Original Article
Fluoxetine enhances cellular chemosensitivity to Cisplatin in cervical cancer

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Abstract: Cervical cancer is the second most common female malignancy worldwide. Although Cisplatin (DDP) is a commonly used chemotherapy agent for cervical cancer, the development of drug resistance has limited its clinical use. Fluoxetine (FLT), which is widely used to treat depression, has exhibited potent anticancer activity against different cancer cell types. Until now, the effect of Fluoxetine on cervical cancer cells has not been previously studied. The aim of this study was to evaluate the anticancer effect of FLX alone or combing with DDP on human cervical cancer cell, as well as its molecular mechanism. Here, we found that FLX combining with DDP could significantly inhibit the proliferation of HeLa cells in a dose dependent manner, and induce cell cycle arrest at G0/G1 phase and apoptosis in vitro. Moreover, FLX and DDP in combination could suppress the tumor growth in a HeLa xenograft mouse model in comparison to FLX or DDP alone. Molecular mechanism demonstrated FLX combining with DDP not only could suppress the levels of multi-drug resistance genes including GST-π and P-gp, but also affect the levels of apoptotic related genes including down-regulation of the Bcl-2 and up-regulation of caspase-9 and p17. In conclusion, FLX is a highly effective chemosensitizer, and FLX in combination with DDP might be an effective treatment against cervical cancer.

Keywords: Fluoxetine, Cisplatin, apoptosis, cervical cancer

Introduction

Cervical cancer is one of the most common cancer in women worldwide [1]. Although Cisplatin is a commonly used chemotherapeutic agent against cervical cancer, the cancer patients would develop drug resistance quickly and lead to a decline in the five-year survival rate [2]. Therefore, it is necessary to enhance the sensitivity to Cisplatin in cervical cancer cells.

It has been reported that anti-depression treatment not only improved depressive symptoms, but also improved the quality of life and prognosis of cancer patients [3]. Fluoxetine (FLX) is one of the most commonly used drugs in the treatment of depression [4]. Recent studies demonstrated that FLX played key roles in the apoptosis pathway [5-7]. Furthermore, FLX possessed antitumor activity against different cancer cell types [8-10]. FLX could have a combination effect with chemotherapy drug to over-come drug resistance [11, 12]. Many signal conduction pathways, such as the nuclear factor-κB (NF-κB), Wnt/β-catenin and Hedgehog signaling pathways, showed functional disorder in cervical carcinoma [13, 14]. It is possible that FLX not only improves the depression of cancer patients but also acts as a chemosensitizer, which made the research is of great interest for cancer therapy. However, the effect of Fluoxetine on cervical cancer cells remains unknown. In this study, we aimed to assess the anticancer effect of FLX alone or combing with DDP on human cervical cancer cell, as well as its molecular mechanism.

Materials and methods

Cell lines and culture

The human cervical cancer cells HeLa was obtained from the Chinese Academy of Science (Shanghai, China) and cultured in DMEM (Gibco,

NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA) and 1% penicillin/ streptomycin. Cells were cultured in an incubator (Kendro Laboratory, Canada) at 37°C under humidified conditions of 5% CO_2.

Cell proliferation assay

Cell proliferation was measured with a CCK-8 kit (Dojindo, Kumamoto, Japan). Human cervical cancer cells were seeded into 96-well plates in 100 µl of medium containing 10% FBS and incubated at 37°C in 5% CO_2. Then were treated with different concentrations of DDP or FLX (1 uM, 2 uM, 4 uM, 8 uM, 16 uM, 32 uM, 64 uM, 128 uM and 256 uM). After 24 h incubation, the medium was replaced with 90 µl of fresh medium and 10 µl CCK-8 solution was added to each well. Cells were then incubated for 2 h at 37°C in 5% CO_2, afterward absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each experiment was performed in duplicate and repeated in quadruplicate for each condition.

Cell cycle analysis

Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS) analysis. Firstly, 1×10^4 cells were collected and washed with PBS for 5 min by centrifugation at 1000 g. Then, cells were fixed with paraformaldehyde and transparented with 0.5% Triton X-100 for 10 min. After that, cells were resuspended and incubated for 30 min at 37°C in staining solution containing 1.5 µmol/L propidium iodide (P4170; Sigma-Aldrich, St. Louis, MO, USA) and 25 µg/mL RNase. Flow cytometry was carried out on a FACS caliber flow cytometer (BD Biosciences, San Jose, CA, USA). Data acquisition and analysis were carried out using Cell Quest (BD Biosciences).

