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# CLDN1 Increases Drug Resistance of Non-Small Cell Lung Cancer by Activating Autophagy via Up-Regulation of ULK1 Phosphorylation

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Data Interpretation D  
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**Background:** The aim of this study was to investigate the expression of CLDN1 in non-small cell lung cancer (NSCLC) and its mechanism of action in cisplatin resistance.


**Material/Methods:** A total of 55 patients with NSCLC admitted to our hospital between October 2013 and October 2015 were included. NSCLC tissues and tumor-adjacent tissues ( $\geq 5$  cm from tumor edge) were collected. Among the 55 patients, 37 had adenocarcinoma and 18 had squamous cell carcinoma. Quantitative real-time polymerase chain reaction was used to determine mRNA expression, and protein expression was examined using Western blotting. CCK-8 assay was used to determine cell proliferation and Transwell assay was used to detect migration and invasion of the cells. Confocal microscopy was used to observe autophagosomes.

**Results:** Increased CLDN1 expression promoted the development and metastasis of NSCLC. CLDN1 expression in A549/CDDP cells was up-regulated at both transcriptional and translational levels. Reduced CLDN1 expression decreased the drug resistance, proliferation, migration, and invasion abilities of A549/CDDP cells. Decreased CLDN1 expression promoted the apoptosis of A549/CDDP cells. CLDN1 enhanced CDDP drug resistance of A549 cells by activating autophagy. CLDN1 promoted the autophagy of A549 cells by up-regulating the phosphorylation level of ULK1.

**Conclusions:** The present study demonstrates that expression of CLDN1 in NSCLC is up-regulated and it is correlated with clinicopathological features. CLDN1 activates autophagy through up-regulation of ULK1 phosphorylation and promotes drug resistance of NSCLC cells to CDDP.

**MeSH Keywords:** **Autophagy • Carcinoma, Non-Small-Cell Lung • Claudin-1**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/904177>

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## Background

Non-small cell lung cancer (NSCLC) is a common malignant tumor with poor prognosis, and its morbidity and mortality rank the second and first, respectively, in the causes of death in tumor patients [1,2]. China has a high incidence of lung cancer, mainly NSCLC [3]. Although the level of clinical treatment of NSCLC has been greatly improved, the 5-year survival rate of patients with NSCLC is still around 15% [4,5]. Tumor recurrence and metastasis are main causes of death in patients with NSCLC, and chemotherapy drug resistance is one of the key factors leading to recurrence and metastasis of NSCLC [6]. Currently, cisplatin is a major drug used in chemotherapy of NSCLC, and it facilitates cross-linking between heavy metal ions and cell DNA chain through the connection with nuclear protein, ultimately damaging DNA [7,8]. NSCLC cells have high sensitivity to cisplatin at early stages of treatment, but most of the cells become resistant to chemotherapy after several cycles, leading to failure of chemotherapy [9]. However, its mechanism is still unclear. Therefore, a deeper understanding of the mechanism of drug resistance can provide a new strategy for reversing drug resistance and improving tumor therapy.

Tight junctions (TJs) are apical junctional complexes of epithelial and endothelial cells that participate in the formation and maintenance of epithelial cell layer structure [10]. TJs maintain osmotic pressure in tissues, prevent lateral diffusion of membrane proteins and lipids, and keep the differences between top and basal cells [11]. TJs are also involved in cell proliferation, differentiation, and other functions [12]. CLDN1, a member of the CLDN1 family, is one of the key proteins in TJs, and 24 CLDN family genes have been cloned and identified [13,14]. Abnormal expression of CLDN gene damages epithelial permeability barrier, leading to disappearance of epithelial cell polarity and reduced adhesion between cells [15]. The expression of CLDN family genes in tumor tissues is abnormal, and is closely related to tumor proliferation, invasion, metastasis, drug resistance, and prognosis. For example, CLDN5,7,18 genes inhibit the proliferation and metastasis of lung squamous carcinoma cells by inactivating the AKT signaling pathway [16]. CLDN12 has clinical values in evaluating the prognosis of patients with estrogen receptor-negative breast cancer [17]. Moreover, it is reported that CLDN1 is expressed in various tumor tissues, and is closely related to tumor prognosis, proliferation, and metastasis [18,19]. Interestingly, the prognosis of lung adenocarcinoma patients with high expression of CLDN1 is poor, but the that of lung squamous cell carcinoma patients with high expression of CLDN1 is good, suggesting that the function of CLDN1 may be distinct in different types of lung tumors [20,21]. It is still unclear whether CLDN1 is involved in the regulation of cisplatin resistance in NSCLC. In the present study, we constructed a cisplatin-resistant NSCLC cell line, and examined the effect and mechanism of CLDN1 in cisplatin resistance in NSCLC.

## Material and Methods

### Patients

A total of 55 patients with NSCLC admitted to our hospital between October 2013 and October 2015 were included in the present study. NSCLC tissues and tumor-adjacent tissues ( $\geq 5$  cm from tumor edge) were collected. Among the 55 patients, 37 had adenocarcinoma and 18 had squamous cell carcinoma. In addition, 32 patients had lymph node metastasis (N1) and 23 patients had no lymph node metastasis (N0). According to TNM staging standards, 18 patients were classified at stage I, 21 patients were classified at stage II, 8 patients were classified at stage III, and 8 patients were classified at stage IV. None of the patients were treated with chemotherapy or other tumor therapies, or had a history of other tumors. The age range of the patients was 27.5–72 years, with a mean age of 48 years. All procedures were approved by the Ethics Committee of Qingdao University. Written informed consents were obtained from all patients or their families.

### Cells

Non-small cell lung adenocarcinoma cell line A549 and squamous cell carcinoma cell line NCI-H2170 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cells were defrosted at 37°C and cultured in 5 ml RPMI-1640 medium containing 10% fetal bovine serum at 37°C and under 5% CO<sub>2</sub> for 24 h. After 24 h of incubation, the medium was discarded, and 5 ml fresh RPMI-1640 medium supplemented with 10% fetal bovine serum was added for subsequent culture. The medium was replaced every 2 days, and the cells were passaged when reaching 90% confluency. Cells within the first 3–5 passages were used for subsequent tests.

