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Apatinib resensitizes cisplatin-resistant non-small cell lung carcinoma A549 cell through reversing multidrug resistance and suppressing ERK signaling pathway

Z.-L. LIU[1], B.-J. JIN[1], C.-G. CHENG[1], F.-X. ZHANG[1], S.-W. WANG[1], Y. WANG[1], B. WU[2]

[1]Department of Cardiothoracic Surgery, the 1st Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, China
[2]Molecular Test Center, the 1st Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, China

Abstract. – OBJECTIVE: To observe the reversal effect of apatinib on the resistance to cisplatin (DDP) of A549/cisplatin (A549/DDP) cells and its relevant mechanism.

MATERIALS AND METHODS: A549/DDP cells were treated with the control method, apatinib alone, DDP alone and DDP combined with apatinib. The cell proliferation was detected by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the cell clone formation assay. The cell apoptosis was detected by Hoechst 33258 staining and annexin V and propidium iodide (PI) double labeling. The changes in apoptotic proteins, multidrug resistance protein 1 (MDR1) and extracellular signal-regulated kinase (ERK) signaling pathway proteins in each group after treatment were detected by Western blotting.

RESULTS: MTT assay results showed that compared with A549 cells, A549/DDP cells had obvious resistance to DDP. MTT assay and cell clone formation assay revealed that the tumor inhibition rate of the sub-lethal dose of apatinib (10 μM) combined with DDP was higher than that of DDP alone. The apoptosis detection results indicated that the proportion of apoptotic cells in the apatinib (10 μM) combined with DDP group was significantly increased. Western blotting results revealed that compared with that in parental A549 cells, the expression level of MDR1 in A549/DDP cells was significantly increased, and the ERK signaling pathway was activated. In the apatinib combined with DDP group, the levels of cleaved caspase-3, cleaved caspase-9 and B-cell lymphoma-2 (Bcl-2)-associated X (BAX) proteins were significantly upregulated, while the level of Bcl-2 proteins was downregulated. Apatinib could inhibit the expression of MDR1 and the activity of the ERK signaling pathway in a dose-dependent manner.

CONCLUSIONS: Apatinib can restore the sensitivity of A549/DDP cells to DDP by down-regulating the expression level of MDR1 and inhibiting the activity of the ERK signaling pathway.

Key Words:
Apatinib, DDP, Lung cancer, MDR1, Drug resistance.

Introduction

Lung cancer is one of the malignant tumors around the world, the mortality rate of which ranks first. Cisplatin (DDP) is one of the relatively effective chemotherapy drugs for the treatment of lung cancer[1]. However, recurrence and metastasis still occur in many patients with lung cancer after the initial chemotherapy. The main reason for the failure is that the tumor cells develop multiple drug resistance (MDR)[2]. A large number of studies have shown that the over expression of P-glycoprotein [multidrug resistance protein 1 (MDR1)] is the main cause of MDR[3]. MDR1 is encoded by MDR1 gene, which is a member of the largest subfamily of the adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transmembrane transporters and is the most common cause of tumor MDR. P-glycoprotein can act as an energy-dependent drug efflux pump, which affects the characteristics of many clinical chemotherapy drugs[4]. A research has shown that MDR1 is expressed in almost all the tumor cells to different degrees. The chemotherapy drug is an important

Corresponding Author: Zhiliang Liu, MD; e-mail: liuzhiliangdr@163.com

cause inducing the over expression of P-glycoprotein in the treatment process of tumors, increasing the incidence rate of tumor cell MDR. Dong et al[5] showed that MDR1 is significantly and highly expressed in lung adenocarcinoma A549/DDP-resistant cell lines compared with that in parental A549 cells, so it becomes an important target for reversing the tumor resistance. Since the first generation of MDR reversal agents such as verapamil came out, a number of tumor MDR reversal agents for various MDR-related ABC transporters have been applied in the clinical trial phase, but there is still no mature drug being clinically applied.

