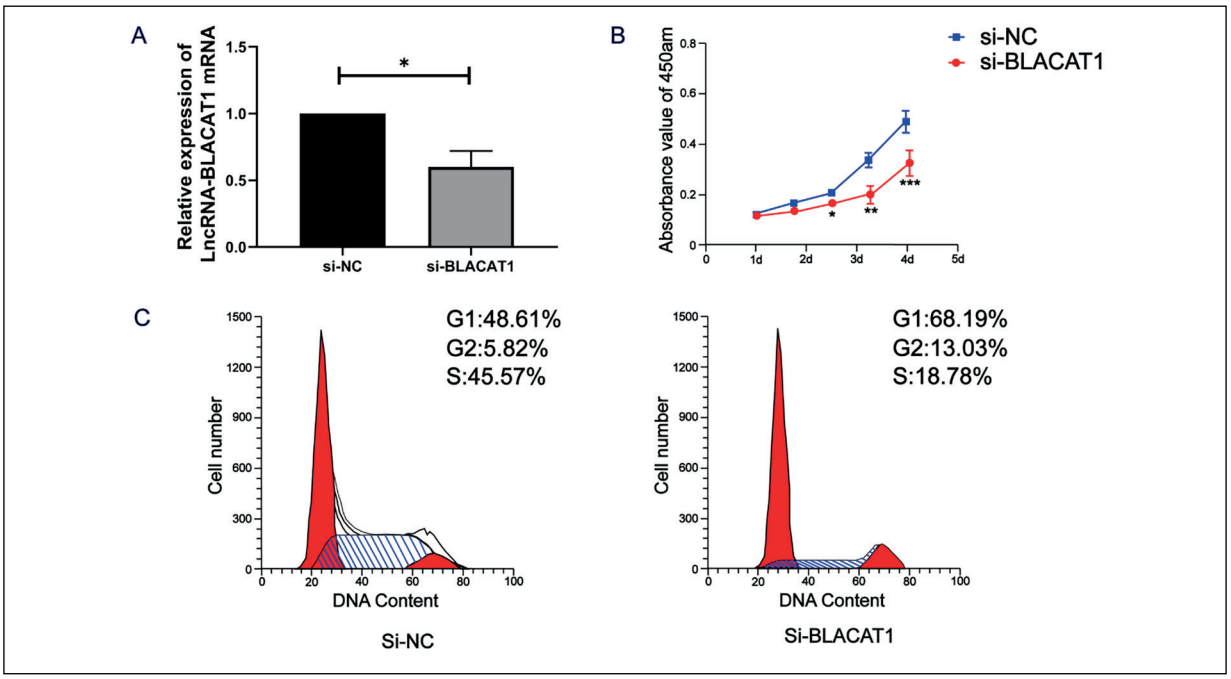

Figure 3. Effect of lncRNA-BLACAT1 on the proliferation of A549/DPP cells. **A,** Compared with that in the si-NC group, the expression of lncRNA-BLACAT1 in si-BLACAT1 group is markedly reduced (p<0.05). **B,** There is an evident difference in the absorbance at 450 nm on the 3rd d between si-NC group and si-BLACAT1 group (p<0.05), and the difference is the most significant on the 5th day (p<0.001). **C,** Compared with that in si-NC group, the G0/G1 phase is prolonged in si-BLACAT1 group (*p<0.05, **p<0.01, ***p<0.001).

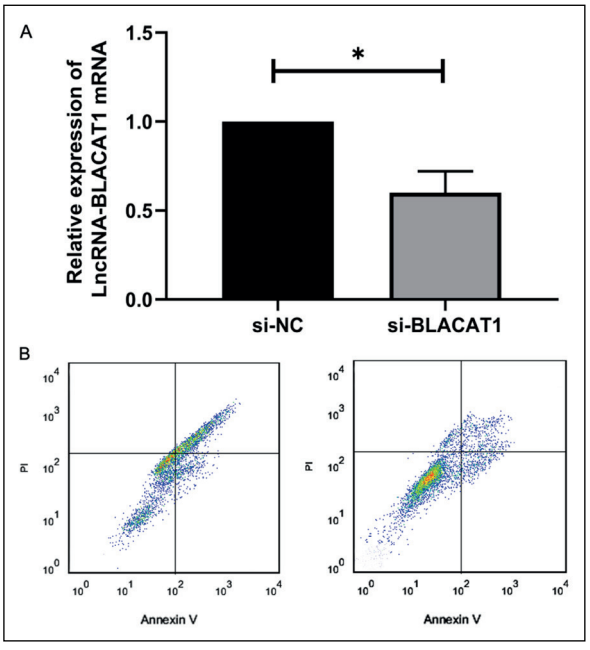

Figure 4. Influence of lncRNA-BLACAT1 on the apoptosis of A549/DPP cells. **A,** Compared with that in si-NC, the expression of lncRNA-BLACAT1 in si-BLACAT1 group is significantly reduced (p<0.05). **B,** In comparison with si-NC group, si-BLACAT1 has notably reduced proportion of apoptotic cells (p<0.05) (*p<0.05, **p<0.01, ***p<0.001).

group, the intercellular distance in the si-BLACAT1 group became larger, and the cell migration ability was evidently reduced (p<0.05) (Figure 5).