To construct a drug-resistant NSCLC cell line (A549/CDDP), 200  $\mu$ l A549 cells in log-phase growth ( $1 \times 10^5$ /ml) were seeded into 96-well plates. Gradient concentrations of CDDP was added onto the wells in triplicate. After cultivation for 24 h, 20  $\mu$ l CCK-8 reagent was added, followed by incubation at 37°C at 5% CO<sub>2</sub> for 1 h. Absorbance was measured at 490 nm, and the half maximal inhibitory concentration (IC<sub>50</sub>) was calculated.

Intermittent impact method was used to construct A549/CDDP cells. After incubation with medium containing CDDP (1  $\mu$ g/ml) for 24 h, dead cells were washed away, and fresh RPMI-1640 complete medium was added. When the cells were stable, the same concentration of CDDP was added for impact, which was repeated until tumor cells were able to stably proliferate in RPMI-1640 complete medium containing 0.5  $\mu$ g/ml CDDP. The IC<sub>50</sub> value of A549/CDDP cells was calculated, and compared with the IC<sub>50</sub> of A549 cells for CDDP. When  $IC_{50}^{A549/CDDP}/IC_{50}^{A549} \geq 5$ , the cells were harvested for subsequent tests.

## Transfection

One day before transfection, log-phase cells ( $2 \times 10^5$ ) were seeded onto 24-well plates containing serum-free RPMI-1640 medium supplemented with 10% fetal bovine serum. On the next day, the cells were observed under a microscope. When reaching 70–80% confluency, the cells were transfected with siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 1.5  $\mu\text{l}$  siR-CLDN1 (25 pmol/ $\mu\text{l}$ ) or siR-NC (25 pmol/ $\mu\text{l}$ ) and 1  $\mu\text{l}$  Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were added into 2 individual vials each containing 50  $\mu\text{l}$  Opti Memi medium. Five minutes later, the liquids in the 2 vials were mixed before standing still for 20 min at room temperature. Then, the mixture was added onto the cells for an incubation of 6 h. After changing to fresh RPMI-1640 medium supplemented with 10% fetal bovine serum, the cells were cultured under normal condition for 48 h before use. CLDN1 overexpression lentivirus vector LV-GFP-RFP-LC3B (Hanbio, Shanghai, China) was also used to infect A549/CDDP or A549 cells (MOI=20). After 72 h, the cells were collected for further use.

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

Homogenized tissues (50 mg) and cells ( $10^6$ ) were mixed with 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. After lysis, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription using PrimeScript RT Regent Kit (Takara, Dalian, China) from 1  $\mu\text{g}$  RNA and stored at  $-20^\circ\text{C}$ .

To test the expression of CLDN1 mRNA, SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China) was chosen, using GAPDH as internal reference. The qRT-PCR reaction system (20  $\mu\text{l}$ ) contained 5  $\mu\text{l}$  cDNA, 10  $\mu\text{l}$  Mix, 0.5  $\mu\text{l}$  upstream primer (5'-ATGACCCAGTCAATGCCAG-3'), 0.5  $\mu\text{l}$  downstream universal primer (5'-GCTGGAAGGTGACAGGTTTG-3'), and 4  $\mu\text{l}$  ddH<sub>2</sub>O. The PCR protocol was initial denaturation at  $95^\circ\text{C}$  for 3 min; 40 cycles of denaturation at  $95^\circ\text{C}$  for 30 s and annealing at  $60^\circ\text{C}$  for 30 s (ABI SteponePlus; Thermo Fisher Scientific, Waltham, MA, USA). The  $2^{-\Delta\Delta\text{Ct}}$  method was used to calculate the relative expression of CLDN1 mRNA against internal reference. Each sample was tested in triplicate.

## Cell-counting kit (CCK)-8 assay

Cells were seeded into 96-well plates at a density of  $1 \times 10^4$  cells per well in triplicate. For every 24 h, the cells were incubated with CCK-8 reagent (Beyotime, Shanghai, China) for 30 min. Absorbance at 490 nm was read on a microplate reader

(168–1000; Model 680, Bio-Rad, Hercules, CA, USA) at 24 h, 48 h, and 72 h, and proliferation curves were plotted using absorbance values at each time point.

## Flow cytometry

To examine cell cycles, cells were seeded into 24-well plates at a density of  $1 \times 10^5$  cells per well in triplicate. After being incubated overnight (16 h), the cells were subjected to flow cytometry for the determination of cell cycle changes according to the manufacturer's manual (BD Cycletest Plus DNA Reagent Kit, BD Bioscience, Franklin Lakes, NJ, USA).