The mitogen-activated protein kinase (MAPK) pathway is a bridge that binds extracellular stimuli to intracellular gene expression, which plays an important role in tumor drug resistance[6-8]. At present, most studies have concluded that the excessive activation of extracellular signal-regulated kinase (ERK) is positively correlated with the drug resistance of many tumors. Its mechanism may be to regulate the expressions of MDR1 genes and P-glycoprotein. Kisucka et al[9] found that the MDR of mouse leukemia cell line L1210/vincristine (VCR) is related to the sustained activation of ERK, which in turn increased expression level of P-glycoprotein, finally decreasing the concentration of intracellular VCR. However, specific inhibitors of ERK can inhibit ERK activity, thereby increasing intracellular drug concentration and reversing L1210/VCR drug resistance. All of the above studies have shown that MAPK pathway may be involved in the regulation of the MDR of P-glycoprotein, so the study and development of MAPK pathway inhibitors have become breakthrough points of studies on the reversal of drug resistance.

Apatinib mesylate is a new small molecule anti-angiogenic agent that highly and selectively inhibits the activity of vascular endothelial growth factor receptor-2 (VEGFR-2) tyrosine kinase and blocks the signal transduction that binds VEGF to its receptor, thereby strongly inhibiting tumor angiogenesis and playing an anti-tumor role[10]. Registered clinical trials of apatinib at Phase I, II and III have shown that clear and objective curative effects and significant survival benefits can be achieved when apatinib is used in patients with advanced gastric cancer who have undergone standard chemotherapy failure[11]. Mi et al[12] demonstrated that apatinib can increase the accumulation of doxorubicin and rhodamine 123 in the highly expressed P-glycoprotein or breast cancer resistant proteins in cells, thus significantly increasing the toxicity of the drug to cells. Based on the above research, this work aimed to explore the reversal effect of apatinib on the DDP resistance of lung cancer, thus providing some ideas for clinical reversal treatment of lung cancer resistance.

Materials and Methods

Materials

Anti-BAX, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-ERK, anti-p-ERK, anti-MDR1 and anti actin antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Hoechst and annexin V/propidium iodide (PI) staining kits were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China), and other experimental reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Cell Culture

A549/DDP cells were purchased from the Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and DDP-resistant A549/DDP cells were produced by the induction of DDP concentration gradient. The above cells were placed in an incubator at 37°C with 5% CO_2 for culture with the minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution, 1% non-essential amino acid as the culture medium.

Induction Methods for Drug-resistant Cells

A549/DDP cells were induced by long-term co-incubation with parental A549 cells and DDP solution at gradient concentrations. Initially, A549 cells were co-incubated with 1 μM DDP for 48 h, and then the dead cells were removed from the culture medium. After that, A459 cells were digested, passed on, and cultured in the fresh cell culture medium. After the cells were well adhered to the wall, DDP was added, and the final concentration of DDP in the medium was 2 μM. The cells were further incubated with DDP for 48 h. Repeat culture and passage of cells induced the production of drug-resistant cells by drugs at gradually increased concentrations, and ultimately obtained A549/DDP cells that could grow

in the environment with 50 μM DDP. Besides, 50 μM DDP was used for continuous induction for 3 months so as to maintain its resistance to stability.

Detection of Cell Activity

5×10^3 cells were seeded in 96-well plates and were incubated. Next, the cells were adherent to the wall, they were co-incubated with DDP at gradient concentrations for 48 h, and were cultured *in vivo* to the logarithmic growth phase. The adherent cells were digested by 0.25% axillary enzyme, and then prepared as a single cell suspension, counted, and centrifuged. The cell concentration was adjusted to 5×10^4/mL, and cells were added to 96-well plates at 100 μL/well. After that, cells were cultured in an incubator at 37°C with 5% CO_2 for 24 h. Drugs at the corresponding concentration were added, and after 48 h incubation, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) detection was conducted.