Apoptosis assay

Cell apoptosis was detected by Annexin V-FITC assay. Apoptotic cell death was quantified by flow cytometry with Annexin V-FITC and propidium iodide (PI) staining. BD Annexin V Staining Kit (Cat: 556420; BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's instructions. Briefly, cells were washed two times with cold PBS and then cells were resuspended in 1 × binding buffer at a concentration of ~1×10^6 cells/ml. Then 100 µl of the

solution (~1×10^5 cells) was transferred to a 5 mL culture tube. Annexin V and PI (Propidium Iodide) 5 µl/test was added. Cells were gently mixed and incubated for 15 min at RT in the dark. Stained cells were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA).

Western blot analysis

Cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and the protein concentrations were quantified using the bicinchoninic acid (BCA) assay method. Equal amounts of proteins (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat milk at room temperature for 1 h and then incubated with anti-Bcl-2, caspase-9, p17, GST-π and P-gp antibody, respectively at 4°C overnight, and followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibody (1:5,000, Beyotime Institute of Biotechnology) at 37°C for 1 h. The blotted protein bands were exposed to and visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL). Developed films were digitized by scanning, and β-actin was used as an internal reference for the analysis of protein expression.

Tumor xenograft mouse models and DPP or FLX treatment

Animal care and experimental protocols were approved by the Animal Ethics Committee of Beijing Tongren Hospital. Tumor-bearing mice were size-matched and divided into groups. The weights of mice were similar within each treatment cohort. To establish cervical cancer xenografts in nude mice, Hela cells (1×10^6) were subcutaneously injected into nude mice (6 mice/group) under anesthesia. For FLX or DDP treatment of cervical cancer-bearing mice, treatment was started 7 days after inoculation and mice were randomly assigned to receive a daily oral dose of 3 mg/Kg DDP or 1 mg/Kg FLX. The mice were monitored every other days for 5 weeks. Tumor volumes were measured three to four times weekly with a caliper and calculated as the formula: V=0.5 × (Length × Width²).

Immunohistochemistry

Immunohistochemistry was used to detect the levels of Bcl-2, caspase-9, p17, GST-π and P-gp
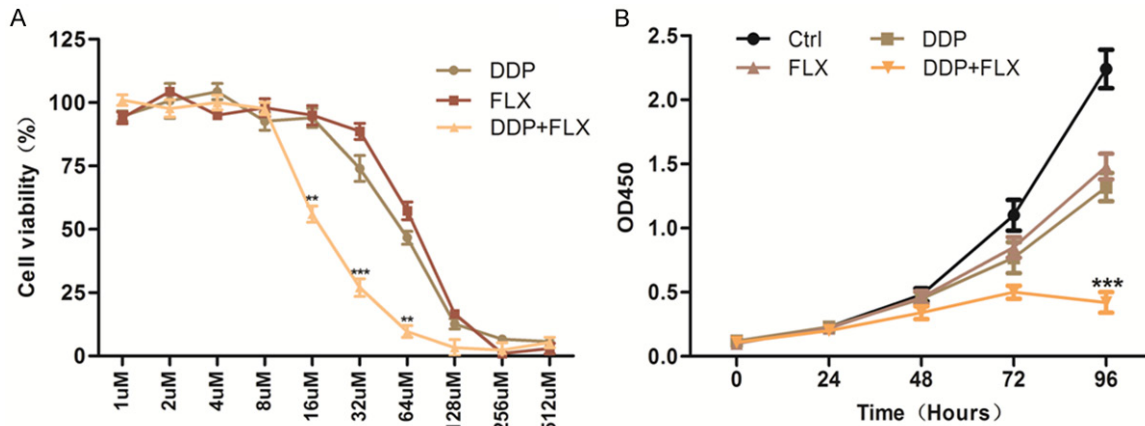
Figure 1. FLX, DDP and their combination inhibit cervical cancer cell proliferation. Cells were treated with different concentration (1-512 μM) of FLX or DDP alone or in combination for 24 hours. Cell viability was analyzed by CCK-8 assay. A. Cell growth curve. B. Inhibitive rate by bar chart.

