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Original Article

Curcumin combined with cis-platinum promote the apoptosis of human colorectal cancer HT29 cells and mechanism

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Abstract: Colorectal carcinoma is one of the common malignant carcinoma that severely harms the public health. Curcumin (Cur) exhibits anti-tumor, antioxidant, anti-inflammatory and antiviral effects, which result in the promotion of the apoptosis of cancer cells. The present study aimed to investigate the effect of cis-platinum (DDP) combined with Cur on the apoptosis of colorectal carcinoma cells, and to elucidate its underlying mechanism of action. The cell viability of human colorectal cancer HT29 cells was detected by MTT after DDP treatment. The effect of DDP/Cur co-treatment on cell proliferation and apoptosis were investigated using CCK-8 assay and flow cytometry assay. HT29 cells growth was significantly inhibited after DDP treatment and DDP combined with Cur treatment for 24 h. In our results, DDP in combination with Cur significantly promoted the apoptosis of HT29 colorectal carcinoma cell in a dose dependent manner. Bcl-2-associated X protein (Bax) expression was promoted and B-cell lymphoma-2 (Bcl-2) expression was suppressed, In addition, Notch1, Notch1 intracellular domain (NICD1) and hairy and enhancer of split 1 (Hes-1) were decreased dramatically in HT29 cells treated with Cur combined with DDP. The effect of Cur combined with DDP treatment was better than the effect of DDP treatment. It showed that DDP combined with Cur promote the apoptosis of HT29 cells via regulating the expression of related apoptotic genes and inhibiting the activation of Notch1 signaling pathway.

Keywords: Curcumin, cis-platinum, apoptosis, Notch1, colorectal carcinoma

Introduction

According to the statistics, colorectal cancer incidence ranked top three in 2011 [1]. In recent years, with the continuous improvement of Chinese people's living standards and change of lifestyle and diet, the morbidity rate of colorectal carcinoma is still occurring at a rate that is rapidly beating, by almost 2 to 4 times, mortality rate of colorectal has increased by about 35% [2]. The standardized combined treatment is mainly adopted for colorectal carcinoma patients, which is done by surgery together with chemotherapy and radiotherapy, but it has limited effect, and average more than 600,000 cancer patients has died of colorectal cancer a year [3]. DDP as a broad spectrum antitumor drug is the complex of platinum (Pt), efficient to a series of tumors [4, 5]. But one study showed that DDP resistance was found in the majority of patients within one year, it means that chemotherapy resistance of tumor is one of the critical factors which lead to the chemotherapy failure, therefore, how to further improve the drug efficacy and chemo-sensitivity, this is a problem which have been explored for long times by people [6].

Traditional Chinese medicine for various cancers in China has a long history, some research confirmed that traditional Chinese medicine extract perform well in anti-tumor [7]. Cur is phenolic substance of lower molecular weight extracted from Curcuma [8], which is of interest because it is the most active of the Curcuma, and have revealed a wide range of biological activities, including anti-inflammatory, anti-carcinogenic, anti-atherogenic, anti-lipid peroxide and anti-infection activities [9-12]. Cur has dual mechanism of chemopreventive agents that can inhibit the tumor initiation by preventing carcinogenic substances activation as well as

the cancer cell proliferation in promotion stage of carcinogenesis [13-15]. The synergistic effects of Cur with chemotherapeutic agents has been a hot point for research. Previous studies suggested that Cur enhanced the effectiveness of chemotherapy and reversed multidrug resistance (MDR) by the different functional mechanism [16]. Cur with little side-effect and well tolerated as anti-cancer drug, can promote the apoptosis of various cancers cell, such as lung carcinoma cell, breast cancer cell, prostate cell and other cells [17-19]. Therefore, the purposes of this research is to investigate whether Cur could enhance the cell-killing effect of DDP on colorectal carcinoma cell the mechanisms involved.

