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Original Article

CHOP overexpression sensitizes human non-small cell lung cancer cells to cisplatin treatment by Bcl-2/JNK pathway

Li-Le Wang[1], Rui-Cheng Hu[1], Ai-Guo Dai[2], Shuang-Xiang Tan[1], Ming Xu[3], Chun-Chu Kong[1], Yun-Rong Chen[1], Dai-Yan Fu[1]

[1]Department of Respiratory Medicine, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha 410016, China; [2]Department of Respiratory Diseases, Medical School, Hunan University of Chinese Medicine, Changsha 410208, China; [3]Department of Gastroenterology Medicine, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha 410016, China

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Abstract: C/EBP homologous protein (CHOP), a 29 kDa cellular protein, plays a role in regulating tumor proliferation, differentiation, metabolism, cell death, and in tumor resistance to chemotherapy. Non-small cell lung cancer (NSCLC) is a tumor of the respiratory system and drug resistance is prevalent among NSCLC clinical cell cultures. Herein, our study elucidated the effect of CHOP on NSCLC cells with cisplatin resistance and its mechanism. In a NSCLC cell line with cisplatin-resistance, CHOP expression was decreased, compared with A549 cells. Overexpression of CHOP decreased the cell viability and enhanced cell apoptosis in the cells treated with cisplatin. Expression of CHOP also inhibited the cell proliferation and metastasis. CHOP increased the therapeutic effect of cisplatin on NSCLC cells through the Bcl-2/JNK pathway. In summary, CHOP regulated cisplatin resistance in cells of NSCLC by promoting the expression of apoptotic proteins and inhibiting the Bcl-2/JNK signaling pathway, indicating the antitumor effects of CHOP.

Keywords: NSCLC, CHOP, cisplatin, Bcl-2, JNK, apoptosis

Introduction

Lung cancer threatens human life and health, with an increased number of diagnosis worldwide, and 80% of lung cancer cases are non-small cell lung carcinoma (NSCLC) [1-3]. NSCLC is characterized by a high degree of invasiveness, and metastasis is prone to occur early. Most patients have formed distant metastases or regional lymph node metastases, and the current therapy strategies, such as surgery, radiotherapy, and chemotherapy, are often accompanied by relapse and drug resistance [4-7].

Cisplatin, an anti-tumor drug, is commonly used in various solid tumor treatments [8]. The comprehensive multidisciplinary treatment of cisplatin-based combination chemotherapy for cancer has been achieved, and chemotherapy for lung cancer may prolong the survival of patients [9]. However, due to the side effects and drug resistance, failure of tumor chemotherapy may occur and this limits the widespread use of platinum drugs [10]. Therefore, the mechanism of cisplatin-resistance and related protein regulation needs further investigation.

C/EBP homologous protein (CHOP), as also called growth arrest and DNA damage protein inducible gene 153 (GADD153) [11], was first discovered in the DNA damage response. CHOP is expressed normally at a low level in the cytoplasm of eukaryotic cells. Under stressful stimuli, CHOP aggregates and is expressed in the nucleus of eukaryotic cells [12, 13] and then activates the apoptotic pathway by affecting Bcl-2 protein expression, with cell oxidation reactions or caspase-12 cleavage [14]. Many studies have shown the role that CHOP plays in cell apoptosis [15]. By activating the AP1 and

AARE1 regions in the CHOP promoter, CHOP is activated to promote its transcription and expression, thereby stimulating the downstream signaling protein Bcl-2 [16]. The decrease of Bcl-2 expression leads to the death of tumor cells and achieves anti-tumor efficacy.

Herein, A549 and A549/DDP cells were used to investigate the effects of CHOP in NSCLC cells. Our results show anti-chemotherapy resistance effects of CHOP overexpression in NSCLC, as CHOP could sensitize A549/DDP cells to cisplatin treatment. This might be achieved by Bcl-2/JNK signaling regulation. Thus, CHOP may exert antitumor effects on cisplatin-resistant NSCLC cells.

Materials and methods

Cell culture

Human normal NSCLC cells (A549) and cisplatin-resistant NSCLC cells (A549/DDP) were were incubated with Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The incubator was maintained at 37°C in moist air with 5% CO_2. When the cells reached about 90% fusion density, the culture was ended with 0.25% trypsin.