in samples from Hela xenograph models. The samples were routinely fixed in formalin and embedded in paraffin. Sections of 4 μm thickness were cut from paraffin-embedded tissue blocks and mounted on silanized slides. Following dewaxing and rehydration, the sections were antigen retrieved with ethylenediamine tetraacetic acid or citric acid, incubated with 3% H_2O_2 for 10 min and blocked with 5% bovine serum albumin for 20 min. The specimens were then incubated with the primary antibodies (anti-Bcl-2 1:500, anti-caspase-9 1:500, anti-p17 1:800, anti-GST-π 1:1000, and anti-P-gp 1:600) for 24 h at 4°C. Color was developed using the diaminobenzidine method. Negative were treated identically except without primary antibodies. Staining was independently assessed by two experienced pathologists. Images were scanned and acquired using an OLYMPUS PM20 automatic microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard derivation. Statistical significance of the data was analyzed by the two-tail Student's t-test or ANOVA or the Chi-square test. A P<0.05 was considered statistically significant.

Results

Combined effects of FLX and DDP on cell viability in HeLa cells

HeLa cells were exposed to FLX/DDP alone or combination to investigate their effect on the proliferation of Hela cells. Firstly, we found that FLX in combination with DDP could significantly inhibit cellular proliferation (**Figure 1A**). Although high concentration of FLX (128 uM) or DDP (64 uM) alone induced significant cell death, 16 uM fluoxetine in combination with 16 uM DDP could suppress cell growth (**Figure 1B**). The results indicated that FLX could use as a chemosensitizer for anticancer drugs.

Combined effect of FLX and DDP on cell cycle and cell apoptosis

To unravel the mechanism of cell growth inhibition induced by FLX/DDP alone or combination, we examined the cell cycle and apoptosis by FACS analysis, respectively. The results showed FLX combining with DDP could significantly induce G0/G1 phase arrest (73%) comparing with FLX (58%) or DDP (60%) treating group (**Figure 2A**). Furthermore, we also found the percentage of apoptotic cells was 48.3% when treated with FLX and DDP for 48 h, which was significantly higher than that with single treatment of FLX (38.1%) or DDP (31.5%) (P<0.05) (**Figure 2B**).

Combined effect of FLX and DDP on tumor growth in nude mice bearing HeLa xenografts

