

Original Article

Caspase-8 knockdown suppresses apoptosis, while induces autophagy and chemo-sensitivity in non-small cell lung cancer cells

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Abstract: Purpose: Drug resistance remains a major cause of relapse and therapeutic failure in non-small cell lung cancer (NSCLC). The purpose of this investigation is to explore the relationship between caspase-8 level and chemo-sensitivity, as well as its underlying mechanism in NSCLC cells. Methods: NSCLC cell line, A549 cells was used to investigate the influence of caspase-8 on the biological behavior *in vitro*. The abundance of caspase-8 in A549 cells was manipulated by transfection lentivirus containing specific caspase-8 short hairpin RNA (sh-caspase-8) and caspase-8 overexpressed plasmid. Cell viability and the percentage of apoptotic cells was quantified using cell counting kit-8 (CCK-8) assay and flow cytometry following Annexin V-FITC/PI staining, respectively. The formation of acidic vesicle organelles (AVOs) was examined by acridine orange staining and visualized under a fluorescence microscope. The mRNA and protein levels of relative genes were determined by qRT-PCR and western blotting. Results: Our results indicated that cells infected with sh-caspase-8 exhibited high knockdown efficiency. Knockdown of caspase-8 significantly reduced apoptosis of A549 cells. As evidenced by the decreased number of apoptotic cells and the reduction of Bcl-2/bax ratio. Interestingly, caspase-8 knockdown also enhanced autophagy in A549 cells. Additionally, knockdown of caspase-8 reduced the doxorubicin, carboplatin, cisplatin, and etoposide sensitivity towards A549 cells. Conclusion: In summary, our results revealed that knockdown of caspase-8 could promote cell growth and autophagy, while reduce chemo-sensitivity and apoptotic cell death. These finding suggest caspase-8 might serve as a potential target to improve the chemo-sensitivity for NSCLC patients in clinical setting.

Keywords: Caspase-8, NSCLC, apoptosis, autophagy, chemo-sensitivity

Introduction

Nowadays, cancer is a major public health problem and is the second leading cause of death worldwide followed cardiovascular disease. According to the recent cancer statistics, there are 1,806,590 new cancer cases and 606,520 cancer deaths in the United States in 2020. Among various human malignant tumors, lung cancer represents the most commonly diagnosed cancer and the leading cause of cancer death. The number of new cases and deaths were approximately 228,820 (25% of total cases) and 135, 770 (45% of total cancer deaths) in 2020, respectively [1]. Based on distinct pathological patterns, lung cancer was mainly divided into two types, small cell lung

cancer and non-small cell lung cancer (NSCLC) [2]. NSCLC is the most frequently diagnosed and accounts for 85% cancer of lung. In clinical setting, the conventional treatment regime for NSCLC patients mainly includes surgery resection, radiotherapy, chemotherapy and immunotherapy [3-5]. However, the overall survival rate for NSCLC patients was still unsatisfactory due to the inherent and/or acquired resistance, as well as the individual heterogeneity of immunotherapy [6, 7]. Therefore, it is urgent to identify novel strategies that could address the chemotherapy resistance question.

Caspase-8 is a cysteine-aspartate specific protease, which has been proven to participate in various cellular functions, such as cell apopto-

Table 1. Primers for qRT-PCR

Gene Name	Sequence	Length
GAPDH-Forward	AAAGGGTCATCATCTCTG	80
GAPDH-Reverse	GCTGTTGTCATACTTCTC	
Caspase 8-Forward	GAAGATAATCAACGACTATG	94
Caspase 8-Reverse	TTCATCTATCCTGTTCTCT	

sis, anoikis, autophagy and pyroptosis, necroptosis, and T cells differentiation [8]. Besides, numerous studies reported that caspase-8 also involved in the development of chemo-resistance in cancer therapeutic [9, 10]. However, few studies have investigated the influence of caspase-8 on the chemo-sensitivity of doxorubicin, carboplatin, cisplatin, and etoposide against NSCLC cells.

In current study, we investigated whether the abundance of caspase-8 was correlated with the chemo-sensitivity. It was found that caspase-8 knockdown effectively promoted cell growth and inhibited apoptotic cell death. Moreover, our results demonstrated that caspase-8 knockdown could induce the development of chemo-resistance in NSCLC cells.

Materials and methods

Reagents

Roswell Park Memorial Institute (RPMI)-1640 culture medium (#11875-093), fetal bovine serum (FBS) (#10099-141), penicillin and streptomycin and L-Glutamine (#190241) were purchased from GIBCO BRL (Grand Island, NY, USA). Adriamycin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin (#SC-5170), carboplatin (#YZ-100322), Etoposide (#SE87000), Cell counting kit-8 (CCK-8), and Annexin V-FITC Apoptosis Detection Kit were purchased from Solarbio (Shanghai, China). Negative control (NC), caspase-8 vector, sh-NC, sh-caspase-8 were all obtained from GenePharma Co., Ltd (Suzhou, China). Primary antibodies against LC3B (#18725-1-AP, 1:2000), p62 (#18420-1-AP, 1:1000), and Bax (#50599-2-Ig, 1:2000) were purchased from Proteintech Group, Inc (Wuhan, China). GAPDH (#P30008, 1:1000) was purchased from Abmart Inc (Shanghai, China). While Bcl-2 (#7074, 1:2000) was obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell line and culture

Human NSCLC cell line A549 was purchased from Kunming Cell Bank of the Chinese Academy of Sciences (Kunming, China). A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin, and 3 mM L-Glutamine (#1894153). The incubation condition was 37 °C and 5% CO₂, in a humidified environment. Cells in logarithmic growth phase were used for subsequent experiments.