Cell viability assay

The cells (cell density 5×10^4) were incubated in 96-well plates for 24 h, and then treated with chemotherapy drugs. After treatment, 20 μL 5 mg/mL MTT solution was added in each well and incubated for 2~4 h. 100 μL DMSO was added to dissolve Formazan after supernatant was removed. The absorbance at 490 nm (optical density value, OD value) was detected using microplate reader (BIO-RAD, USA). An estimated concentration of 50% growth inhibition (IC_{50}) was produced for each drug based on the relative survival curve. The inhibition (%) = $(1\text{-}OD_{treated}/OD_{control})$ 100.

RT-qPCR

Total mRNA was extracted from cell lines by Trizol (Ambion, Austin, TX, USA), cDNA reverse and PCR-amplified as described in previous reports [17]. mRNA expression data were calculated relative to GAPDH, using the $2^{-\Delta\Delta Ct}$ method. Primers were: CHOP F: 5'-GGGCATCACCTCCTGTCTGTCTC-3'; CHOP R: 5'-TCCCCTCCTCAGCATGTGCAG-3'; GAPDH F: 5'-AAGTTCAACGGCACAGTCAA-3'; GAPDH R: 5'-TACTCAGCACCAGCATCACC-3'.

Western blot

Cells were lysed by RIPA buffer (Thermofisher Scientific, USA). The total protein was quantified by BCA method, and the upstream protein in each well was 50 μg. After protein separation, transfer and incubation with 5% skim milk, the membrane containing proteins was incubated with primary antibody and HRP-conjugated secondary antibodies at room temperature for 1 h. The antibody complexes were detected by Enhanced electrochemiluminescence (ECL) Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA). The protein band intensity was determined by densitometry using ImageJ software. The quantification of proteins was relative to loading controls and control group.

Cell transfection

The cells were transfected with siRNA-NC, siRNA-CHOP, pcDNA3.1-NC and pcDNA3.1-CHOP (Qiagen, Valencia, CA, USA) respectively. The siRNA sequences for CHOP were 5'-GGAAGAACUAGGAAACGGA-3' and antisense, 5'-UCCGUUUCCUAGUUCUUCC-3'. For overexpression of CHOP, the sequences were chop-F: CGGAATTCTGTTAAAGATGAGCGGGTGG and chop-R: CGGGATCCGGCTGAAATGGAAGTGCTGG. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the instructions [17].

Cell apoptosis detection

Apoptosis assay kit (Sigma, St. Louis, MO, USA) and flow cytometry (BD Biosciences, CA, USA) were used to determine the cell apoptosis according to the instructions [18]. The apoptotic rate was also calculated by FlowJo software (Tree Star Inc. Ashland, USA). The apoptotic cells were the cells in Annexin V-positive areas, including late apoptosis (Annexin V^+/PI^+) and early apoptosis (Annexin V^+/PI^-).

Wound healing assay

Cells (6×10^5) were added in 6 well plates for 12 h. Then, we used a sterile pipette tip to make a scratch in the surface of these wells,

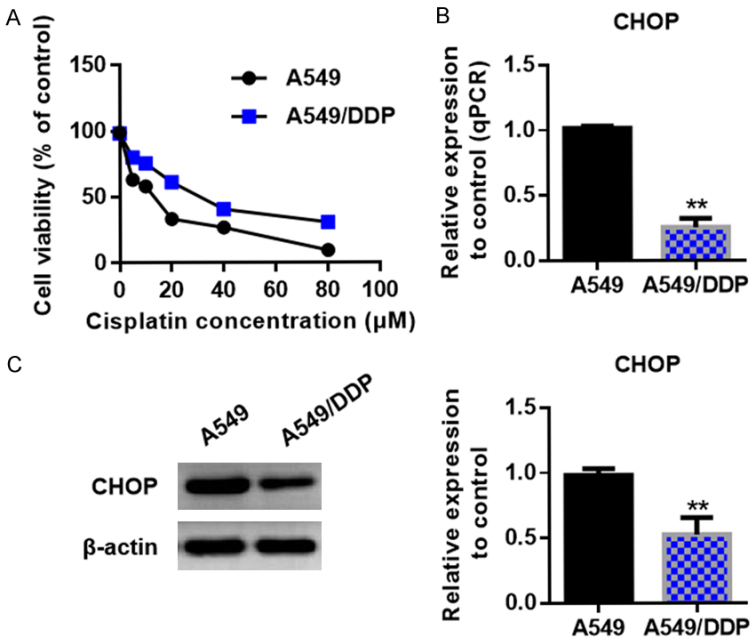

Figure 1. CHOP expression was decreased in A549/DDP cells. A. The cell viability was measured by MTT in cells treated with different concentration of cisplatin. B. mRNA expression of CHOP was determined by RT-qPCR. C. Expression of CHP protein was detected by western blot.

and each well was washed with PBS. Pictures of these cells were taken immediately after the scratch was made and 48 h later. The cells were observed and photographed under a microscope (Nikon, Japan). The relative migration rate of cells was obtained by counting the percentage of wound healing area.