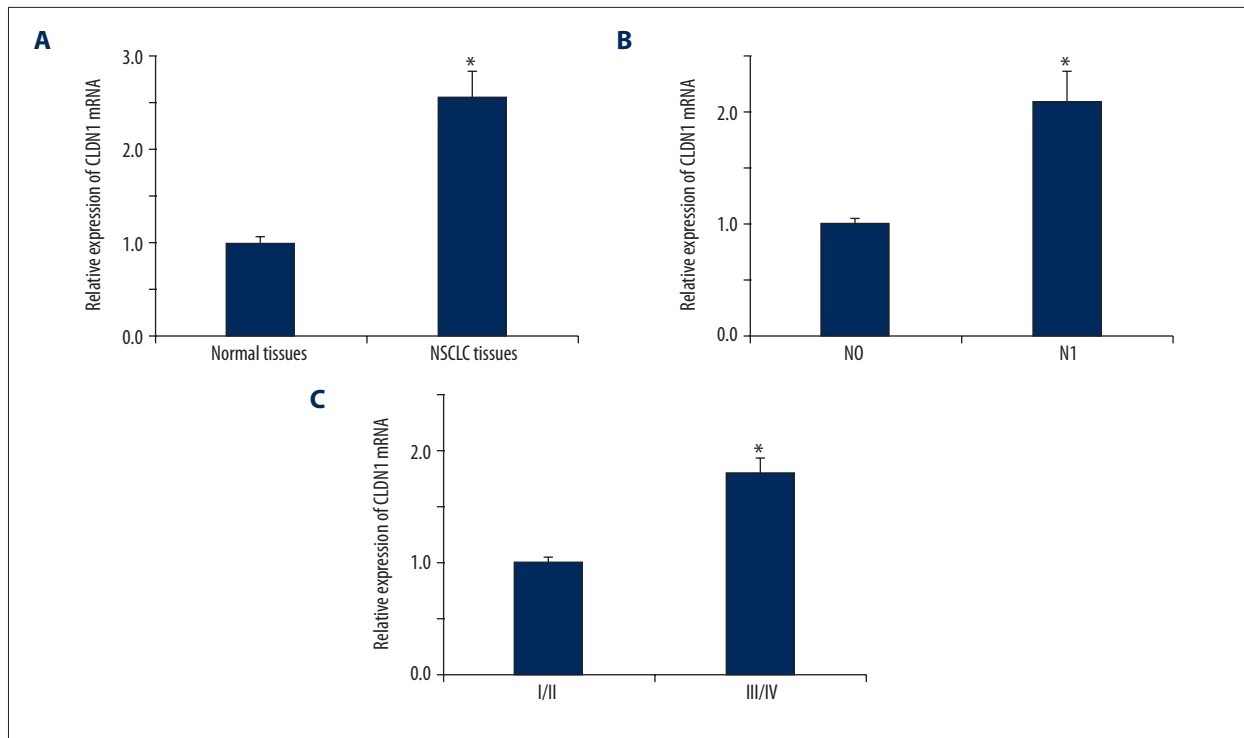
To detect apoptosis, cells were seeded into 24-well plates at a density of  $1 \times 10^5$  cells per well in triplicate. After adhesion, the cells were incubated with RPMI-1640 medium containing 0.5  $\mu\text{g}/\text{ml}$  CDDP for 48 h. Then, cell apoptosis was detected by flow cytometry according to the manufacturer's manual (FITC Annexin V Apoptosis Detection Kit, BD Bioscience, Franklin Lakes, NJ, USA).

## Transwell assay

Cells were resuspended into serum-free RPMI-1640 medium at a density of  $5 \times 10^5/\text{ml}$ . Then, 200  $\mu\text{l}$  cell suspension was added into the upper chamber (Merck Millipore, Billerica, MA, USA). In the lower chamber, 500  $\mu\text{l}$  RPMI-1640 complete medium was added, followed by incubation for 24 h. After Giemsa staining for 1 min, the number of cells that passed through the membrane was counted under a microscope.

## Western blotting

Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600  $\mu\text{l}$ ; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, and 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) and 1% phenylmethylsulfonyl fluoride were thoroughly mixed with cells, and then the cells were lysed at  $4^\circ\text{C}$  overnight. On the next day, lysed cells were centrifuged at 12 000 rpm for 15 min. Protein samples (50  $\mu\text{g}$ ) were then mixed with 5 $\times$  sodium dodecyl sulfate loading buffer before denaturation in a boiling water bath for 10 min. Afterwards, the samples (5  $\mu\text{l}$ ) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 1 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human CLDN1 polyclonal primary antibody (1: 1,000; Abcam, Cambridge, UK) and rabbit anti-human GAPDH primary antibody (1: 5,000; Abcam, Cambridge, UK) at  $4^\circ\text{C}$  overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 5 times of 5 min each, the membranes were



**Figure 1.** CLDN1 mRNA expression in non-small cell lung cancer (NSCLC) and its clinical significance. **(A)** Relative expression of CLDN1 in NSCLC tissues and tumor-adjacent normal tissues. \*  $P < 0.05$  compared with normal tissues. **(B)** Relative expression of CLDN1 in patients with lymphatic metastasis (N1) or without lymphatic metastasis (NO). \*  $P < 0.05$  compared with patients without lymph node metastasis. **(C)** Relative expression of CLDN1 in patients at TNM stages I/II and III/IV. \*  $P < 0.05$  compared with stage I/II.

incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1: 10 000; Abcam, Cambridge, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 5 times for 5 min each. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of CLDN1 protein was expressed as CLDN1/GAPDH ratio.

### Confocal microscopy

Cells ( $1 \times 10^5$ ) were seeded onto round culture plates (60 mm diameter). Lv-GFP-RFP-LC3B was added for transfection to achieve  $MOI=20$ . After 72 h, medium was discarded, and the cells were washed with phosphate-buffered saline 3 times. Then, the cells were fixed with 4% formaldehyde for 10 min before being washed with phosphate-buffered saline twice. The cells were then observed under a confocal microscope (SP8; Leica, Wetzlar, Germany).

### Statistical analysis

The results were analyzed using SPSS 16.0 statistical software (IBM, Armonk, NY, USA). All measurement data were expressed as means  $\pm$  standard deviations. Intergroup comparison was performed using the paired t-test.  $P < 0.05$  was considered statistically significant.

## Results

### Increased CLDN1 expression promotes the development and metastasis of NSCLC

To measure the expression of CLDN1 mRNA, qRT-PCR was performed. The data showed that CLDN1 mRNA expression in NSCLC tissues was significantly higher than that in tumor-adjacent tissues ( $P < 0.05$ ) (Figure 1A). In addition, CLDN1 mRNA expression in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis ( $P < 0.05$ ) (Figure 1B). CLDN1 mRNA expression in patients at TNM stages III and IV was significantly higher than that in patients at stages I and II ( $P < 0.05$ ) (Figure 1C). The

**Table 1.** IC50 and fold of drug resistance of A549 and A549/CDDP cells.

	CDDP IC50 (1 µg/ml)
A549	1.34±0.06
A549/CDDP	7.11±0.25
Fold of drug resistance	5.30±0.31

results suggest that increased CLDN1 expression promotes the development and metastasis of NSCLC.