Detection of Cell Apoptosis

The A549/DDP cells in the logarithmic growth phase were digested and cultured in 6-well plates. 4 wells were selected and added with drugs at the corresponding concentration each for incubation for 24 h. The cells were digested and taken out for incubation with fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-) fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min at room temperature. The flow cytometry was used for detection, which showed a cell scatter plot with four quadrants. The results of flow cytometry were analyzed by the flow cytometry software. The experiment was repeatedly conducted for 3 times.

Clone Formation Experiments

Cells in the logarithmic growth phase were inoculated in 6-well plates at 400/well. After incubation for 24 h, cells were incubated and cultured in the cell culture medium containing the corresponding concentration of drugs for 48 h, when the cells were well spread adherent to the wall. After that, the cells were further cultured in the culture medium not containing drugs for 12 days, washed with phosphate-buffered saline (PBS), fixed by 10% formaldehyde and stained by Giemsa stain; finally, the pictures were taken. The cell colony formed by more than 50 cells was regarded as a clone. The experiment was repeatedly conducted for 3 times.

Hoechst 33342 Staining

A549 cells in the logarithmic growth phase were inoculated in 6-well plates. After the adherence to the wall, cells were treated with the treatment methods in the control group, the 10 μM apatinib group, the 16 μM DDP group and 10 μM apatinib combined with 16 μM DDP group for 24 h. The supernatant was discarded, and cells were washed with PBS twice. 1 mL Hoechst 33242 fluorescent stain was added, and after the cells were incubated at 37°C for 15 min, the fluorescent stain was discarded. After PBS washing, cells were observed and pictures were taken under an inverted fluorescence microscope. The experiment was repeatedly conducted for 3 times.

Western Blotting

The cells were washed with pre-cooling PBS, and then fully lysed with radioimmunoprecipitation assay (RIPA) cell lysate. The supernatant was removed after the centrifugation. After proteins were quantified, 30 μg total protein sample was mixed with 5×sodium dodecyl sulfate (SDS) protein loading buffer and denatured at 100°C for 5 min. The loading sample received SDS polyacrylamide gel electrophoresis (PAGE) at 350 mA, after which the gelled and activated polyvinylidene difluoride (PVDF) membrane was placed in the transfer film for membrane transfer at the constant current for 2 h. Then, the PVDF membrane was removed and sealed in 5% skim milk powder for 1 h; primary antibodies were added for incubation at 4°C overnight. The membrane was washed with Tris-buffered saline with Tween 20 (TBST), added with horseradish peroxidase (HRP)-labeled secondary antibodies, and then incubated at room temperature for 1 h. After that, TBST was used for washing, and pictures were developed. ImageJ software was applied to analyze the gray value. The expression level of actin represented the relative expression level of the target protein. The experiment was repeatedly conducted for 3 times.

Statistical Analysis

All statistics were performed using Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 20.0. The *t*-test was performed for the comparison of continuous data, and analysis of variance (ANOVA) for the comparisons between groups. Least Significant Difference (LSD) was used as its post hoc test. $p < 0.05$ suggested the differences were statistically significant.

Results

Apatinib Promoted the Sensitivity of A549/DDP Cells to DDP

Firstly, in order to confirm the drug resistance of the induced A549/DDP-resistant cells, MTT assay was used to detect the inhibitory effects of different concentrations of DDP on parental A549 cells and A549/DDP cells, respectively. MTT analysis results showed that the half maximal inhibitory concentration (IC50) of DDP to A549 was 12 μM and to A549/DDP cells was 120 μM. Compared to A549 cells, A549/DDP cells showed significant drug resistance (Figure 1A). In order to explore the inhibitory effect of apatinib alone on A549/DDP cells, different concentrations of apatinib were used to be co-incubated with A549/DDP cells. The results revealed that apatinib showed a certain dose-independent inhibitory effect (Figure 1B). In order to further investigate whether apatinib synergistically facilitated the sensitivity of A549/DDP-resistant cells to DDP, apatinib at a non-lethal dose combined with DDP at gradient concentrations was co-incubated with A549/DDP cells for 48 h. Of note, apatinib and DDP exhibited synergistic inhibition effect on A549/DDP cell growth with the increase of dose. The results indicated that 10 μM apatinib combined with 16 μM DDP reflected a high synergistic inhibition rate (Figure 1C). The subsequent cell clone formation experiment further validated the synergistic effect. The results revealed that the number of cells formed by clones in the apatinib combined with DDP group was significantly lower than that in the apatinib group or DDP group, suggesting that apatinib combined with DDP can significantly inhibit the cell clone formation (Figure 1DE).