To further investigate the inhibitory effects of FLX/DDP alone or combination in vivo, we employed the Hela xenograft model. The result demonstrated that FLX and DDP in combination group revealed the greatest inhibitory effects on tumor growth (**Figure 3A**). After 28 days, the mice were sacrificed. As shown in
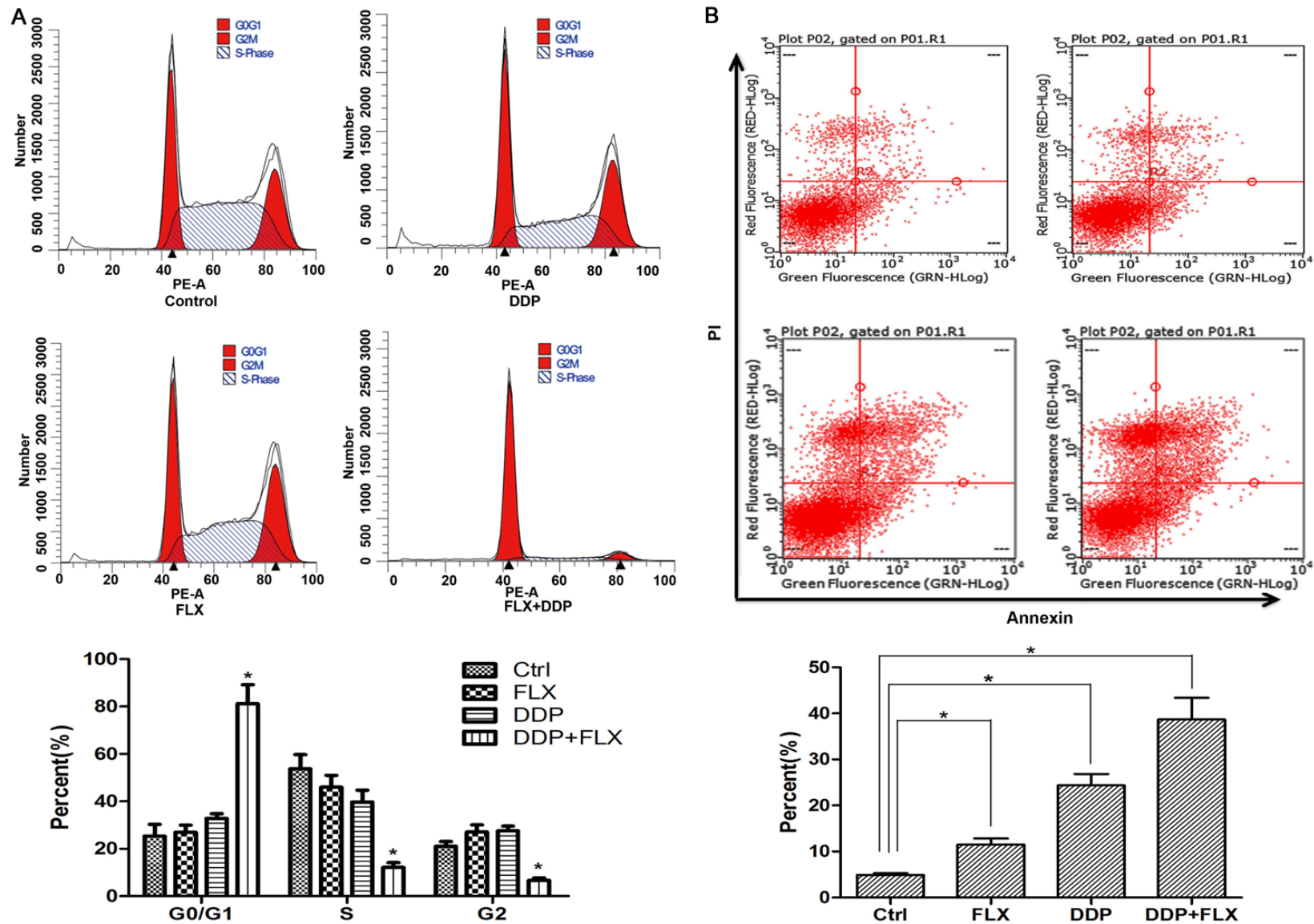
Figure 2. FLX, DDP and their combination affect cell cycle and apoptosis. A. The percentage of G0/G1 phase cells was significantly higher when cells are treated with FLX, DPP or their combination (P<0.05 vs. control group). B. Apoptotic analysis by flow cytometry showed that the combination group resulted in an increase in the number of early apoptotic cells compared with FLX or DDP group in Hela cells.
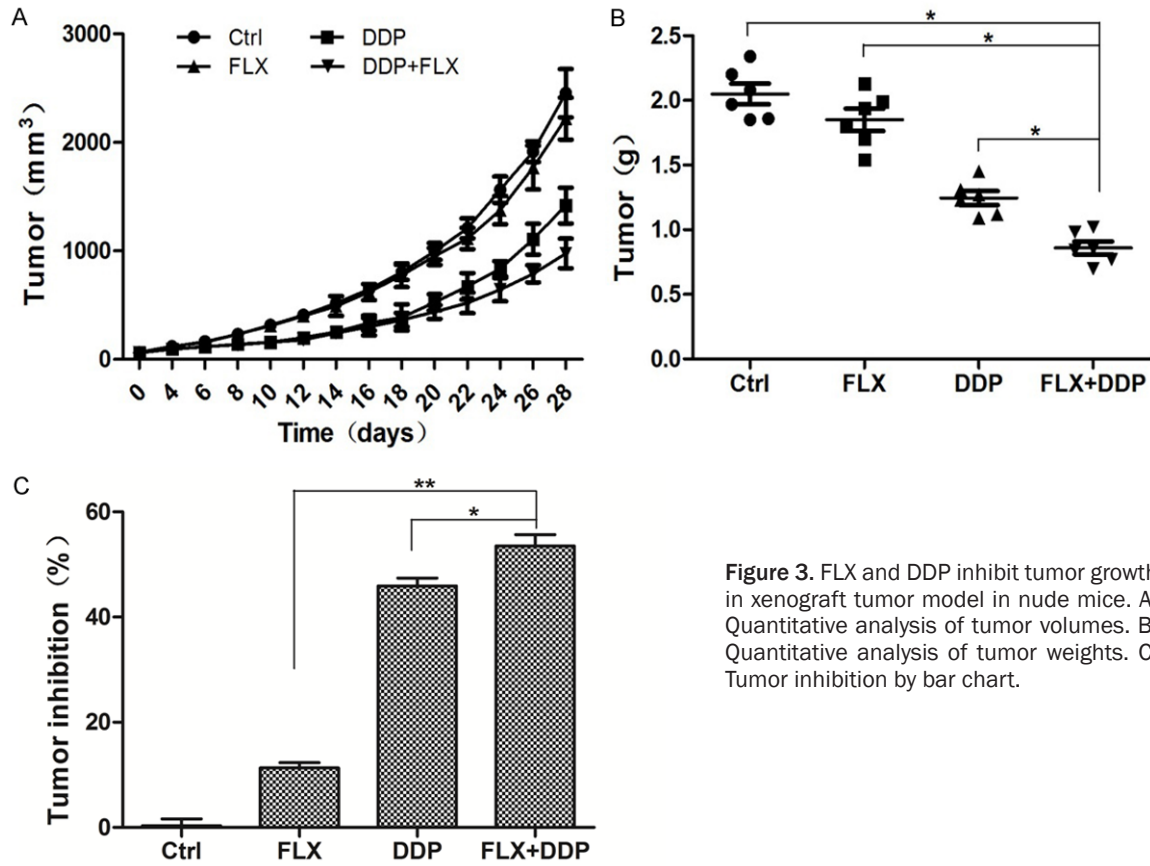
Figure 3. FLX and DDP inhibit tumor growth in xenograft tumor model in nude mice. A. Quantitative analysis of tumor volumes. B. Quantitative analysis of tumor weights. C. Tumor inhibition by bar chart.