Materials and methods

Cell line and reagents

The human colorectal carcinoma cell lines HT29 was purchased from Tongpai Bio-Technique Co. Ltd. (Shanghai, China). All cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), which were grown at 37°C in a humidified 5% CO_2 atmosphere. Cisplatin for injection (freeze-dried) was purchased from QILU PHARMACEUTICAL Co. Ltd. (Shandong, China). Cur were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell treatment and cell viability assay

HT29 cells were seeded in 96-well plates at an initial density of 1×10^3 cells/well for 24 h. The cells were then treated with DDP (1-60 μmol/L) for another 12, 24 and 48 h. Cell viability was determined by MTT dye reduction assay as described previously [20]. After DDP treatment, 20 μL of MTT (5 mg/mL; Amresco, USA) solution was added to each well, and the culture was incubated for another 4 h at 37°C. The supernatant was discarded after centrifugation, and then 150 μL of DMSO was added to each well and ten-minute oscillations with a low-speed. The optical density (OD) were read at 570 nm by a microplate reader (BIO-RAD, USA), and inhibition rate were counted according to average OD values.

CCK8 assay

CCK8 assay was performed using Cell Counting Kit-8 (CCK)-8 Kit (Beyotime, China) as described previously [21]. Cur was diluted with dimethylsulfoxide (DMSO) (Sigma, USA) before they could be used. Human HT29 cells were seeded at a density of 5×10^3 cells/well into 96-well plates in 100 mL culture medium, and the cell were then cultured for 24 h, DDP were added to the medium to the final concentrations (5 μmol/L), after the cells were cultured for another 1 h, and the cells treated with 6 different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) for another 24 h. Then, 10 μL of CCK8 assay solution was added to each well, and the culture was incubated for another 1 h at 37°C. The opticaldensity (OD) values were read at 570 nm by a microplate reader (BIO-RAD, USA). Experiments were repeated at least three times each time in triplicate.

Apoptosis analysis

The apoptosis level of HT29 cells was measured by flow cytometric assay. The overall cell apoptosis detection procedures were similar to described previously with slight modification on the cell numbers [22]. In brief, the ethanol-fixed HT29 cells (1×10^6 cells/well). The cells were harvested into centrifuge tubes after DDP and DDP combined with the different concentrations of Cur treatment, which were washed and detached in 2 mL phosphate-buffered saline (PBS) with 2 mM Ethylene Diamine Tetraacetic Acid (EDTA) and centrifuged at 15,000 × g for 5 min, and the suspended cells were stained with 250 μL of hypotonic fluorochrome solutioninclouding PBS, 50 μg PI, 0.1% sodium citrate and 0.1% Triton X-100 and RNase A (100 U/ml) (BD PharMingen) for 30 min in the dark at room temperature. Measurements were made using a flow cytometer (BD Influx; BD Biosciences, Franklin Lakes, NJ, USA). B3 quadrant represented viable cells; B2 quadrant and B4 quadrant represented apoptotic cells.

RNA isolation and RT-PCR

After HT29 cells treated with DDP and DDP combined with the different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L, with 0.1% DMSO vehicle as the control) for 24 h, according to the manufacturer's instructions, total RNA was extracted from cells using total RNA isolation kit (Solarbio Life Sciences, Beijing, China) and was quantified by spectro-

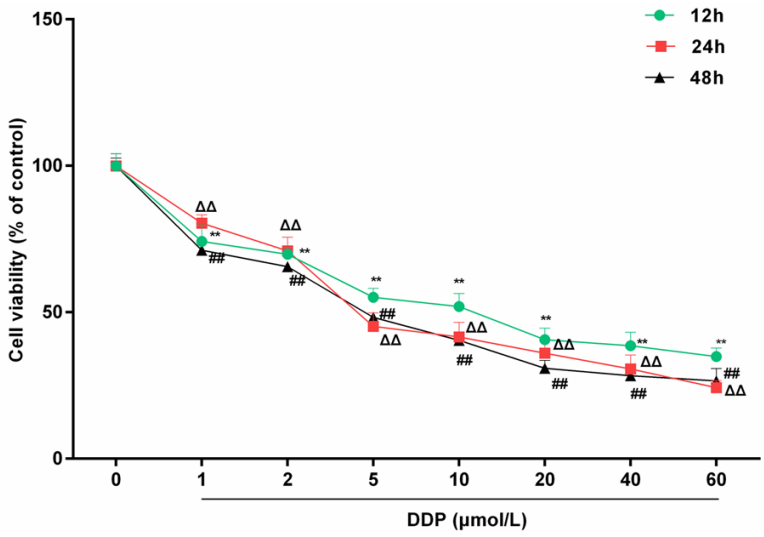

Figure 1. Effect of DDP on the cell viability of human HT29 cells. Cells were treated with different doses of DDP (0, 1, 2, 5, 10, 20, 40 and 60 μmol/L) for 12, 24 and 48 h, and MTT was performed to identify the cell viability. Data were presented as mean ± SD, n=3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. control.