### A549/CDDP cell line resistant to cisplatin is successfully constructed

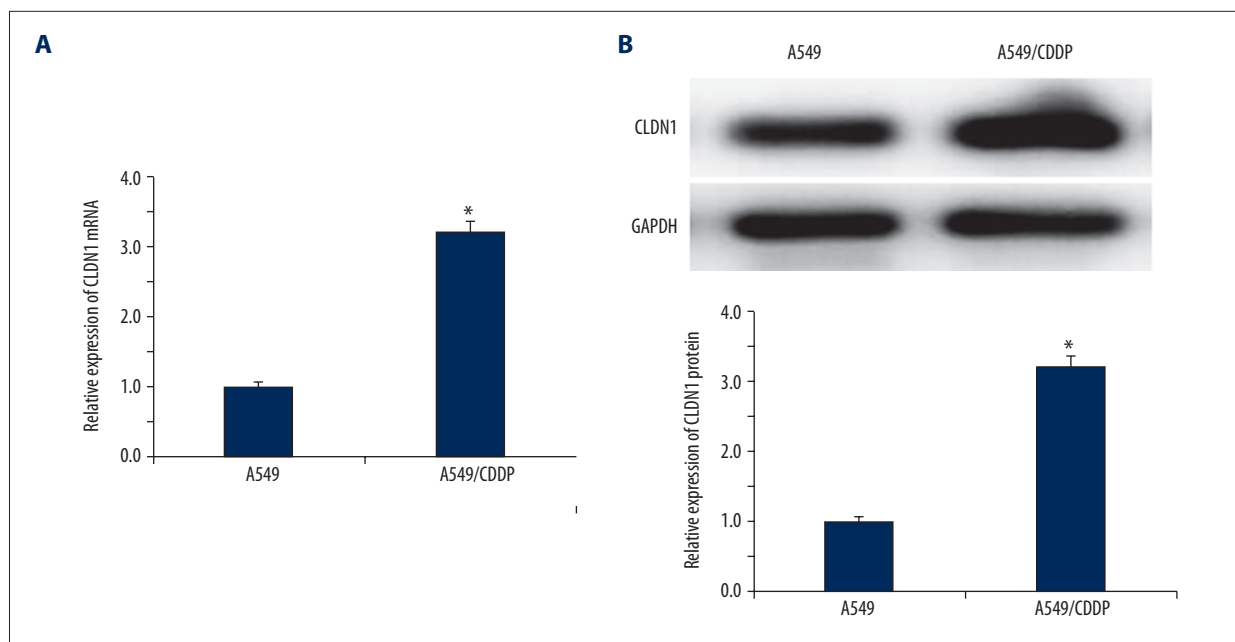
To construct A549/CDDP cells, A549 cells were cultured in the presence of 1 µg/ml CDDP for 6 months, and CDDP IC50 of A549 cells and A549/CDDP cells was determined. The data showed that IC50 of A549/CDDP cells was significantly higher than that of A549 cells ( $P<0.05$ ) (Table 1). The results indicate that a A549/CDDP cell line resistant to cisplatin was successfully constructed.

### CLDN1 expression in A549/CDDP cells is up-regulated at both transcriptional and translational levels

To measure the expression of CLDN1 mRNA and protein in A549/CDDP cells, qRT-PCR and Western blotting were used. qRT-PCR showed that CLDN1 mRNA levels in A549/CDDP cells were significantly higher than that in A549 cells ( $P<0.05$ ) (Figure 2A), and Western blotting showed that CLDN1 protein expression in A549/CDDP cells was significantly increased compared with that in A549 cells ( $P<0.05$ ) (Figure 2B). The results suggest that CLDN1 expression in A549/CDDP cells is up-regulated at both transcriptional and translational levels.

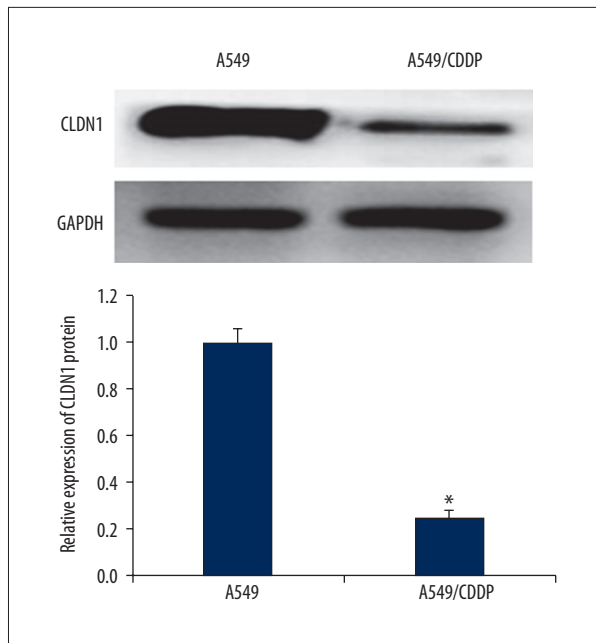
### Reduced CLDN1 expression decreases the drug resistance, proliferation, migration, and invasion abilities of A549/CDDP cells

To further investigate the effect of CLDN1 on the drug resistance of A549/CDDP, siRNA was used to down-regulate the expression of CLDN1 in A549/CDDP cells. The data showed that siRNA interference reduced the expression of CLDN1 protein in A549/CDDP cells (Figure 3). IC50 of A549/CDDP cells with reduced CLDN1 expression was significantly higher than that of A549 cells ( $P<0.05$ ), but significantly lower than that of A549/CDDP cells transfected with siR-NC ( $P<0.05$ ) (Table 2). CCK-8 assay showed that the absorbance of A549/CDDP cells



**Figure 2.** Relative expression of CLDN1 in A549 cells and A549/CDDP cells. (A) Relative expression of CLDN1 mRNA. \*  $P<0.05$  compared with A549 cells. (B) Relative expression of CLDN1 protein. \*  $P<0.05$  compared with A549 cells. Intermittent impact method was used to construct A549/CDDP cells. After incubation with medium concentration of CDDP (1 µg/ml) for 24 h, dead cells were washed away and fresh RPMI-1640 complete medium was added. When the cells were stable, the same concentration of CDDP was added for impact, which was repeated until tumor cells were able to stably proliferate in RPMI-1640 complete medium containing 0.5 µg/ml CDDP.