Cell transfection

To manipulate the expression level of caspase-8 in A549 cells, we transfected A549 cells with or without Overexpression control lentivirus (MOI: 40), caspase-8 overexpressed lentivirus (MOI: 40), shRNA control lentivirus (MOI: 40), shRNA-caspase-8 lentivirus (MOI: 40) for 48 h. Following that, transfection efficiency was confirmed under an Inverted fluorescence Microscope (#TE 2000V, Nikon corporation, Japan) and detected the mRNA level of caspase-8 in each group using qRT-PCR. Transfection was performed using lentivirus.

Measurement of cell viability

Cell viability of cultured cells was detected using CCK-8. Briefly, A549 cells, caspase-8 overexpressed A549 cells, and caspase-8 knockdown A549 cells were seeded in triplicates in 96-well plates at 6000 cells/well and cultured for 24 h, then were exposed to various concentrations of adriamycin, cisplatin (#SC5-170), carboplatin (#YZ-100322), etoposide (#SE87000) for another 24 h. Cell culture media were changed into fresh media containing 10% (v/v) CCK-8 reagent at indicated times. After incubation for 2 h, the absorbance at 450 nm of each well was measured on a microplate reader.

Quantitative real time polymerase chain reaction (qRT-PCR)

The transfection efficiency of caspase-8 shRNA and caspase-8 vector was confirmed by qRT-PCR. Briefly, after transfection for caspase-8 shRNA, scramble RNA, caspase-8 vector, empty vector for 48 h, total RNA was isolated from cultured A549 cells using Trizol reagent (#15596026, Lifetech). Following that,