Figure 3B, FLX, DDP and combined therapy group effectively reduced the tumor weights with the inhibition rates of 10.9%, 46.6% and 53.7%, respectively.

FLX altered the levels of genes related to multi-drug resistance and apoptosis

Based on the above results, we further examined the levels of multi-drug resistance genes (GST-π and P-gp) and apoptotic related genes (Bcl-2, caspase-9, p17) in Hela cells treated with FLX/DDP or in combination. As shown in **Figure 4A**, FLX and DDP combination significantly downregulated GST-π, P-gp and Bcl-2 level, and enhanced the expression of caspase-9 and p17. Next, we collected HeLa Xenografts tissue samples from animal treatment experiment and assessed the expression of related genes. Consistence with the results of Western blot, the staining intensity of multi-drug resistance-associated protein (GST-π and P-gp) and Bcl-2 was weak, while the levels of caspase-9 and p17 were significantly increased in combining treatment group compared to FLX or DDP single treatment group (**Figure 4B**).

Discussion

Cisplatin is a widely used chemotherapy agent for cervical cancer, while multi-drug resistance (MDR) is the main obstacle of cancer chemotherapy. A chemosensitizer is expected to overturn the poor response of drug-resistant cells to anticancer drugs and at the same time to have little or no effect on drug-sensitive cells [15]. Fluoxetine (FLX), which is widely used to treat depression, has exhibited potent anticancer activity against different cancer cell types. Until now, the effect of FLX on cervical cancer cells has not been previously studied.