Apatinib Combined with DDP Synergistically Facilitated the Apoptosis of A549/DDP Cells

Many investigations have shown that drug-resistant cells have resistance to apoptosis, so in order to study the mechanism of apatinib combined with DDP in inhibiting A549/DDP, the effect of the combination of the two drugs on apoptosis was further explored. Firstly, the flow cytometry was used to detect the apoptosis rates of the control group, apatinib group, and DDP group (Figure 2A). Data showed that the apoptosis rate

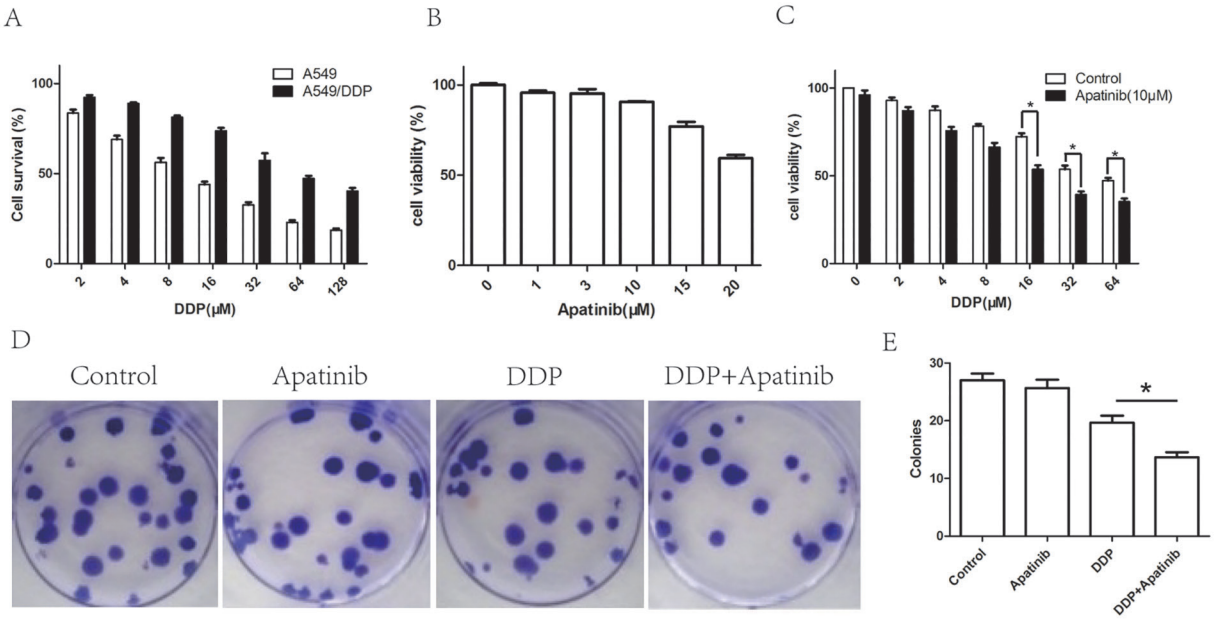

Figure 1. Apatinib sensitizes the cytotoxic effect of DDP in A549/DDP cells. ***A,*** A549 and A549/DDP cells were treated with indicated concentrations of DDP for 48 h. ***B,*** A549/DDP cells were treated with indicated concentrations of Apatinib for 48 h. ***C,*** A549/DDP cells were treated with 10 μM Apatinib combined with indicated concentrations of DDP for 48 h. Cell viability was tested by MTT assay. The data were expressed as mean ± SD obtained from 3 independent experiments. ***D,*** Representative photos of colonies in control, 10 μM Apatinib, 16 μM DDP, Apatinib combined with DDP treated group. ***E,*** Number of colony formed per dish after indicated reagents treated. Three independent experiments were performed. *$p < 0.05$.