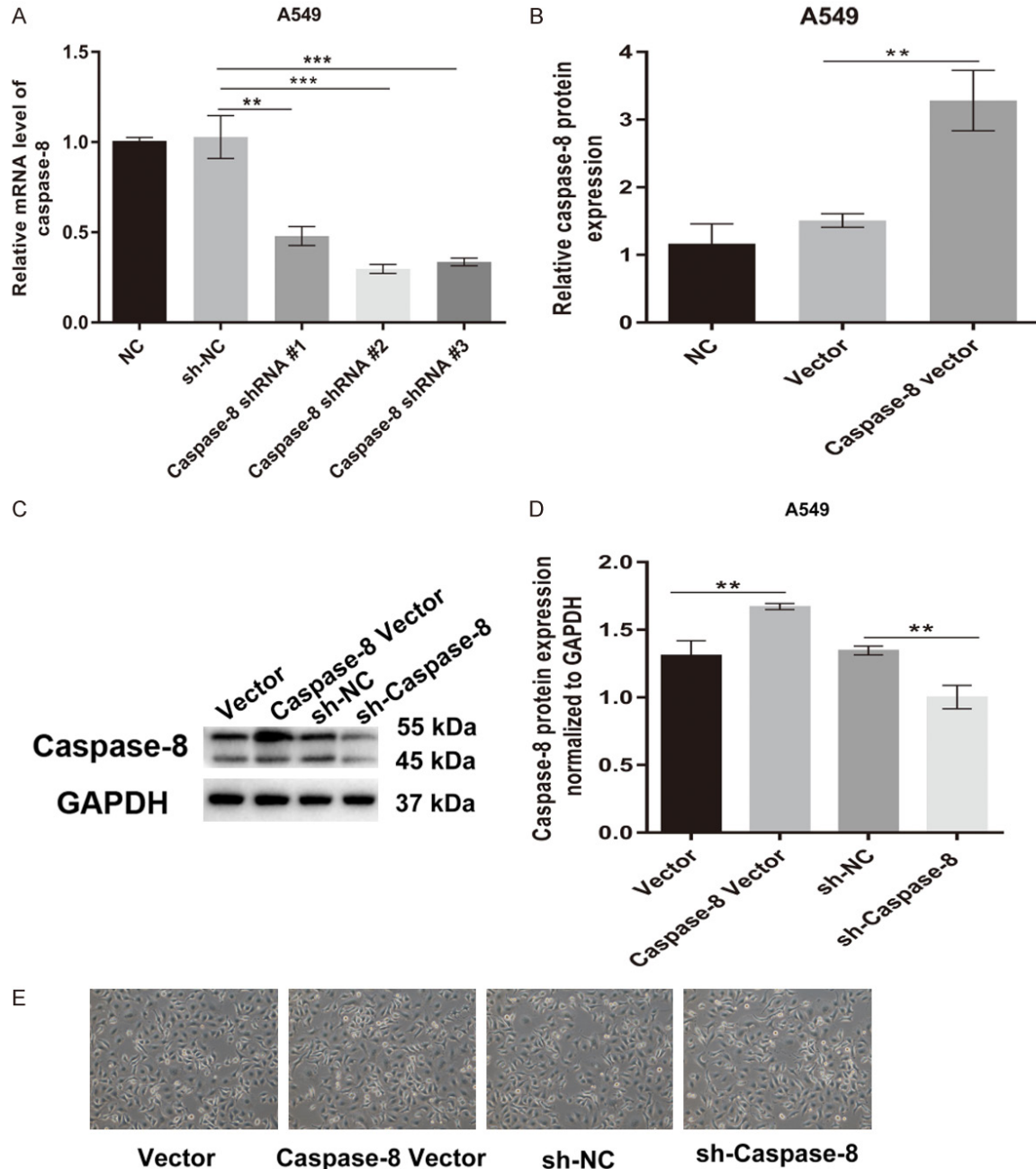


Figure 1. Knockdown of caspase-8 in A549 cells. (A) The relative expression levels of caspase-8 in cells were analyzed by using qRT-PCR after being transfected sh-NC, caspase-8 shRNA #1, caspase-8 shRNA #2, and caspase-8 shRNA #3 for 48 h. $**P<0.01$, $***P<0.001$. (B) qRT-PCR detected caspase-8 expression levels in normal A549 cells, empty vector transfected cells, and caspase-8 vector transfected cells. $**P<0.01$, $***P<0.001$. (C) The protein levels of caspase-8 in normal A549 cells, empty vector transfected cells, and caspase-8 vector transfected cells were examined by using western blotting. GAPDH was selected as internal reference. (D) Protein expression levels of caspase-8 in caspase-8 vector group cells were increased, while reduced in sh-caspase-8 group. $**P<0.01$. (E) Morphological changes of A549 cells in different transfected-groups were observed using an inverted phase-contrast microscopy (magnification: 100 $\times$). One-Way ANOVA following LSD test was used in (A). Two-tailed Student's t test was used in (B and D).

total RNA were reverse transcription into complementary DNA (cDNA) by using RevertAid-TM First Strand cDNA Synthesis Kit (#k1662,

Fermentas). Then the relative expression mRNA level of caspase-8 was quantified using SYBR Green master mix (#KK4601, KAPA). The prim-