In this study, we firstly evaluate the anticancer effect of FLX alone or combing with DDP on human cervical cancer cell. The results showed that FLX in combination with DDP could significantly inhibit cellular proliferation. The results indicated that FLX could used as a chemosensitizer for anticancer drugs, which was similar to other chemosensitizers [16]. Further FACS analysis showed FLX combining with DDP could significantly induce G0/G1 phase arrest and apoptosis. Previous studies also confirmed that
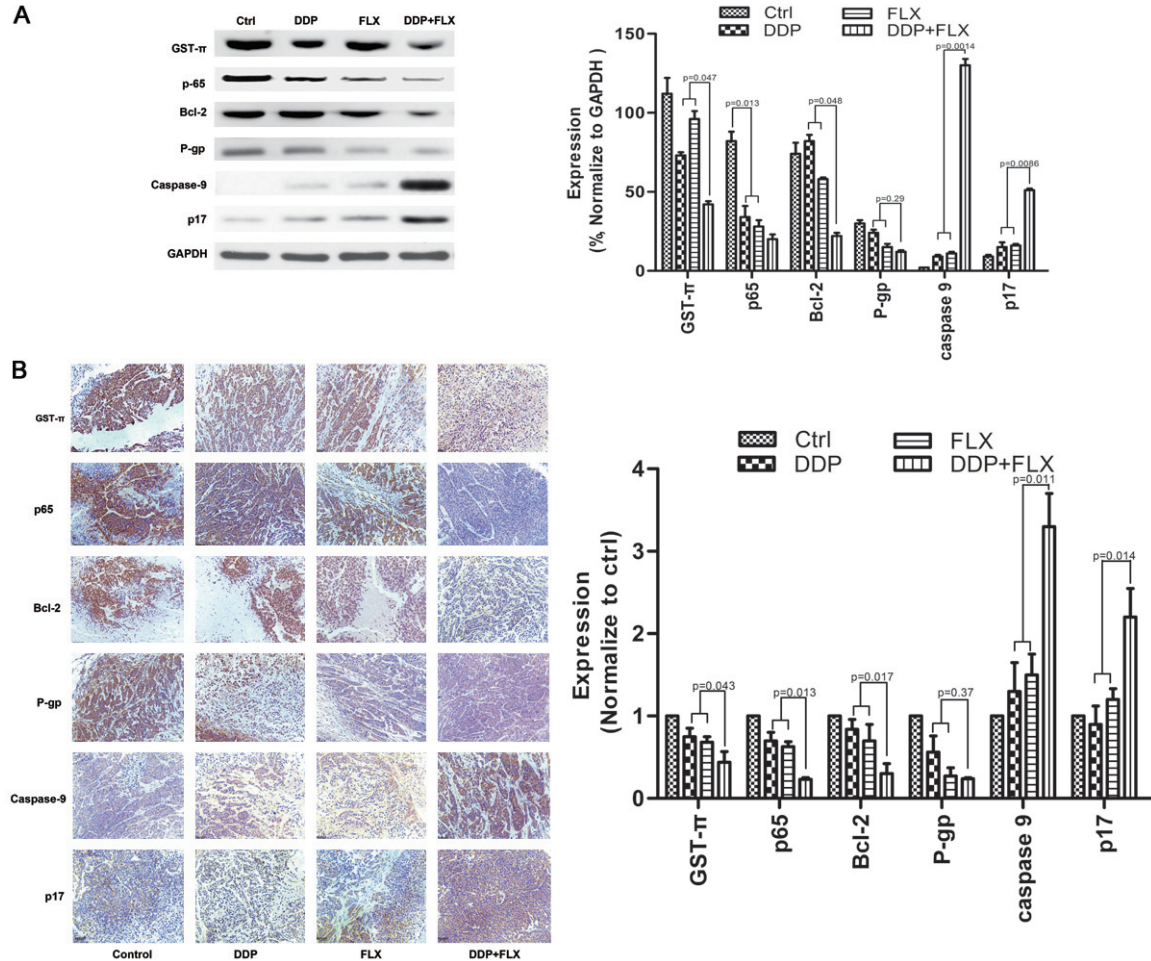
Figure 4. Effect of FLX and DPP on the expression of multidrug resistance and apoptosis related genes in HeLa cells and tumor tissues of xenograft nude mice. A. Levels of multi-drug resistance genes (GST-π and P-gp) and apoptotic related genes (Bcl-2, caspase-9, p17) were analyzed by western blotting in HeLa cells. B. IHC analysis of multi-drug resistance genes (GST-π and P-gp) and apoptotic related genes (Bcl-2, caspase-9, p17) in animal treatment experiment (200 ×).

FLX delayed cell cycle progression independently of ROS production and DNA damage in human colon cancer cells in vitro [17], and blocked cell cycle at G0/G1 phase in breast cancer cells [18]. To further investigate the inhibitory effects in vivo, we employed the Hela xenograft model and found that FLX and DDP in combination group effectively reduced the tumor weights.

During cancer chemotherapy, cancer cells were capable of developing resistance to cytotoxic agents. The related mechanisms are involved in MDR, including increasing drug efflux (P-gp) and activation of detoxifying systems (GST-π). Cancer chemotherapy always induce to cell apoptosis [19]. Inhibition of Bcl-2 family pro-

teins could induce apoptosis and activation of caspase-9 and p17. Based on the above results, we further examined the levels of multi-drug resistance genes (GST-π and P-gp) and apoptotic related genes (Bcl-2, caspase-9, p17) in Hela cells treated with FLX/DDP or in combination. The results showed that FLX and DDP combination significantly downregulated GST-π, P-gp and Bcl-2 level, and enhanced the expression of caspase-9 and p17. To further validate the results, we also detected their levels in HeLa Xenografts tissue samples from animal treatment experiment, and found that the staining intensity of multi-drug resistance-associated protein (GST-π and P-gp) and Bcl-2 was weak, while the levels of caspase-9 and p17 were significantly increased in combining treat-

ment group compared to FLX or DDP single treatment group.

In conclusion, this study firstly demonstrated that FLX could be used as a chemosensitizer, and FLX in combination with DDP might be an effective treatment against cervical cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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