Determination of migration

Transfected or control cells were placed in the upper layer of the transwell compartment at 20 μM of cisplatin (15663-27-1, Meilun Biotechnology Co., Ltd., Dalian, China). The conditioned medium was added to the lower layer of the transwell chamber. The unpenetrated cells were swabbed off with cotton swabs, and the bottom side of the invaded surface was fixed with cold methanol to immobilize the cells which were stainedwith crystal violet. We counted the cells in the field under a microscope (Nikon, Japan).

Statistical analysis

All results were performed for three independent experiments and calculated by SPSS 18.0 software; Student's *t*-tests were used for between group comparisons of the means of quantitative data, and $*P < 0.05$ was considered significant.

Results

CHOP expression decreased in cisplatin-resistant NSCLC cells

Compared with the normal NSCLC cell line A549, cytotoxicity of cisplatin was decreased significantly in A549/DDP, which indicated that the viability inhibition ability of cisplatin was suppressed in A549/DDP cells (**Figure 1A**). In A549 and A549/DDP cells, the expression of CHOP was determined transcriptionally and translationally. Compared with A549 cells, CHOP mRNA and protein was decreased in A549/DDP cells (**Figure 1B**, **1C**). This suggests that CHOP expression was downregulated in NSCLC cells with cisplatin-resistance. The results suggested an antitumor role played by CHOP in NSCLC cells.

Upregulation of CHOP increased sensitivity of NSCLC cells to cisplatin

CHOP overexpression plasmid was used to transfect cells to make CHOP overexpressed and they were co-incubated with cisplatin. The CHOP overexpression plasmid significantly upregulated the protein expression level of CHOP (**Figure 2A**). After determining the cell viability and IC_{50} in the cells treated with cisplatin, overexpression of CHOP reduced the IC_{50} of cisplatin (**Figure 2B**). This indicated that CHOP expression sensitized NSCLC cells to cisplatin treatment. The effects of CHOP expression on apoptosis NSCLC cells were also determined. Overexpression of CHOP promoted cisplatin-induced cell death (**Figure 2C**), suggesting that CHOP promotes cisplatin-induced apoptosis.

CHOP knockdown decreases sensitivity of NSCLC cells to cisplatin

CHOP siRNA transfection was used to decrease the expression of CHOP (**Figure 3A**). CHOP knockdown decreased the cell viability inhibi-