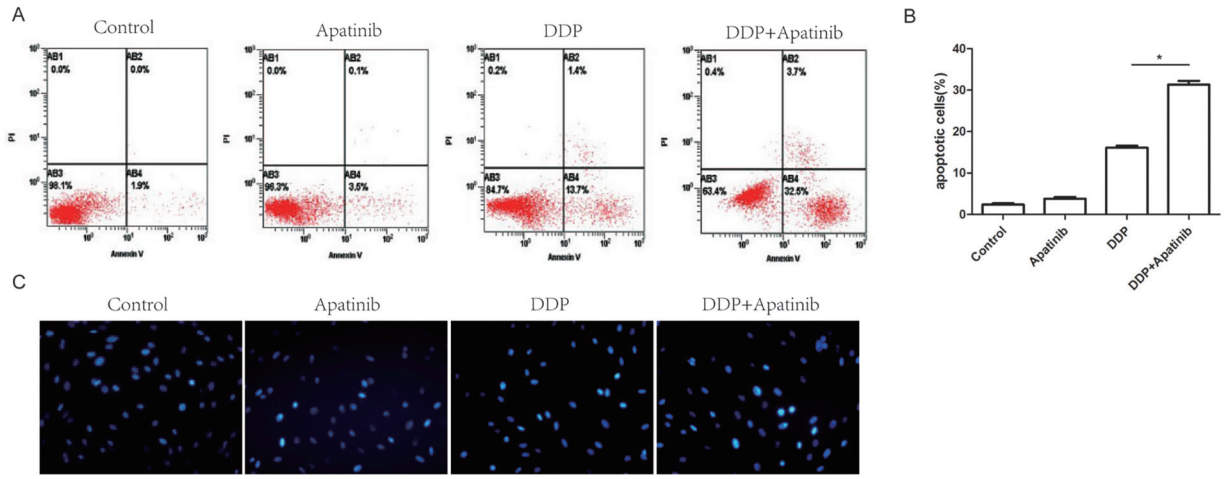

Figure 2. Apatinib sensitizes A549/DDP cells to DDP induced apoptosis. ***A-B,*** A549/DDP cells were treated with MEM, 10 μM Apatinib, 16 μM DDP or the combination of DDP and Apatinib for 24 h. Cells were analyzed by Annexin V/PI staining. Data shown are representative of 3 independent experiments. Data are presented as means ± SD (n = 3). *$p < 0.05$. ***C,*** A549/DDP cells were cultured with MEM, 10 μM Apatinib, 16 μM DDP or the combination of DDP and Apatinib for 48 h. Cell apoptosis was observed by Hoechst 33342 staining.

of the apatinib group was 3.6%, that of the DDP group was 15.1%, and that of the combined treatment group rate reached as high as 36.2%, indicating that 10 μM apatinib alone did not significantly inhibit the apoptosis, but the apoptosis rate was significantly increased after the combined treatment with apatinib and DDP (Figure 2B), which were consistent with the results of Hoechst 33342 staining. Compared with the single-drug treatment group, there were more apoptotic cells in the combined treatment group (Figure 2C).