photometry (Eppendorf, Gremany). Total RNA were reverse transcribed into cDNA using cDNA First Strand Synthesis kit (Thermo, USA). The RT-PCR conditions were performed according to the manufacturer's instructions of TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) included an initial denaturation for 30 sec at 95°C, followed by 40 cycles of denaturation for 5 sec at 95°C, annealing for 30 sec at 60°C and extension at 72°C for 30 sec using ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA) in triplicate. The specific primer sequences for each gene was listed as the follows: 5'AGGATCGAGCAGGGCGAATG3' and 5'TCAGCTTCTTGGTGGACGCA3' for Bax (product: 84 bp); 5'CCACCTGTGGTCCACCTGAC3' and 5'GGCTGGACATCTCGGCGAA3' for Bcl-2 (product: 85 bp), 5'CTCCTCGCAGTGCTTCCAGA3' and 5'TCCACGGTCTCACTCTGCAC3' for Notch1 (product: 117 bp), 5'AGATAGCTCGCGGCATTCCA3', 5'CAGCACACTTGGGTCTGTGC3' for Hes-1 (product: 121 bp) and 5'AGCTCACTGGCATGGCCTTC3' and 5'CGCCTGCTTCACCACCTTCT3' for GAPDH (product: 116 bp). All of primers were synthesized By Shanghai Sangon Company (Shanghai, China). Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantification, and all samples were normalized to GAPDH, which was used as an endogenous control.

Protein lysate preparation and Western blotting

HT29 cells treated with DDP and DDP combined with the different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) and were washed with ice-cold PBS, suspended in lysis buffer on ice for 30 min and centrifuged at 15,000 × g for 10 min. 10 mg protein lysates were loaded onto the SDS-PAGE gel (Beyotime Institute of Biotechnology, Haimen, China) and transferred onto the PVDF membrane (KAILV MEMBRANE TECHNOLOGY Co., Ltd, Hangzhou, China). The blots were quantified by Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to measure the density of the bands. The following antibodies were used: Anti-Notch1 (1:500 dilution) purchased from Abcam (USA), anti-NICD1 (1:1000 dilution) purchased from GeneTex (UK) and anti-Hes-1 (1:800 dilution)purchased from Affymetrix (USA); anti-Bax (1:200 dilution) and anti-Bcl-2 (1:100 dilution) purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-GAPDH (1:3,000 dilution; Beyotime) was used as a loading control.

Statistical analysis

All experiments were performed in triplicate and the results were presented as the mean ± standard deviation. All raw data were analyzed with IBM SPSS 18 software (IBM SPSS, Armonk, NY, USA). Two-sided Student's t-test was performed to determine the significant differences between the treated cell and non-treated cell. $P < 0.05$ and $P < 0.01$ were considered to indicate a statistically significant difference.

Results

Effect of DDP on the cell viability in human HT29 cells

HT29 cells treated with different concentrations of DDP (1, 2, 5, 10, 20, 40 and 60 μmol/L) for 12, 24 and 48 h, the cell viability was determined with MTT analysis. The results are shown