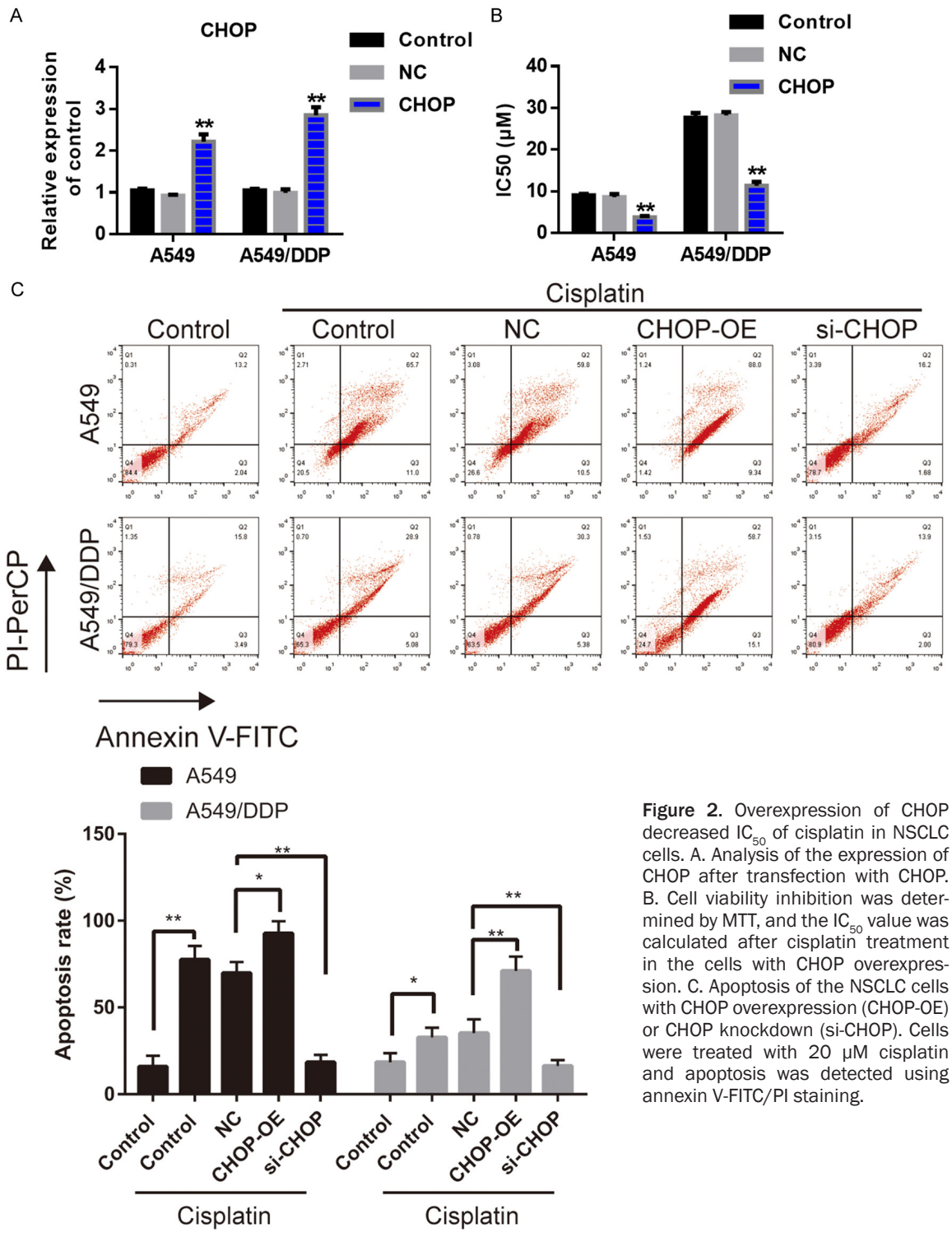

Figure 2. Overexpression of CHOP decreased IC_{50} of cisplatin in NSCLC cells. A. Analysis of the expression of CHOP after transfection with CHOP. B. Cell viability inhibition was determined by MTT, and the IC_{50} value was calculated after cisplatin treatment in the cells with CHOP overexpression. C. Apoptosis of the NSCLC cells with CHOP overexpression (CHOP-OE) or CHOP knockdown (si-CHOP). Cells were treated with 20 μM cisplatin and apoptosis was detected using annexin V-FITC/PI staining.

tion ability of cisplatin in NSCLC cells (**Figure 3B**). Besides, CHOP knockdown also reduced the cell death rates induced by cisplatin in NSCLC cells (**Figure 2C**), suggesting that inhibition of CHOP expression reduced the sensitivity of NSCLC cells to cisplatin.

Upregulation of CHOP enhanced cisplatin's ability to inhibit proliferation and metastasis of NSCLC cells

The role that CHOP expression played in cell proliferation and metastasis was examined.