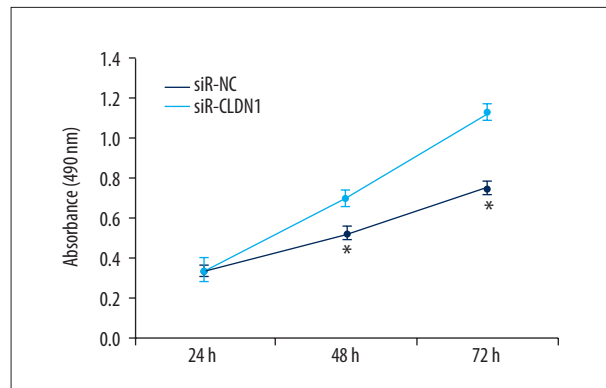




**Figure 3.** Relative expression of CLDN1 protein in A549/CDDP cells transfected with siR-NC or siR-CLDN1. Western blotting was performed to measure protein expression. \*  $P < 0.05$  compared with siR-NC group.

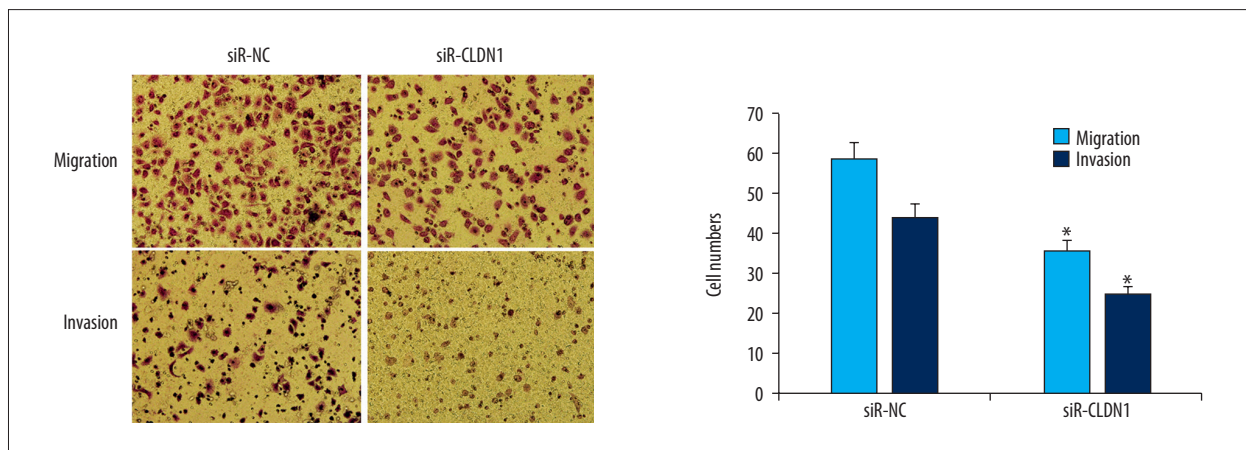
**Table 2.** Fold of drug resistance of A549/CDDP cells after silencing CLDN1 expression.

	Fold of drug resistance
A549/CDDP with siR-CLDN1	$3.06 \pm 0.16$
A549/CDDP with siR-NC	$5.03 \pm 0.24$
A549	$1.07 \pm 0.13$