LncRNA-BLACAT1 Regulated the Expression of Cyclin D1

Western blotting (WB) was performed to detect the changes in Cyclin D1 in si-NC group and si-BLACAT1 group. Compared with si-NC group, si-BLACAT1 group had markedly lowly expressed Cyclin D1 (p<0.05), suggesting that lncRNA-BLACAT1 is able to positively regulate the Cyclin D1 expression (Figure 6).

Discussion

The curative effect of DDP-based chemotherapy is limited by congenital and acquired drug resistance. The mechanism of DDP resistance is very complex, so there is no strategy based on a single mechanism to overcome DDP resistance[16,17]. The mechanism of DDP resistance was classified into four categories: (1) pre-target resistance triggered by prevention from the binding of

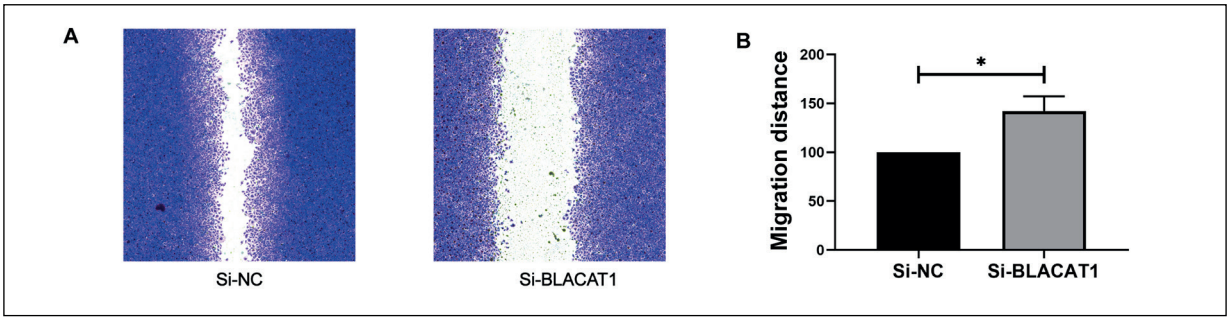

Figure 5. Cell migration detected by cell wound healing assay. **A,** Cell migration changes in si-NC group and si-BLACAT1 group. (magnification: 40×) **B,** Compared with that in si-NC group, intercellular distance in si-BLACAT1 group is significantly increased ($p<0.05$) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

DDP to DNAs by reducing cell accumulation or enhancing binding to cytoplasmic components; (2) target resistance resulting from tolerance or repair of DDP-DNA adducts; (3) post-target resistance caused by several changes or defects in signaling pathways after DDP damage to DNAs; and (4) off-target resistance, whose mechanism does not directly involve the signal initiated by DDP, but can enable cells to avoid DDP-induced cell death[17]. It has been found that various proteins, genes or pathways are involved in the resistance to DDP[18,19], but any mechanism is considered to be crucial. Therefore, a strategy based on a single mechanism to overcome DDP resistance is unlikely to be effective, and the extensive interaction between the cytoplasm and nucleus and DDP, as well as the multifactorial nature of drug resistance, drives the development of a more systematic approach, leading to the establishment of an overall model to explain the development of DDP resistance in NSCLC cells in the near future[20]. In this study, a bottom-up method was chosen to compare NSCLC cells sensitive to DDP with those resistant to DDP. Firstly, 483 cases of NSCLC tissues and 374 cases of cancer-adjacent tissues were analyzed in GEO database, and it was found that lncRNA-BLACAT1 was significantly highly expressed in NSCLC and was associated with poor prognosis. In the treatment of NSCLC, cells are prone to be resistant to DDP, a chemotherapeutic drug. LncRNAs are related to chemotherapy drug resistance, so the role and mechanism of lncRNA-BLACAT1 differentially expressed in NSCLC in DDP resistance were investigated in this study. The NSCLC cell line A549, DDP-resistant NSCLC cell line A549/DPP, and normal lung epithelial cell line BEAS-2B were selected, and the expression level of lncRNA-BLACAT1 in cells was detected using real-time fluorescence qPCR technology. The results manifested that compared with that in BEAS cells, the expression level of lncRNA-BLACAT1