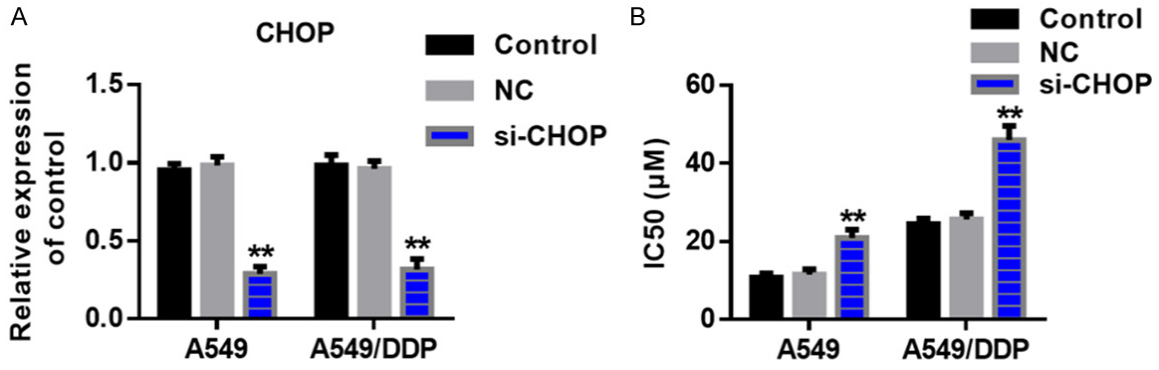

Figure 3. CHOP knockdown decreased the IC_{50} of cisplatin in NSCLC cells. A. Analysis of the expression of CHOP after transfection with CHOP. B. Cell viability inhibition was determined by MTT, and IC_{50} value was calculated after cisplatin treatment in the cells with CHOP knockdown.

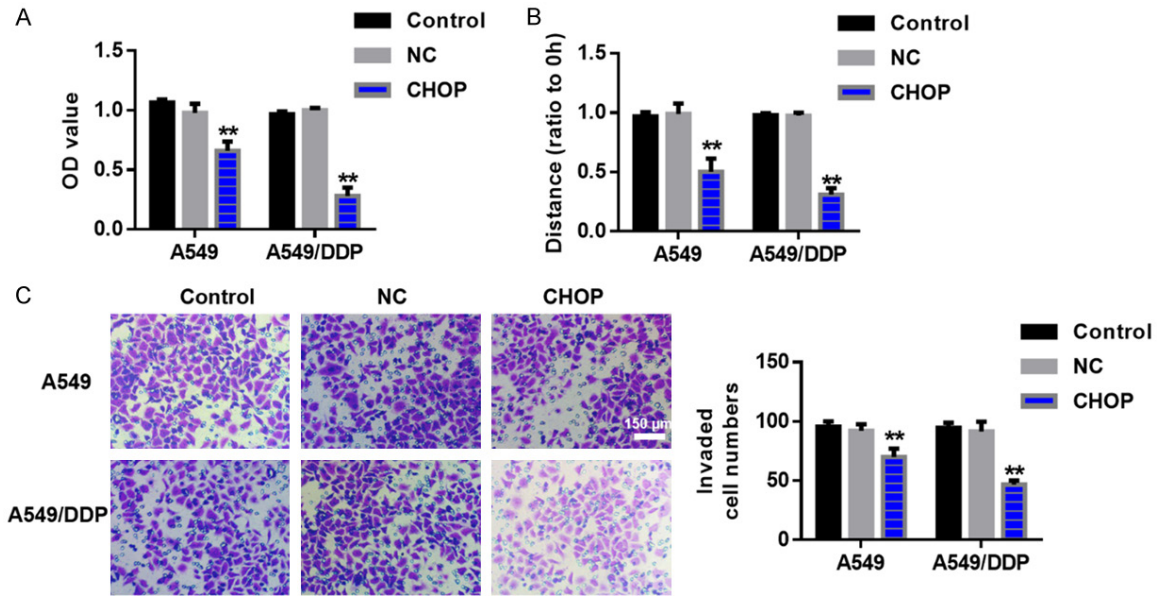

Figure 4. Up-regulated CHOP enhances cisplatin's ability to inhibit NSCLC cell proliferation, migration, and invasion. A. Effect of up-regulated CHOP on NSCLC cell proliferation after cisplatin treatment. B. Effect of up-regulated CHOP on NSCLC cell migration after cisplatin treatment. C. Effect of up-regulated CHOP on NSCLC cell invasion after cisplatin treatment (bar: 150 μm).

The results showed that when CHOP was overexpressed, cisplatin inhibited cell proliferation significantly (**Figure 4A**). The inhibition of cisplatin on the ability of cell migration and invasion was also enhanced by the overexpression of CHOP (**Figure 4B**, **4C**). In contrast to overexpression of CHOP, CHOP knockdown decreased the inhibitory ability of cisplatin on cell proliferation of NSCLC cells (**Figure 5A**). The inhibitory effects of cisplatin on cell migration and invasion were also weakened with the knockdown of CHOP (**Figure 5B**, **5C**). This suggested that CHOP is involved in regulating the inhibitory effects of cisplatin on cell proliferation and metastasis of NSCLC cells.