Apatinib Combined with DDP Promoted the Changes in the Expression of Apoptosis-related Proteins

The expression of apoptosis-related proteins plays an important role in the apoptotic signal transduction. In order to verify the proapoptotic effect of apatinib combined with DDP on A549/DDP cells from the protein expression level, Western blotting was applied to explore the changes in the expression of apoptosis-related proteins after drug treatments. The results showed that compared with those in the two single-drug treatment groups, the levels of cleaved caspase-3, cleaved caspase-9 and BAX proteins were up-regulated in the combined treatment group, while the level of BCL-2 proteins was significantly down-regulated. Cleaved caspase-3, cleaved caspase-9, and BAX proteins, belong to pro-apoptotic proteins, which play key roles in regulating the mitochondrial apoptosis pathway. Bcl-2 proteins play negative roles in the regulation of apoptosis, and its high expression can inhibit cell apoptosis. This study confirmed that apatinib combined with DDP could promote the apoptosis of A549/DDP cells by up-regulating the expression levels of cleaved caspase-3, cleaved caspase-9, BAX proteins and other pro-apoptotic proteins and down-regulating the expression level of Bcl-2 (Figure 3 AB).

Apatinib Inhibited the Expression of A549/DDP-resistant Proteins and the Activation of the ERK Pathway

The high expression of drug-resistant proteins is one of the main causes of drug resistance in tumor cells. Drug-resistant cell lines are often accompanied by high expression of drug-resistant proteins. The expressions of MDR1 in parental A549 cells and A549/DDP cells were detected by Western blotting. The results showed that MDR1 was obviously and highly expressed in A549/DDP cells, indicating that MDR1 proteins played key roles in the resistance of A549/DDP cells to DDP. A relevant research has shown that the activation of ERK is also involved in the formation of drug resistance, so blocking MAPK could partially reverse drug resistance[13]. Therefore, the expressions of ERK in the two kinds of cells were demonstrated. The results revealed that ERK and p-ERK are significantly and highly expressed in A549/DDP cells. In view of the above results, we will focus on MDR1 and ERK signaling path-

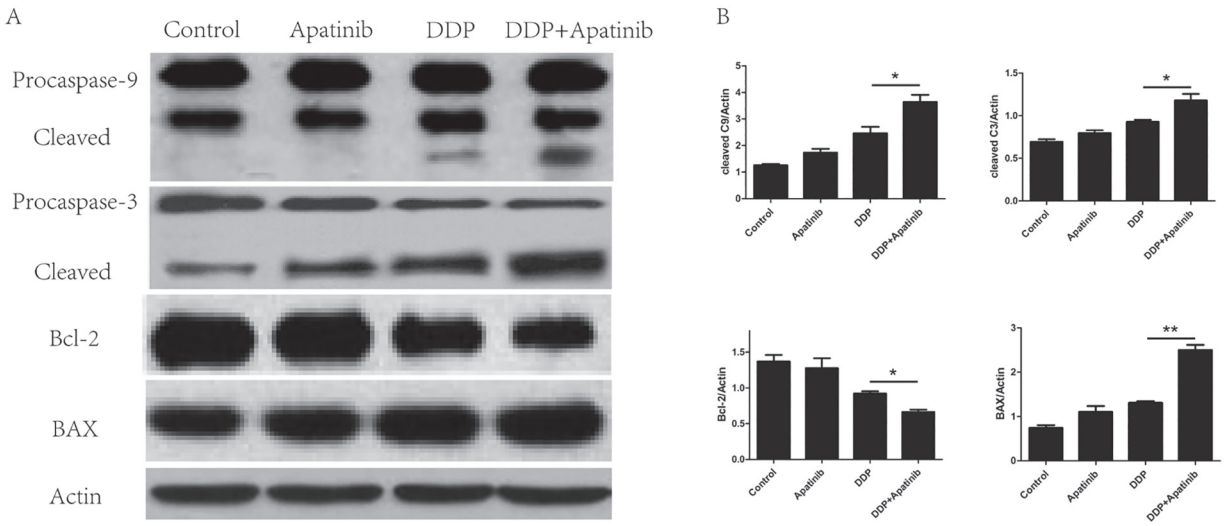

Figure 3. Apatinib promoted the changes in the expression of apoptosis-related proteins induced by DDP. (A, B) A549/DDP cells were treated with MEM, 10 μM Apatinib, 16 μM DDP or the combination of DDP and Apatinib for 24 h, and the expression level of target proteins (procaspase-9, cleaved caspase-9, procaspase-3, cleaved caspase-3, Bcl-2, BAX) measured by Western blot. Data shown are representative of 3 independent experiments. Data are presented as means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