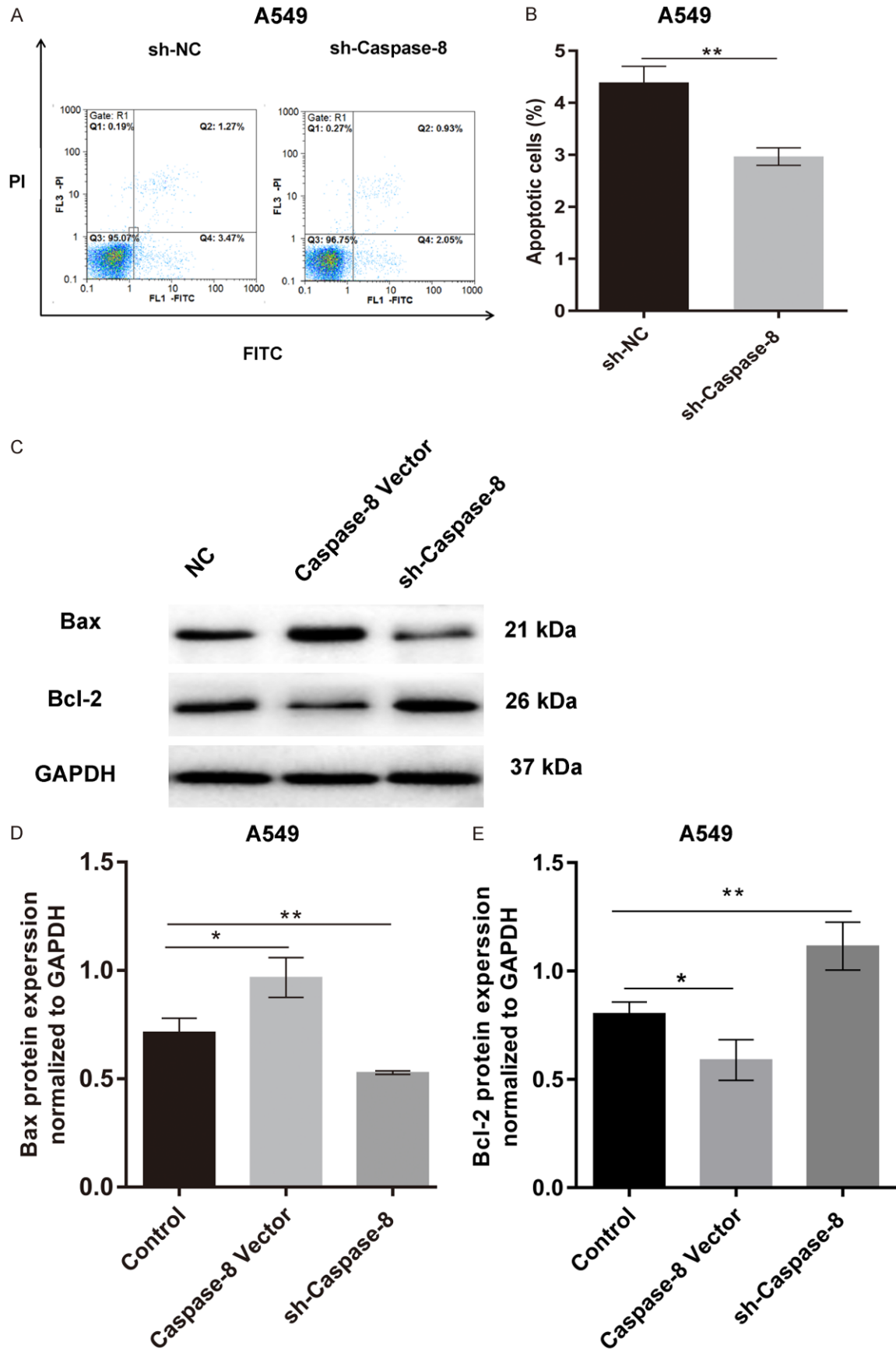


Figure 2. Knockdown caspase-8 suppresses cell apoptosis in A549 cells. (A) The number of apoptotic cells in sh-NC group and sh-caspase-8 group was quantified using Flow cytometry after Annexin V-FITC/PI staining. (B) Cell apoptosis of A549 cells in sh-caspase-8 group is markedly lower than that in sh-NC group. $^{**}P<0.01$. (C) The expression levels of apoptosis-related genes (Bax and Bcl-2) in NC group, caspase-8 vector group, and sh-caspase-8 group were detected using western blotting. GAPDH was selected as internal reference. (D) The protein expression of Bax increased in caspase-8 vector transfected cells, while reduced in sh-caspase-8 transfected cells, compared with NC group. $^{*}P<0.05$, $^{**}P<0.01$. (E) The protein expression of Bcl-2 reduced in caspase-8 vector transfected cells and increased in sh-caspase-8 transfected cells, compared with NC group. $^{*}P<0.05$, $^{**}P<0.01$. Two-tailed Student's t test was used in (B, D, and E).

ers were designed using Beacon Designer 7.90 and synthesized by invitrogen (Guangzhou, China). The primer sequences of caspase-8 were presented in **Table 1**.

Western blotting analysis

For detection of proteins expression levels in this study, western blotting was performed according to standard protocols. Briefly, following desired treatment as described, cells were harvested and lysed with RIPA buffer (Solarbio, Beijing, China) supplemented with 1000:1 protease inhibitor PMSF (Beyotime, Jiansu, China). The protein density was quantified using commercial Bicinchoninic acid (BCA) detection kit. Then proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes (Bio-Rad) and incubated with caspase-8 (1:2000), Bax (1:1000), and Bcl-2 (1:1000), LC3B (1:2000) and anti-P62 (1:1000) antibodies overnight at 4°C. The protein signals were detected using ECL chemiluminescent substrate and quantified using Gel Doc 2000 (BioRad, USA). GAPDH was served as an internal reference.

Cell apoptosis analysis

For cell apoptosis analysis, A549 cells in logarithmic growth phase were collected and plated in six-well plate at the density of 4×10^5 per well. Following incubation for indicated time, cells were collected and stained with 5 μ l Annexin V-FITC and 10 μ l propidium iodide (PI) for 15 min at dark room according to the manufacture's protocols. Following that, the ratios of apoptotic cells were quantified using flow cytometry.

Acridine orange staining

Acridine orange staining was conducted to assess the effect of caspase-8 knockdown on the development of acidic vesicle organelles (AVO) in A549 cells. In brief, cells were washed

with 1×PBS for three times. Then, cells were stained with 0.01% acridine orange (Solarbio, China) for 5 min and observed under a red filter fluorescence microscope (BX53, OLYMPUS, Tokyo, Japan).

Statistical analysis

All data were expressed as the mean $\pm$ standard errors. SPSS (Version 22.0; IBM, Armonk, NY, USA) and GraphPad Prism (Version 7.0; La Jolla, CA, USA) were used for statistics analysis and prepared graph. The differences between two groups or multiple groups were compared using Two-tailed Student's t test or One-Way ANOVA following LSD test. A *P* value less than 0.05 was considered statistically significant.

Results

Knockdown and overexpression of caspase-8 in vitro

Given the important role of caspase-8 in cell apoptosis, autophagy, and Chemo-sensitivity, we then manipulated the expression levels of caspase-8 in A549 cells by transfection caspase-8 overexpressed lentivirus and shRNA-caspase-8 lentivirus. qRT-PCR and western blotting were employed to determine transfection efficiency. As presented in **Figure 1A**, the relative expression of caspase-8 at mRNA levels were significantly reduced in three shRNA-caspase-8 lentivirus compared with vector control ($P<0.01$). Transfection efficiency for caspase-8 shRNA#1, #2, and #3 were 53%, 71%, and 67%, respectively. Consequently, shRNA#2 was chosen in further investigation. Moreover, **Figure 1B** and **1C** presented the up-regulation of caspase-8 in A549 cells after being transfected with caspase-8 vector compared with control ($P<0.01$). Consistently, western blotting analysis also confirmed that caspase-8 expression was significantly elevated in caspase-8 vector transfected group, while reduced in sh-caspase-8 infection group ($P<0.01$) (**Figure 1C**