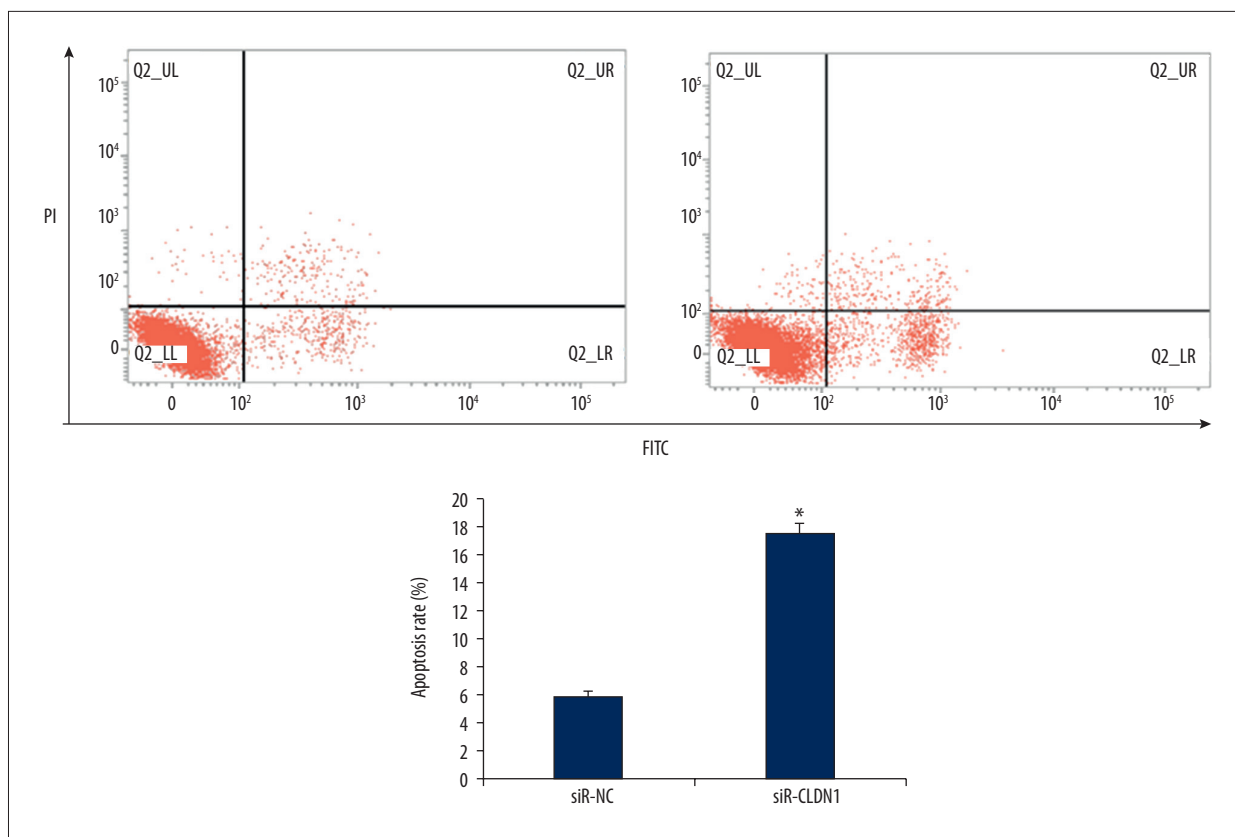


**Figure 4.** Effect of silencing of the expression of CLDN1 on the proliferation of A549/CDDP cells. Cells were seeded into 96-well plates at a density of  $1 \times 10^4$  cells per well in triplicate. For every 24 h, the cells were incubated with CCK-8 reagent (Beyotime, Shanghai, China) for 30 min. Absorbance at 490 nm was read on a microplate reader (168-1000; Model 680, Bio-Rad, Hercules, CA, USA) at 24 h, 48 h, and 72 h, and proliferation curves were plotted using absorbance values at each time point. \*  $P < 0.05$  compared with siR-NC group.

with silenced CLDN1 expression was significantly lower than that of A549/CDDP cells transfected with siR-NC ( $P < 0.05$ ) (Figure 4). Migration and invasion Transwell assays showed that the numbers of A549/CDDP cells with reduced CLDN1 expression that crossed the chamber membrane were significantly lower than that of A549/CDDP cells transfected with siR-NC ( $P < 0.05$ ) (Figure 5). These results indicate that reduced CLDN1 expression decreases the drug resistance, proliferation, migration, and invasion abilities of A549/CDDP cells.



**Figure 5.** Effect of silencing of the expression of CLDN1 on the migration and invasion of A549/CDDP cells. Transwell assay was used to determine the migration and invasion ability of cells. \*  $P < 0.05$  compared with siR-NC group.



**Figure 6.** Effect of silencing of the expression of CLDN1 on the apoptosis of A549/CDDP cells. Flow cytometry was used to detect the apoptosis rate of cells. \*  $P < 0.05$  compared with siR-NC group.

### Decreased CLDN1 expression promotes the apoptosis of A549/CDDP cells

To investigate the apoptosis of A549/CDDP cells with silenced CLDN1 expression at 24 h after induction with CDDP (0.5  $\mu\text{g/ml}$ ), flow cytometry was carried out. The data showed that the apoptotic rate of A549/CDDP cells with silenced CLDN1 expression was higher than that of A549/CDDP cells treated with siR-NC ( $P < 0.05$ ) (Figure 6). The results suggest that decreased CLDN1 expression promotes the apoptosis of A549/CDDP cells.

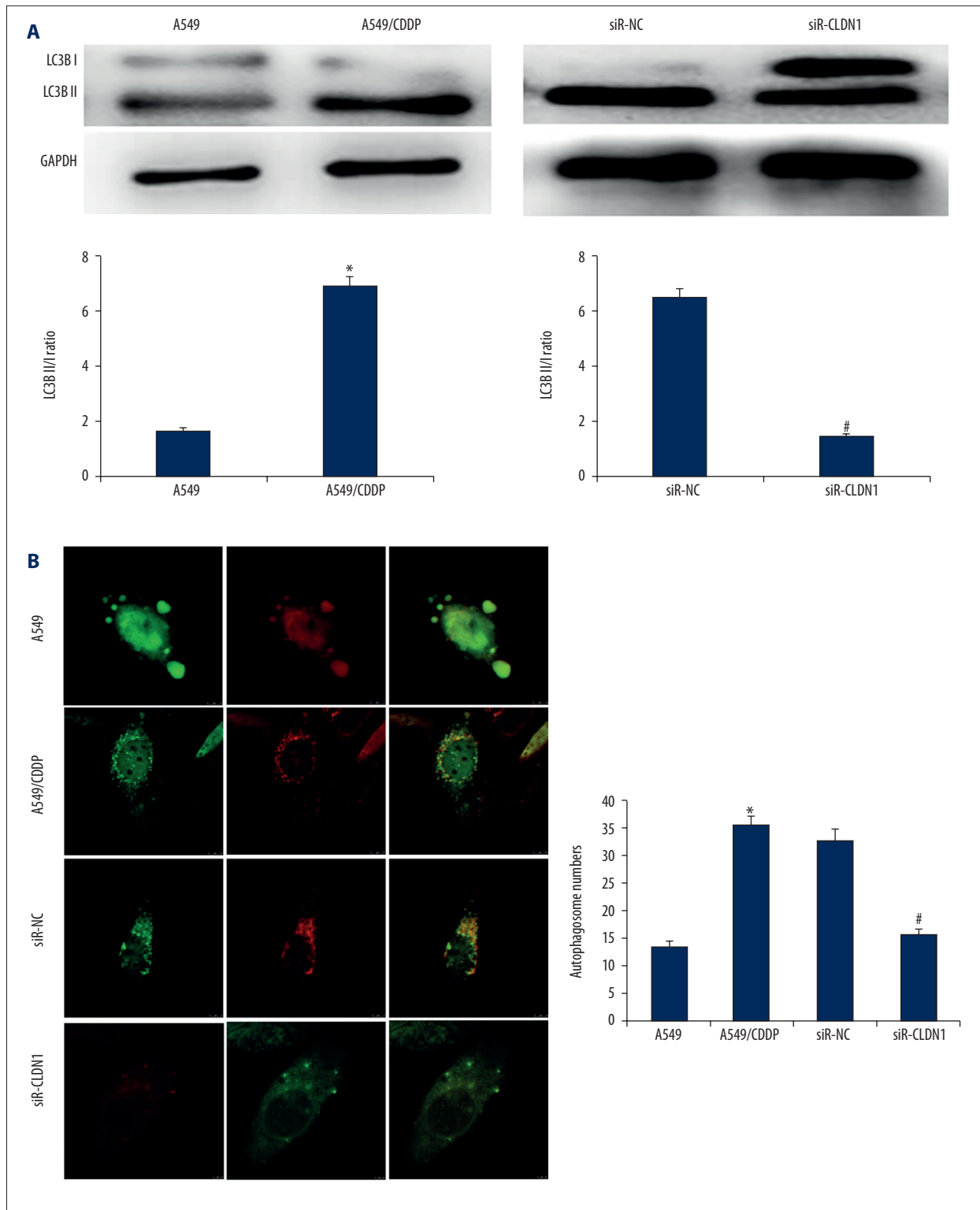
### CLDN1 enhances CDDP drug resistance of A549 cells by activating autophagy