ways. In this study, whether apatinib reversed the drug resistance of lung cancer by acting on MDR1 or ERK signaling pathways, was investigated. Western blotting results showed that apatinib was able to inhibit MDR1 and ERK signaling pathways in A549/DDP cells in a dose-dependent

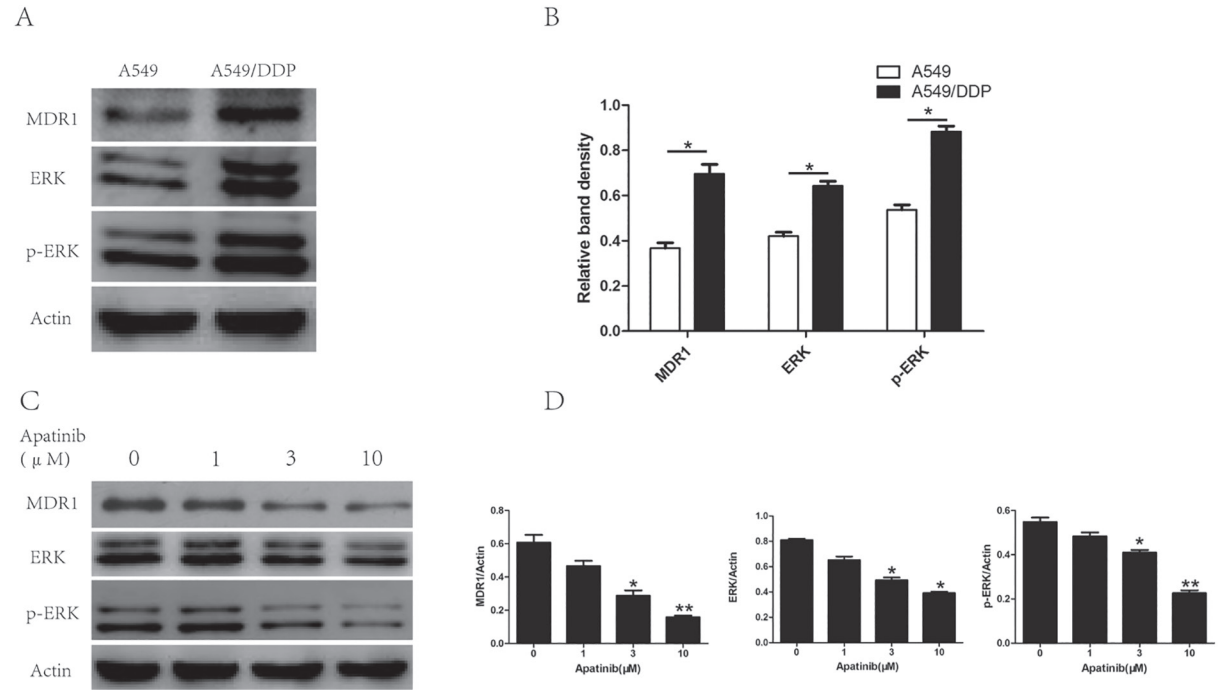

Figure 4. Effect of apatinib on the expression of target proteins (MDR1, ERK, p-ERK) in A549/DDP cells. ***A-B,*** The expression level of MDR1, ERK, and p-ERK in A549 and A549/DDP cells. ***C-D,*** A549/DDP cells were treated with indicated doses of Apatinib for 24 h, and the expression level of target proteins (MDR1, p-ERK, ERK) measured by Western blot. Data shown are representative of 3 independent experiments. Data are presented as means ± SD (n = 3). *$p < 0.05$,**$p < 0.01$.

manner (Figure 4CD), indicating that apatinib can restore the sensitivity of A549/DDP cells to DDP by down-regulating MDR1 and ERK signaling pathways.