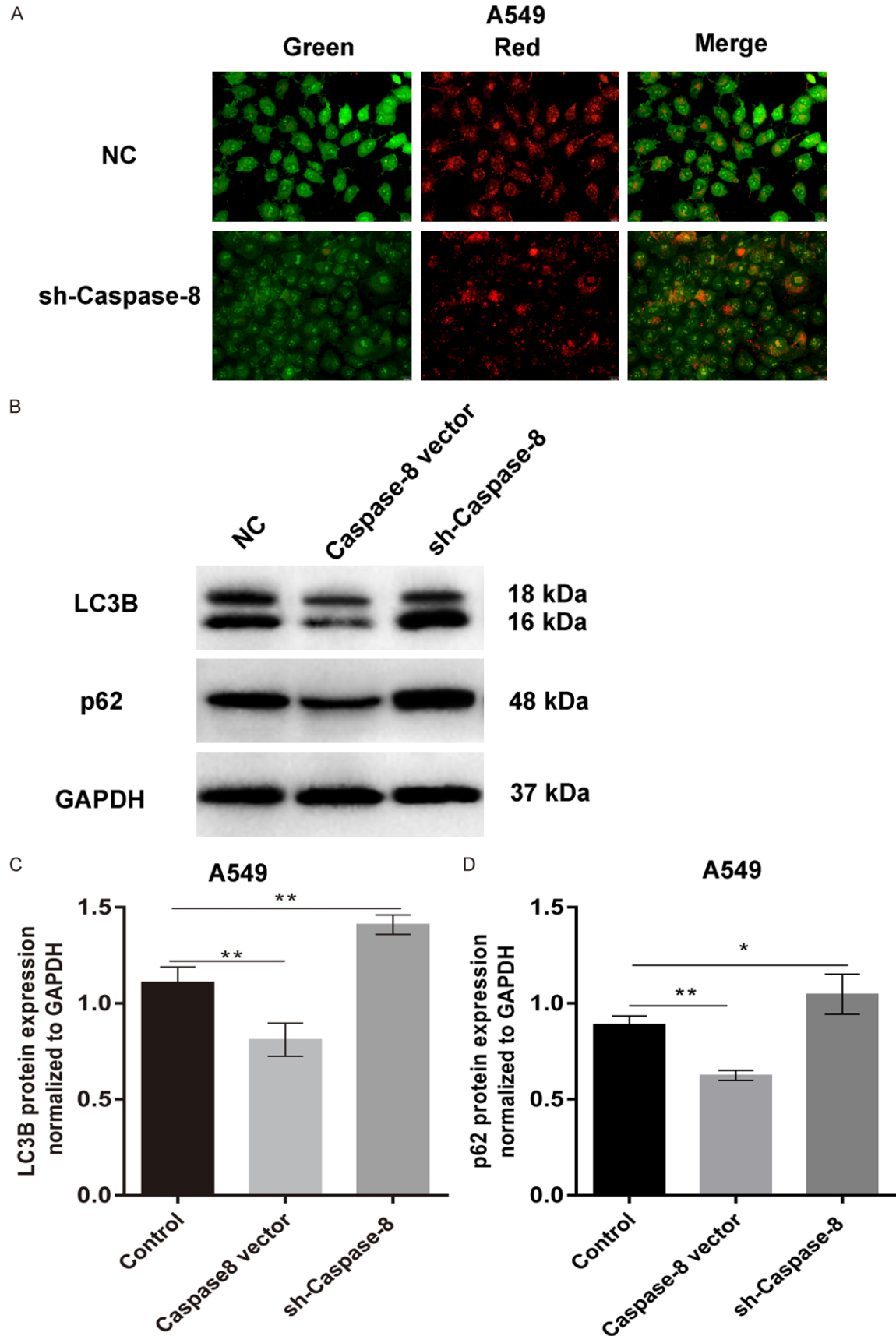


Figure 3. Knockdown caspase-8 enhances autophagy in A549 cells. (A) Knockdown of caspase-8 in A549 cells significantly increased the formation of acidic vesicular organelles (AVOs). The formation of AVOs was detected using acridine orange (AO) staining and visualized under a fluorescence microscope (Magnification: 40×). Yellow and red puncta represents AVOs. (B) The expression levels of autophagy-related genes (LC3B and p62) in NC group, caspase-8 vector group, and sh-caspase-8 group were detected using western blotting. GAPDH was selected as internal reference. (C) The ratio of LC3B I/II was significantly increased in caspase-8 vector group cells, and reduced in sh-caspase-8 cells, compared with NC group. **P<0.01. (D) The Protein level of p62 was reduced in caspase-8 vector transfected cells, while increased in sh-caspase-8 transfected A549 cells compared with NC. *P<0.05, **P<0.01. Two-tailed Student's t test was used in (C and D).

and **1D**). Additionally, we also observed the morphological changes of A549 cells using microscopy. **Figure 1E** showed that the number of nonadherent cells markedly elevated in caspase-8 vector group, while reduced in sh-caspase-8 group, when compared with control.

Knockdown of caspase-8 suppresses cell apoptosis in A549 cells

Additionally, we also detected the influence of caspase-8 knockdown on cell apoptosis. Flow cytometry results revealed that the number of apoptotic cells in caspase-8 shRNA transfected group (4.74%) was significantly reduced compared with the vector group (2.98%) (P<0.01) (**Figure 2A** and **2B**). It has been well-established that Bax and Bcl-2 are two crucial regulatory genes in cell apoptosis. Thus, we also determined the expression levels of Bax and Bcl-2 in A549 cells after transfection with sh-caspase-8 and caspase-8 vector (**Figure 2C**). Western blotting analysis results demonstrated that knockdown caspase-8 could effectively elevate Bax level, while reduce Bcl-2 expression. Caspase-8 overexpression exhibited opposite effects (P<0.01) (**Figure 2D** and **2E**). Collectively, these findings suggest that knockdown caspase-8 inhibits apoptosis of A549 cells.

Knockdown of caspase-8 promotes autophagy in A549 cells

Accumulating evidence reveals that autophagy and apoptosis are highly interconnected [11]. Multilevel crosstalk between autophagy and apoptosis in aspects of common regulators, mutual inhibition of these processes, the stimulation of apoptosis by autophagy or autophagic proteins has been validated [12]. Therefore, we also detected the influence of caspase-8 knockdown on autophagy process in cells. Firstly, we examined the formation of acidic vesicular organelles (AVOs) by using acridine orange (AO) staining. AO staining showed that

knockdown of caspase-8 promoted the formation of AVOs in A549 cells, as supported by the increased of yellow and red puncta (**Figure 3A**). Furthermore, the relative protein expression of autophagy-related proteins was examined by western blotting. As depicted in **Figure 3B-D**, the levels of LC3B and p62 were obviously increased in cells transfection with sh-caspase-8 than that in control (P<0.05, P<0.01). In contrast, caspase-8 overexpression significantly reduced LC3B and p62 levels in A549 cell. To sum up, these results suggest that caspase-8 knockdown promotes autophagy in A549 Cells.