CHOP regulates sensitivity of cisplatin to A549/DDP cells through BCL-2/JNK signaling pathway

Finally, the molecular mechanism by which CHOP sensitized A549/DDP cells to cisplatin was explored. After overexpression of CHOP, proteins in the Bcl-2/JNK signaling pathway were determined. In A549/DDP cells, overexpression of CHOP inhibited expression of Bcl-2

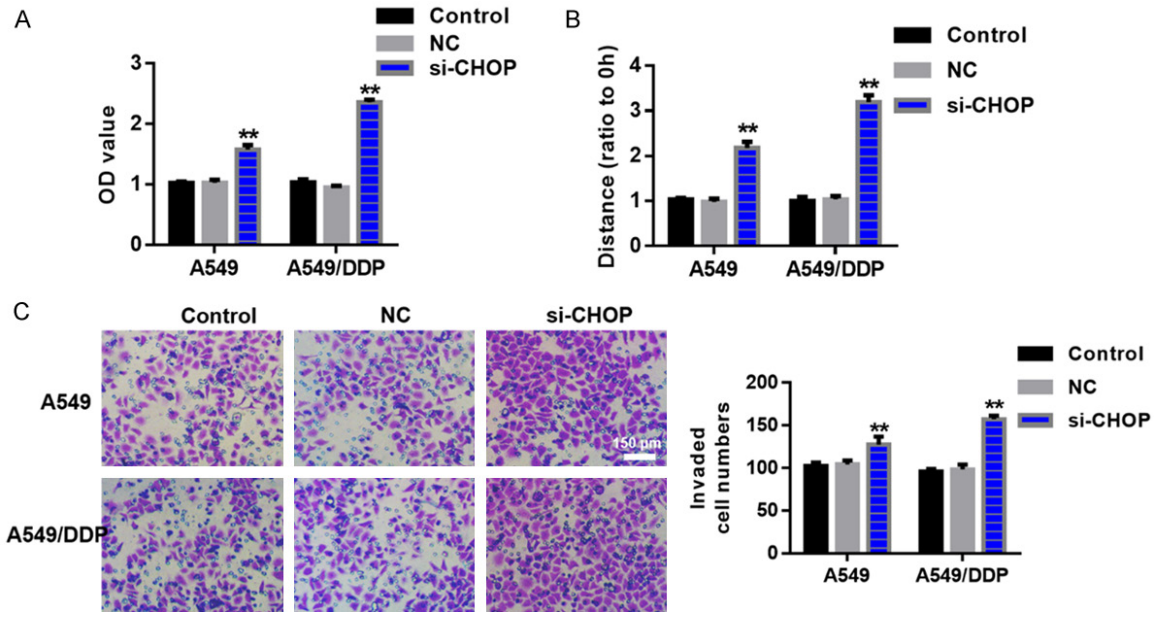

Figure 5. Down-regulation of CHOP decreases cisplatin's ability to inhibit NSCLC cell proliferation, migration, and invasion. A. Effect of knockdown of CHOP on proliferation of NSCLC cells after cisplatin treatment. B. Effect of knockdown of CHOP on migration of NSCLC cells after cisplatin treatment. C. Effect of knockdown of CHOP on the invasion of NSCLC cells after cisplatin treatment (bar: 150 μm).