To test whether CLDN1 enhances drug resistance, proliferation, migration, and invasion of A549 cells by activating autophagy, Western blotting and confocal microscopy were used. Western blotting showed that the ratio of LC3B II/I in A549/CDDP cells was significantly higher than that in A549 cells ( $P < 0.05$ ). In addition, the ratio of LC3B II/I in A549/CDDP cells in the siR-CLDN1 group was significantly reduced compared with that in A549/CDDP cells in the siR-NC group ( $P < 0.05$ ) (Figure 7A). Confocal microscopy of autophagocytic vacuoles after transfection with GFP-RFP-LC3B lentivirus showed that the number

of autophagosomes in A549/CDDP cells was significantly higher than that in A549 cells ( $P < 0.05$ ). In addition, the number of autophagosomes in A549/CDDP cells of the siR-CLDN1 group was significantly lower than that in A549/CDDP cells of the siR-NC group ( $P < 0.05$ ) (Figure 7B). Of note, addition of autophagy agonist Rapamycin increased the fold change of drug resistance of A549/CDDP cells in siR-CLDN1 group, while co-incubation with autophagy inhibitor 3-MA reduced the fold change of drug resistance of A549/CDDP cells (Table 3). The results indicate that CLDN1 enhances CDDP drug resistance of A549 cells by activating autophagy.

### CLDN1 promotes the autophagy of A549 cells by up-regulating the phosphorylation level of ULK1

To identify whether CLDN1 regulates autophagy via ULK1, we determined the phosphorylation level of ULK1 gene, which is crucial for autophagy, using Western blotting. The data showed that reduced CLDN1 expression down-regulates the phosphorylation level of ULK1 in A549/CDDP cells ( $P < 0.05$ ), while over-expression of CLDN1 increases the phosphorylation level of ULK1 in A549/CDDP cells ( $P < 0.05$ ) (Figure 8). The results suggest that CLDN1 promotes the autophagy of A549 cells by up-regulating the phosphorylation level of ULK1.



**Figure 7.** Effect of autophagy on CDDP resistance of A549 cells. **(A)** Expression of LC3B I and II and their ratio in A549 cells, A549/CDDP cells, and A549/CDDP cells transfected with siR-NC or siR-CLDN1. Western blotting was used to determine the expression of LC3B I and II. **(B)** Morphology and number of autophagosomes in A549 cells, A549/CDDP cells, and A549/CDDP cells transfected with siR-NC or siR-CLDN1. Confocal microscopy was used to observe autophagosomes. \*  $P < 0.05$  compared with A549 cells; #  $P < 0.05$  compared with siR-NC group.



**Table 3.** Fold of drug resistance of A549/CDDP cells with different autophagy activities.

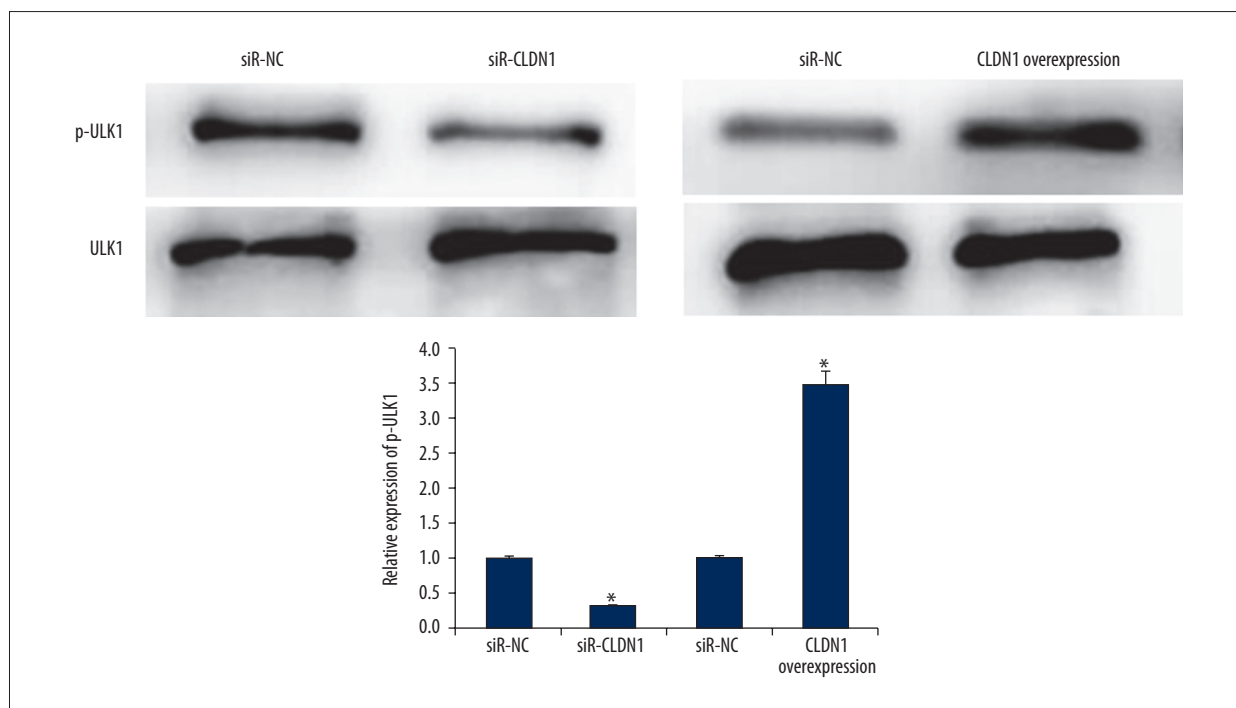
	Fold of drug resistance
A549/CDDP	5.6±0.67
A549/CDDP+3-MA	3.11±0.38
A549/CDDP with siR-CLDN1	3.76±0.27
A549/CDDP with siR-CLDN1+Rapa	5.87±0.62