Caspase-8 knockdown reduces doxorubicin, carboplatin, cisplatin, and etoposide sensitivity in A549 cells

Clinically, chemotherapy is one of the most important therapeutic regimen for NSCLC patients [13, 14]. Thus, we also investigated the influence of caspase-8 knockdown on the sensitivity of doxorubicin, carboplatin, cisplatin, and etoposide against A549 cells. Cells were exposed to various concentrations of doxorubicin, carboplatin, cisplatin, and etoposide for 24 h, then cell viability and IC₅₀ (half maximal inhibitory concentration) values were calculated using CCK-8 assay and Regression-probit. As presented in **Figure 4A**, the IC₅₀ value of doxorubicin towards A549 cells was notably elevated after being transfected with sh-caspase-8 (IC₅₀=24.250 μM), when compared with control (IC₅₀=11.484 μM). Interestingly, no obvious change on the IC₅₀ of doxorubicin against A549 cells and caspase-8 vector transfected cells was found. Additionally, in carboplatin, cisplatin, and etoposide treated cells, the IC₅₀ values in sh-caspase-8 transfected groups were significantly elevated, while reduced in caspase-8 groups (carboplatin, NC group: IC₅₀=66.096 μM, sh-caspase-8 group: IC₅₀=116.216 μM, caspase-8 vector: IC₅₀=51.742 μM; cisplatin; NC group: IC₅₀=6.373 μM, sh-caspase-8 group: IC₅₀=15.545 μM, caspase-8

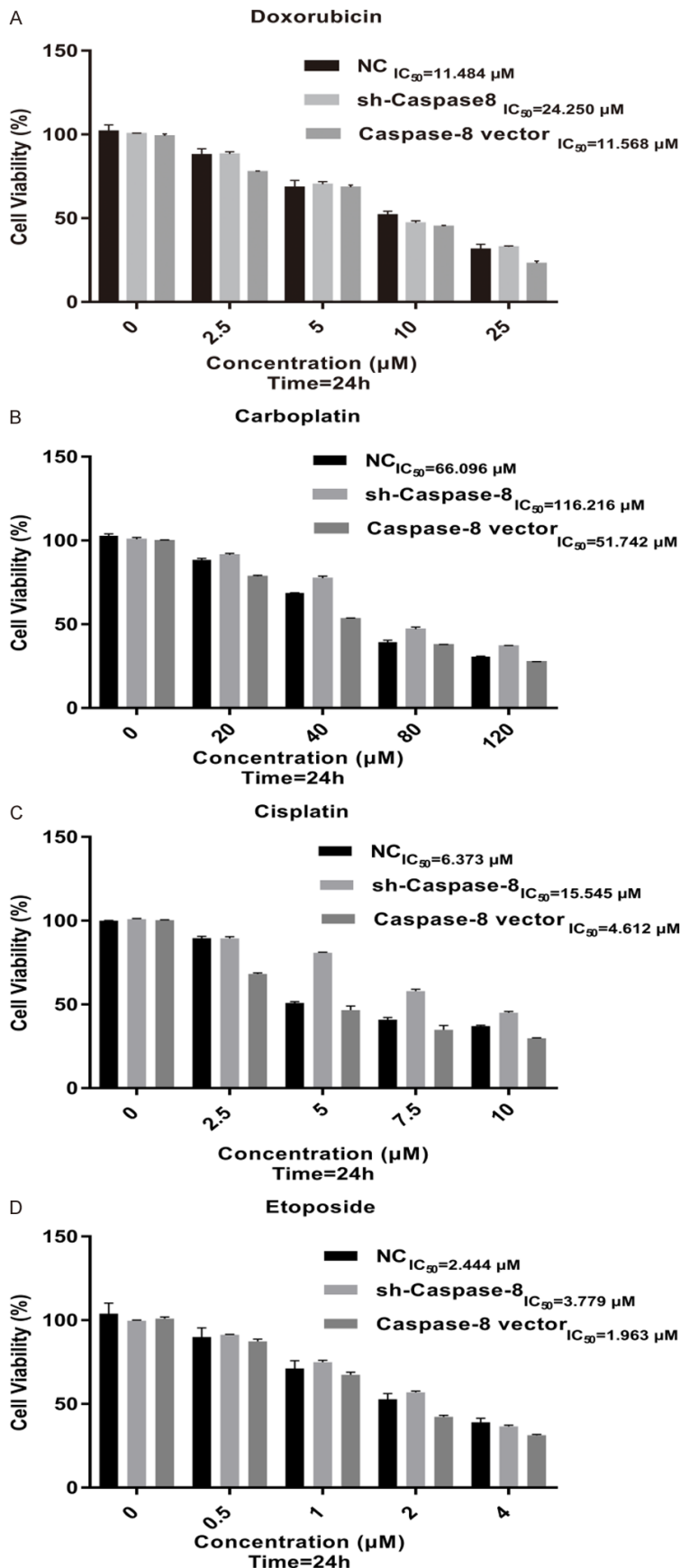


Figure 4. Knockdown of caspase-8 reduces doxorubicin, carboplatin, cisplatin, and etoposide sensitivity in A549 cells. A549 cells were exposed to various concentrations of (A) doxorubicin (0, 2.5, 5, 10, and 25 μM), (B) carboplatin (0, 20, 40, 80 and 120 μM), (C) cisplatin (0, 2.5, 5, 7.5, and 10 μM), (D) etoposide (0, 0.5, 1, 2, and 4 μM) for 24 h. Then cell viability of A549 cells in each groups was determined by using CCK-8 assay. The IC₅₀ (half maximal inhibitory concentration) values in each groups were calculated using Regression-probit.