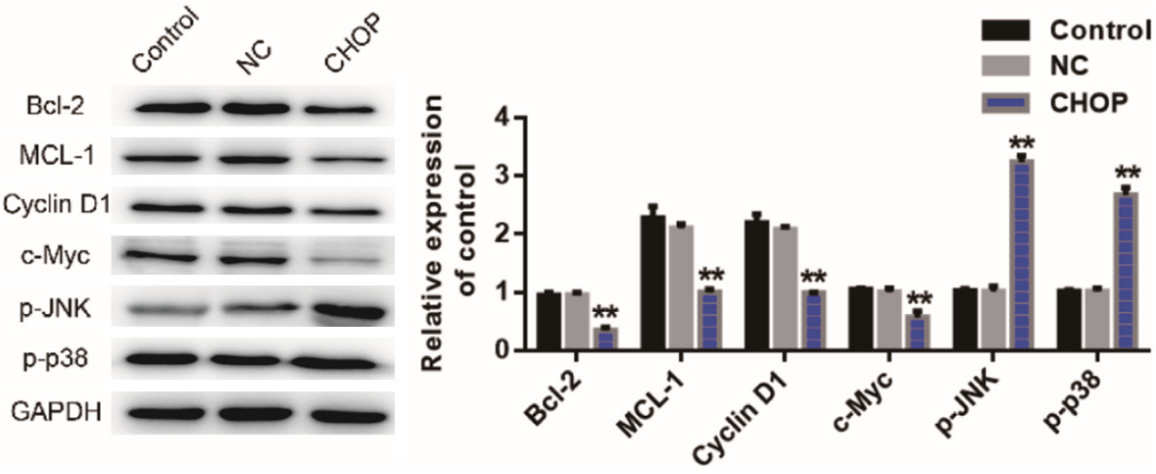

Figure 6. CHOP regulates the BCL-2/JNK signaling pathway. Overexpression of CHOP inhibits Bcl-2, MCL-1, Cyclin D1, c-Myc, p-JNK, and p-p38 expression levels in A549/DDP cells after cisplatin treatment.

and MCL-1 protein, as well as Cyclin D1 and c-Myc. At the same time, overexpression of CHOP up-regulated the expression of p-JNK and p-p38 protein (**Figure 6**), suggesting that CHOP might promote apoptosis by decreasing the expression of BCL-2 and MCL-1, and increasing JNK phosphorylation to enhance the chemotherapy sensitivity of A549/DDP cells to cisplatin.

Discussion

Lung cancer seriously threatens human health [19, 20]. Cisplatin is widely used as an effective chemotherapy drug for lung cancer, while drug resistance in tumor cells limits the use of cisplatin [21]. The development of cisplatin resistance involves multiple mechanisms. Membrane transporter-mediated drug efflux is

one of the important mechanisms leading to cisplatin resistance. Overexpression of P-gp and MRP1 can reduce the drug's effect on lung cancer cells toxic effects [22]. The drug resistance of lung cancer cells caused by chemotherapy is one of the important reasons for the failure of cancer treatment [23]. There are many reasons leading to chemotherapy resistance of cisplatin, among which apoptosis evasion is a major factor leading to the failure of chemotherapy [24]. Recently, it has been reported that CHOP plays a regulatory role in chemotherapy resistance of tumors. Herein, we verified the role of CHOP in sensitizing NSCLC cells to cisplatin treatment, even in cisplatin-resistant cells, showing an antitumor effect. We verified that CHOP expression was suppressed in A549/DDP, the cisplatin-resistant NSCLC cells. Overexpression of CHOP could sensitize the NSCLC cells to cisplatin treatment, including cell viability inhibition, apoptosis induction, proliferation, and metastasis suppression; while CHOP knockdown alleviated the effects of cisplatin. This indicates that CHOP acts as a key factor in the mechanism of cisplatin resistance.

JNK is a mitogen-activated protein kinase (MAPK) in mammalian cells [25]. JNK signaling pathway has been found to be involved in a variety of physiological and pathological processes, including proliferation, apoptosis, and cell cycle [26-29]. The key antitumor roles of JNK and CHOP have also been shown. For example, Kaempferol induces cell death in gastric cancer cells by the JNK/CHOP axis, and corosolic acid promotes drug-resistant prostate cancer cell to undergo apoptosis [30, 31]. In this study, we found that overexpression of CHOP can promote the activation of the JNK pathway, up-regulate the phosphorylation level of JNK, and then play a synergistic role in cisplatin-induced apoptosis. The Bcl-2 family plays a key role in the regulation of apoptotic signals, among which Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein [32]. When Bcl-2 expression is up-regulated and/or Bax expression is down-regulated, the pro-apoptotic effect of cisplatin can be significantly inhibited [18]. Previous studies showed a correlation between Bcl-2 family and cisplatin-induced cell death and related drug resistance [18]. The studies also emphasize the role that Bcl-2 plays as a predictor of cisplatin response; thus, the combination of Bcl-2 inhibitor ABT-263 with cisplatin exerts notable antitumor efficiency on lung cancer treatment [33].

In summary, we verified that CHOP expression was negatively related to cisplatin resistance. Overexpression of CHOP could decrease the apoptosis evasion of A549/DDP, and suppress cell proliferation and metastasis. The role of CHOP in anti-cisplatin resistance may be achieved by regulating JNK pathway and expression of apoptosis-related proteins. These results indicate that CHOP plays a key regulatory role in cisplatin-resistant NSCLC cells, providing a new approach for the treatment of drug resistance in NSCLC.

Disclosure of conflict of interest

None.

Address correspondence to: Rui-Cheng Hu, Department of Respiratory Medicine, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha 410016, China. E-mail: huruicheng@hotmail.com

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