## Discussion

Surgery combined with chemotherapy is one of the major methods for clinical treatment of NSCLC, and cisplatin is one of the most common drugs for chemotherapy after tumor surgery [22]. Tumor cells are very sensitive to CDDP, but they begin to develop drug resistance with chemotherapy [23]. This is an important reason for failures in clinical treatment of malignant tumors. Studies show that drug resistance of tumor cells is closely related to tumor heterogeneity, epigenetics, DNA repair, and gene expression disorders [24–26]. It is of great significance to study the molecular mechanism of tumor drug resistance. In the present study, we discovered that CLDN1 expression in NSCLC tissues is significantly elevated and is

correlated with clinicopathological features. *In vitro* experiments demonstrate that up-regulated CLDN1 expression in A549/CDDP cells increases the phosphorylation level of ULK1, activates cell autophagy, promotes drug resistance of A549/CDDP cells, and facilitates tumor proliferation and metastasis.

TJs are important functional structures in epithelial cells that maintain the epithelial barrier and polarity. TJs are composed of various protein family members, including occludin, claudin, and zo1 [10]. The expression and distribution of TJs in a variety of tumor tissues are abnormal and closely related to the invasion and metastasis of tumors. Ding et al. discovered that CLDN7 promotes the proliferation and metastasis of colon cancer by directly regulating the integrin/FAK signaling pathway [27]. CLDN1 is one of the key proteins in the formation of TJs, playing important roles in tumor recurrence and metastasis. For example, Nakagawa et al. reported that CLDN1 promotes the invasion and metastasis of colon cancer cells, and has a negative correlation with the prognosis of patients [28]. Fortier et al. showed that deletion of Keratin 8 and 18 genes induces the up-regulation of CLDN1, and promotes the proliferation, migration, and invasion of HepG2 tumor cells [29]. Jian et al. discovered that the function of CLDN1 to promote the migration and invasion of osteosarcoma cells is related to its detachment from cell membrane and entrance into the nucleus, suggesting that the intracellular localization of CLDN1 protein is closely related to tumor invasion and metastasis [30].



**Figure 8.** Effect of CLDN1 expression on the phosphorylation level of ULK1. Western blotting was performed to measure the expression of ULK1 and phosphorylated ULK1 in A549/CDDP cells in siR-NC, siR-CLDN1, or CLDN1 overexpression groups. \* P<0.05 compared with siR-NC group.

In addition, Zhou et al. reported that silencing CLDN1 expression inhibits distant migration of breast cancer cells [31]. The high expression of CLDN1 suggests that the prognosis of patients with NSCLC is not good, but whether CLDN1 is associated with CDDP drug resistance is not clear. The present study shows that increased expression of CLDN1 in NSCLC is positively correlated with lymph node metastasis and TNM staging, suggesting that CLDN1 may be an oncogene. In order to further study whether CLDN1 is associated with CDDP resistance, we constructed a CDDP-resistant A549 cell line, A549/CDDP. The A549/CDDP cell line has a drug resistance 4 times higher than that of A549 cells, and is able to grow in medium containing 0.5 µg/ml CDDP. Our data show that CLDN1 expression in A549/CDDP cells is significantly higher than that of A549 cells. Interference of CLDN1 expression by its siRNA reduces drug resistance, proliferation, migration, and invasion, but increases the apoptosis rate of A549/CDDP cells. This suggests that CLDN1 enhances drug resistance of A549/CDDP cells, and alleviates the inhibition of proliferation and metastasis of tumor cells by CDDP.

Autophagy is a process by which cells swallow their own substance or organelles and break down the enveloped contents by forming autolysosomes with lysosomes [32]. In this way, cell metabolism is achieved and organelles are renewed [32]. Inhibition of autophagy enhances the killing effect of CDDP on tumor cells, and it is of great value to determine the mechanism of autophagy for the clinical treatment of cancers [33]. For example, Jin et al. discovered that miR-26 promotes apoptosis and chemosensitivity of hepatocellular carcinoma by inhibiting autophagy [34]. Li et al. showed that miR-199a-5p enhances the sensitivity of osteosarcoma cells to cisplatin by inhibiting autophagy [35]. Our study shows that LC3B II/I ratio of A549/CDDP cells is significantly higher than that of A549 cells, and interference of CLDN1 expression decreases LC3B II/I ratio of A549/CDDP cells. Confocal microscopy shows that the number

of autophagosomes in A549/CDDP cells is significantly higher than that in A549 cells, but in the siR-CLDN1 group it is significantly lower than in the siR-NC group. Inhibition of A549/CDDP cell autophagy by addition of 3-MA significantly decreases the fold change of drug resistance of the cells, but after the promotion of autophagy of A549/CDDP cells in the siR-CLDN1 group by addition of Rapamycin, the fold change of drug resistance is enhanced. The results suggest that CLDN1 can regulate CDDP drug resistance of lung cancer cells via autophagy. ULK1 plays an important role in the regulation of autophagy in tumor cells, and it directly activates autophagy after phosphorylation [36,37]. Our results show that the phosphorylation level of ULK1 in the siR-CLDN1 group is significantly lower than in siR-NC group, while the phosphorylation level of ULK1 in A549/CDDP cells with overexpression of CLDN1 is significantly elevated, suggesting that CLDN1 results in drug resistance by up-regulating ULK1 phosphorylation and autophagy.

## Conclusions

The present study demonstrates that expression of CLDN1 in NSCLC is up-regulated and is correlated with clinicopathological features. CLDN1 activates autophagy through the up-regulation of ULK1 phosphorylation, and promotes drug resistance of NSCLC cells against CDDP. Therefore, CLDN1 is a potential molecular therapeutic target.

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## Conflict of interest

None.

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