vector: IC₅₀=4.612 μM; etoposide: NC group: IC₅₀=2.444 μM, sh-caspase-8 group: IC₅₀=3.779 μM, caspase-8 vector: IC₅₀=1.963 μM). The findings indicate the chemo-sensitivity of A549 cells was reduced after knockdown caspase-8.

Caspase-8 knockdown alleviates doxorubicin, carboplatin, cisplatin, and etoposide-induced apoptosis of A549 cells

Next, we also investigated whether knockdown caspase-8 could influence the apoptotic cell death-induced by chemotherapy. As shown in **Figure 5A**, the percentage of apoptotic cells in each groups were quantified by using flow cytometry. Results indicated that the percentage of apoptotic cells was obviously reduced in caspase-8 knockdown group when compared with control, while overexpression of caspase-8 increased the number of apoptotic cells in all chemotherapy drugs treatment ($P < 0.01$, $P < 0.001$). The percentage of apoptotic cells in each groups were as following: NC group (doxorubicin: $43.29 \pm 0.6239\%$, carboplatin: $41.33 \pm 0.4443\%$, cisplatin: $42.24 \pm 0.8364\%$, etoposide: $40.1 \pm 1.226\%$), caspase-8 vector gr-

Caspase-8 and chemo-sensitivity in NSCLC

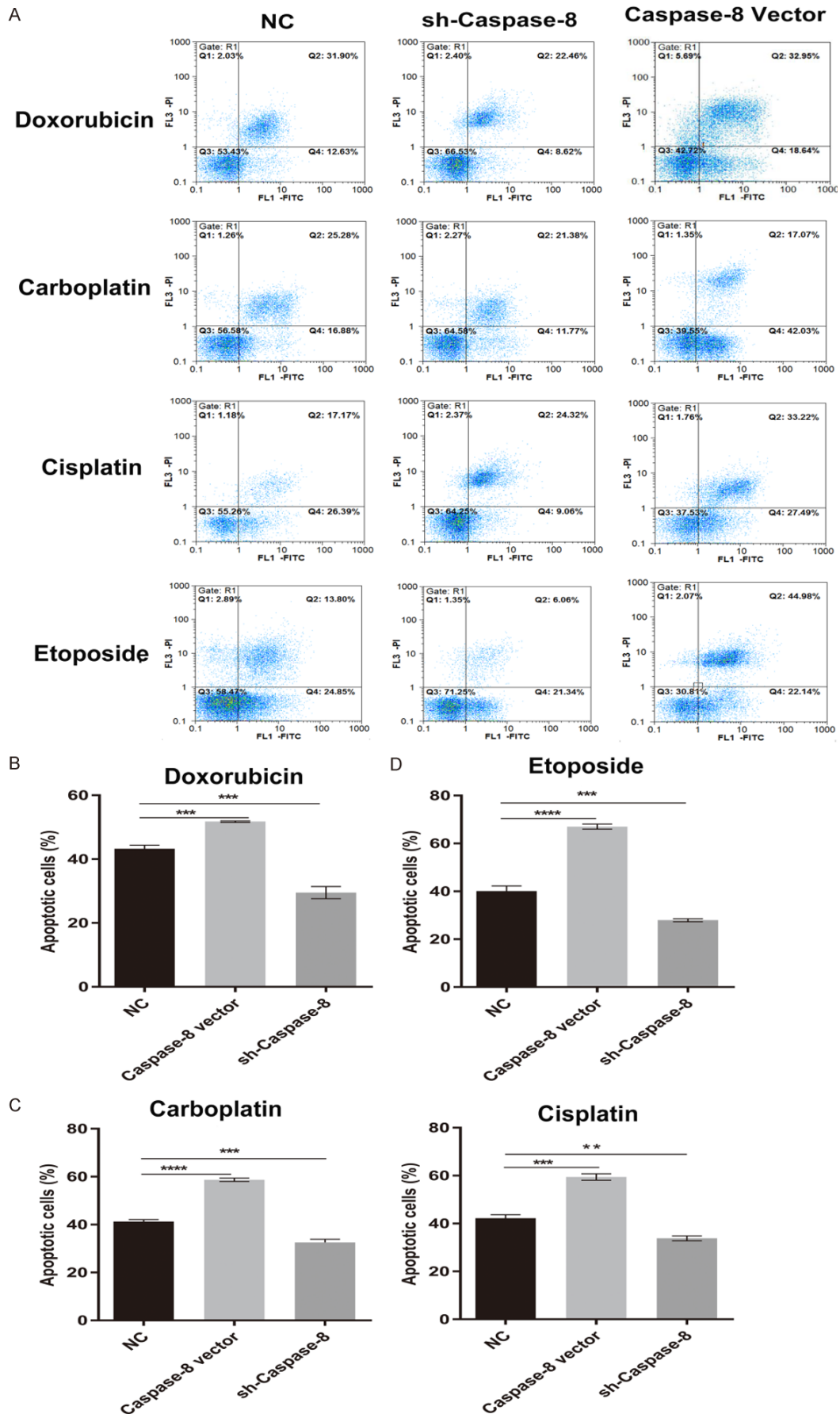


Figure 5. Caspase-8 knockdown significantly reduces apoptotic cell death induced by doxorubicin, carboplatin, cisplatin, and etoposide in A549 cells. (A) Normal A549 cells, caspase-8 vector transfected cells, and sh-caspase-8 transfected cells were treated with indicated concentration of doxorubicin, carboplatin, cisplatin, and etoposide for 24 h. The number of apoptotic cells in each group was quantified using Flow cytometry following Annexin V-FITC/PI staining. (B-D) Caspase-8 knockdown abolished doxorubicin, carboplatin, cisplatin, and etoposide-induced cell apoptosis, while caspase-8 overexpression enhanced it. ** $P < 0.01$, *** $P < 0.001$. Two-tailed Student's *t* test was used in (B-D).

oup (doxorubicin: $51.76 \pm 0.1288\%$, carboplatin: $58.72 \pm 0.4086\%$, cisplatin: $59.43 \pm 0.7571\%$, etoposide: $67.01 \pm 0.6143\%$), and sh-caspase-8 (doxorubicin: $29.54 \pm 1.095\%$, carboplatin: 32.6 ± 0.7407 , cisplatin: 33.8 ± 0.5798 , etoposide: $27.94 \pm 0.3747\%$). Collectively, these results suggest that knockdown caspase-8 could alleviate the killing effect of chemotherapeutic against A549 cells.

Discussion

NSCLC is the most frequently diagnosed lung cancer and with poor survival and serious confounding co-morbidities. Over the past several decades, we have witnessed dramatic advance in cancer treatment, especially molecular targeted therapy and immunotherapy, which leading to longer overall patient survival [15-17]. Nevertheless, the 5-year survival rate (3-17%) and mortality rates were unsatisfactory [18]. Chemo-resistance represents one of the crucial obstacles to improve NSCLC patient's survival and quality of life. Consequently, novel therapeutic strategies are urgently required for addressing Chemo-resistance for NSCLC patients.