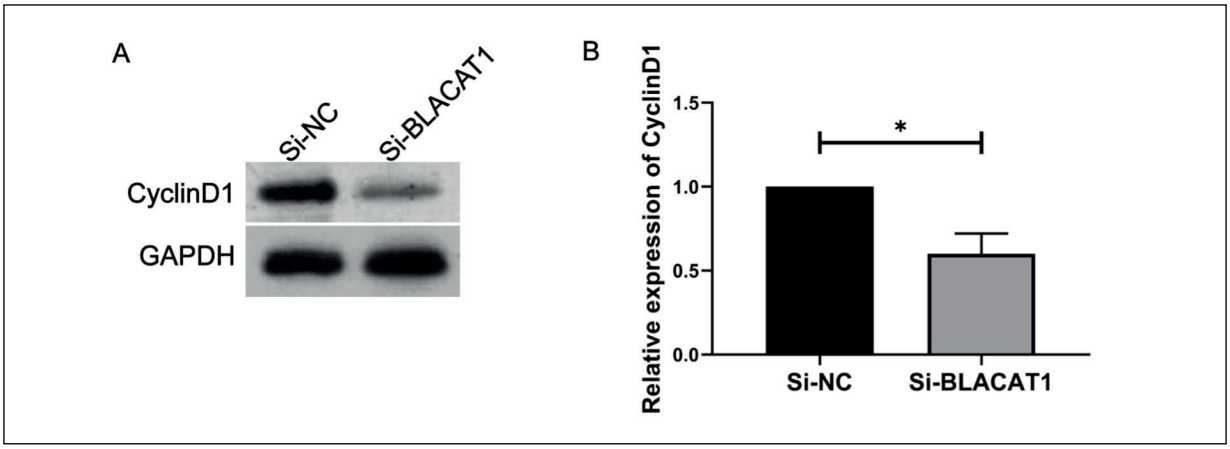

Figure 6. Cyclin D1 changes in si-NC group and si-BLACAT1 group. **A-B,** Compared with si-NC, si-BLACAT1 group has notably lowly expressed Cyclin D1 protein ($p<0.05$) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

was evidently elevated in A549 cells, and in comparison with that in A549 cells, this level was significantly increased in A549/DPP cells. The above results indicate that the expression trend of lncRNA-BLACAT1 in drug-resistant cell lines is identical to that in the tissues. To study the effect of lncRNA-BLACAT1 on the malignant phenotype of DDP-resistant cells, related functional experiments were performed. Specifically, MTS experiment and flow cytometry were adopted to detect the cell cycle. The experimental results revealed that compared with those in the si-NC group, G0/G1 phase was prolonged and S phase (for replication and proliferation) was shortened in the si-BLACAT1 group, suggesting that most cells stay in G0/G1 phase, during which the proliferation and division are blocked. On the 3rd day, the absorbance at 490 nm of cells in si-BLACAT1 was significantly lower than that in the si-NC group, and it was decreased continuously on the 4th and 5th day, which implies that the cells with lowly expressed lncRNA-BLACAT1 grow slowly. Besides, the 24-h wound healing assay results demonstrated that compared with those in the si-NC group, the intercellular distance became larger, and the cell migration ability was weakened after 24 h in the si-BLACAT1 group. In order to eliminate the interference of cell growth rate, the cells were treated with drugs to inhibit cell division. Flow cytometry results verified that the apoptosis rate of A549/DPP cells in si-BLACAT1 group was markedly reduced. Moreover, the functional experimental results showed that inhibiting the expression of lncRNA-BLACAT1 could suppress the malignant phenotype of DDP-resistant NSCLC cells, which might provide a new treatment method for the drug resistance of NSCLC cells. Furthermore, to have a deeper understanding of the mechanism of lncRNA-BLACAT1 in exerting its effect of anti-DDP resistance, the Cyclin D1, a cell cycle protein, associated with lncRNA-BLACAT1 was found. Lowly expressed lncRNA-BLACAT1 can slow down the cell cycle, so the expression of Cyclin D1 in the si-NC group and si-BLACAT1 group was detected. In the si-BLACAT1 group, the expression of Cyclin D1 was prominently lower than that in the si-NC group.

Conclusions

In short, the above results highlighted that lncRNA-BLACAT1 regulates the expression of Cyclin D1, reduces the malignant phenotype of drug-resistant cells, and increases the sensitivity of NSCLC cells to DDP. However, the anti-drug resistance of lncRNA-BLACAT1 has not been verified *in vivo*, so it will be verified later.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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