Discussion

Lung cancer is one of the most common malignant tumors. Platinum drugs are a class of widely used chemotherapy drugs, which show good effects in a variety of malignant tumor treatments. However, the drug resistance of cancer cells developed in the process of lung cancer treatment often leads to the failure of the treatment, so it is an urgent need to look for new drugs that can be used to clinically reverse drug resistance for improving the clinical benefits of patients[14]. One of the mechanisms of tumor cells producing resistance to chemotherapy drugs is that the over expressed MDR1 and other multidrug-resistant proteins increase the efflux of drugs, resulting in reduction of the accumulation of drugs in cells and the effective concentration in the drug target site. Therefore, the study on MDR1 targets has become one of the major research hotspots. However, there is no drug clinically applied for MDR1 and other multidrug-resistant proteins. Studies have shown that ERK pathway is significantly associated with MDR1 expression. The ERK pathway can transport a variety of extracellular stimuli signals to the nucleus through a series of cascade reactions, thus regulating tumor occurrence, proliferation, and apoptosis[15]. It may also result in drug resistance by up-regulating certain drug-resistant genes[16]. Besides, Messersmith et al[13] revealed that effectively blocking the ERK pathway can down-regulate MDR1 genes so as to reverse drug resistance. Apatinib is a new anti-angiogenic small molecule preparation that needs to be orally taken. It selectively binds and inhibits VEGFR-2, thus inhibiting tumor angiogenesis and tumor growth. The animal experiment of Tian et al[17] showed that in mouse models with NCI-H460 transplantable lung cancer and Ls174t transplantable colon cancer, apatinib combined with docetaxel or oxaliplatin/5-fluorouracil can significantly and synergistically inhibit tumor growth. Mi et al[12] revealed that MCF-7/Adr, MCF-7/FLV1000, KBv200 and S1-M1-80 drug-resistant cells were accompanied by the high expression of MDR1 and ATP-binding cassette sub-family G member 2 (ABCG2), whereas apatinib can inhibit the transport activities of MDR1 and ABCG2 and improve the concentration of drugs in cells, ultimately improving the sensitivity of drug-resistant cells to drugs. Studies *in vivo* also revealed that apatinib achieves good synergistic anti-tumor effect on mouse drug-resistant cell transplantation model. In view of the above study, we tried to explore the reversal effect of apatinib on drug resistance of lung cancer and its relevant mechanism. In the work, it was found that apatinib inhibited the expression of MDR1 in A549/DDP cells in a dose-dependent manner, suggesting that apatinib may restore its sensitivity to DDP by inhibiting MDR1-resistant proteins in A549/DDP cells. At the same time, the expression level of ERK in A549/DDP cells was significantly increased compared with that in A549 cells, suggesting that the activation of ERK signals plays an important role in A549/DDP resistance to DDP. Therefore, we explored the effect of apatinib on ERK signals in A549/DDP cells. Notably, it was found that apatinib also inhibited the activation of the ERK signaling pathway in a dose-dependent manner. However, the mechanism of apatinib down-regulating MDR1-resistant proteins, whether MDR1 down-regulation depends on the inhibition of ERK signals, the effect of apatinib on the down-regulation of MDR1 in A549/DDP cells and the inhibition of ERK signals, remain to be further investigated.

Conclusions

We found that apatinib can restore the sensitivity of A549/DDP cells to DDP to a certain extent, and its mechanism may be related to the inhibition of MDR1 and ERK signals by apatinib. This study provides a new exploratory perspective for reversing the drug resistance of lung cancer and a theoretical basis for the selection of therapeutic strategies for clinical drug resistance of lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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