Caspase-8, a cysteine-aspartate specific protease, classically triggers the extrinsic apoptotic pathway, in response to the activation of cell surface Death Receptors (DRs) like FAS, TRAIL-R and TNF-R [19]. In addition, recent large scale studies also reported that caspase-8 also participates in other programmed cell death, such pyroptosis and autophagy [20]. Existing evidence reveals that caspase-8 is a double-edged sword during cancer development and progression [20]. Caspase-8 could exhibit anti-tumor properties through triggering apoptotic cell death. Besides, it also exerts pro-survival function through inhibiting necroptosis [21]. It has been well established that resisting apoptotic cell death is an important hallmarks of cancer cells [22]. Several studies revealed that the depletion of pro-caspase-8 was associated with malignant transformation of tumors. In addition, caspase-8 was frequently low-ex-

pressed in cancers, and therefore contributes to resist cell apoptosis and the development of anti-cancer therapeutics resistance. Thus, caspase-8 might represent a therapeutic target for cancer treatment.

In current study, we found that knockdown of caspase-8 by sh-caspase-8 could effectively suppress apoptotic cell death in A549 cells. Furthermore, our results also confirmed that knockdown caspase-8 alleviate the killing effect of doxorubicin, carboplatin, cisplatin, and etoposide against NSCLC cells, as evidenced by the increased IC_{50} values and decreased apoptotic cells. More recently, accumulating evidence has pointed out that the down-regulation of caspase-8 expression or activity was involved in the development of resistance to chemotherapy. For instance, prostate apoptosis response-4 overcomes chemo-resistance in breast cancer cells by activation caspase-8 [23]. Furthermore, it has been found that IFN-gamma potentiated the chemo-sensitivity of cisplatin, doxorubicin and etoposide towards to medulloblastoma cells through elevating caspase-8 expression and its activity [24]. Consistent with published literature, our results showed that knockdown of caspase-8 induced chemo-resistance in NSCLC cells. Collectively, these findings suggest that activation or up-regulation caspase-8 might represent a promising strategy to confer chemo-resistance in NSCLC.

It has been proved that apoptosis and autophagy share some effector molecules and signaling pathways [8]. Among these, caspase-8 participates in cell apoptosis and autophagy process. A great body of research has demonstrated the regulator role of caspase-8 in autophagy [25]. Such as, our results indicated that caspase-8 knockdown enhanced autophagy in NSCLC cells.

In summary, current finding suggested that caspase-8 knockdown promoted cell growth and autophagy process. Moreover, we also found that the sensitivity of doxorubicin, carboplatin,

cisplatin, and etoposide towards NSCLS cells was notably reduced by caspase-8 knock-down. These data imply caspase-8 might be considered as a potential therapeutic strategy to address chemo-resistance in NSCLC. Despite these mechanistic associations, there are some major limitations in current study. Firstly, the molecular mechanisms that contribute the effect of caspase-8 on chemo-sensitivity are largely an enigma. Moreover, the crosstalk between autophagy and chemo-sensitivity has not been comprehensively addressed. Therefore, further research still needs to examine more closely the links between caspase-8 and chemo-sensitivity in NSCLC.

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Disclosure of conflict of interest

None.

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Caspase-8 and chemo-sensitivity in NSCLC

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