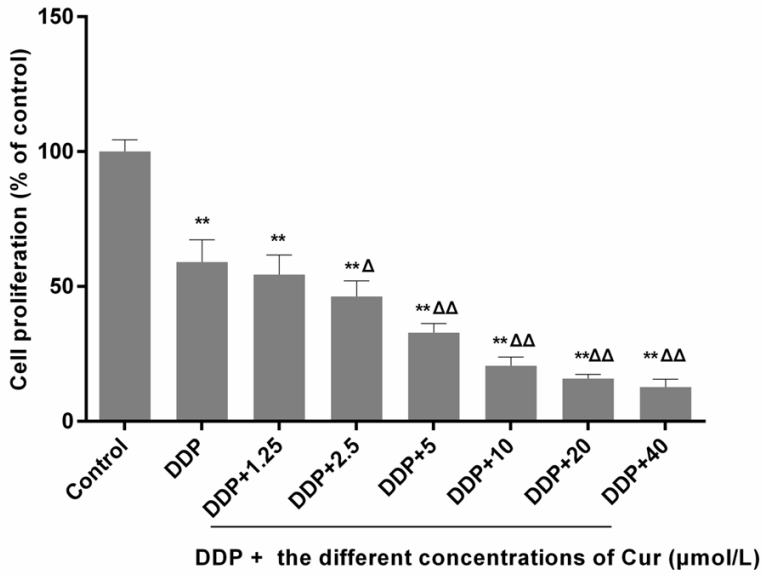

Figure 2. Effect of DDP combined with Cur on the proliferation of human HT29 cells. Cells were treated with different doses of Cur (0, 1.25, 2.5, 5, 10, 20 and 40 μmol/L) for 24 h, and CCK8 was performed to identify the cell proliferation. Data were presented as mean ± SD, n=3, $*P < 0.05$ and $**P < 0.01$ vs. control; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ vs. DDP.

in **Figure 1**. The cell viability was significantly suppressed by DDP treatment with time- and dose-dependent manners. The cell viability was the lowest in HT29 cells treated with different concentrations of DDP (1, 2, 5, 10, 20, 40 and 60 μmol/L) for 24 h compared to HT29 cells treated with different concentrations of DDP (1, 2, 5, 10, 20, 40 and 60 μmol/L) for 12 and 48 h. After 5 μmol/L DDP treatment, the cell viability respectively was 55.12 ± 3.07%, 45.2 ± 4.64% and 48.27 ± 2.35% at 12, 24 and 48 h.

Effect of DDP combined with Cur on cell proliferation in human HT29 cells

HT29 cells pre-treated with 5 μmol/L DDP for 24 h, and then the cells treated with different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) for another 24 h, and harvested cells were used for detection of cell proliferation by CCK-8 analysis. The final results are shown in **Figure 2**. The cell proliferation was dramatically inhibited by DDP treatment and DDP combined with Cur treatment. The cell viability of DDP treatment group was lower than that of DDP combined with Cur treatment groups at a dose dependent manner. The cell proliferation activity reduced from group control to group DDP combined with different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L), and which was 100 ± 4.39%, 58.33 ± 6.32%, 54.35 ± 7.11%, 46.31 ± 5.79%, 32.82 ± 3.51%, 20.58 ± 3.27%, 15.83 ± 1.57% and 12.65 ± 3.03%.

Effect of DDP combined with Cur on the apoptosis in human HT29 cells

Flow cytometry tested the apoptosis of the manipulated cells. The results revealed that the apoptosis level of HT29 cells (**Figure 3**). DDP combined with Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) significantly promoted the cell apoptosis. There were significant differences between DDP group and DDP in combination with Cur, and the apoptosis of DDP combined with Cur treatment groups higher than that of group control and DDP group. B2 quadrant and B4 quadrant represented apoptotic cells, the apoptosis rate was significantly increased from group control to group DDP combined with different concentrations of Cur, followed by 39.9 ± 4.07%, 52.33 ± 4.12%, 58.10 ± 5.21%, 70.23 ± 6.52%, 78.44 ± 3.44%, 81.57 ± 7.56%, 88.33 ± 8.87% and 90.12 ± 5.21% (**Figure 3B**).

Effect of DDP combined with Cur on the apoptosis associated proteins in human HT29 cells

Protein and mRNA expression of Bax and Bcl-2 were detected by Western blot and RT-PCR. In **Figure 4**, Bax expression was significantly increased and Bcl-2 expression was significantly decreased after DDP treatment and DDP in combination with Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) treatment. Bax and Bcl-2 expressions were remarkably regulated by DDP and DDP combined with Cur. DDP in combination with Cur treatment was related more to the regulation of Bax and Bcl-2 than DDP treatment.

Effect of DDP combined with Cur on Notch1 signaling pathway in human HT29 cells

HT29 cells were treated with DDP treatment and DDP in combination with Cur (1.25, 2.5, 5,

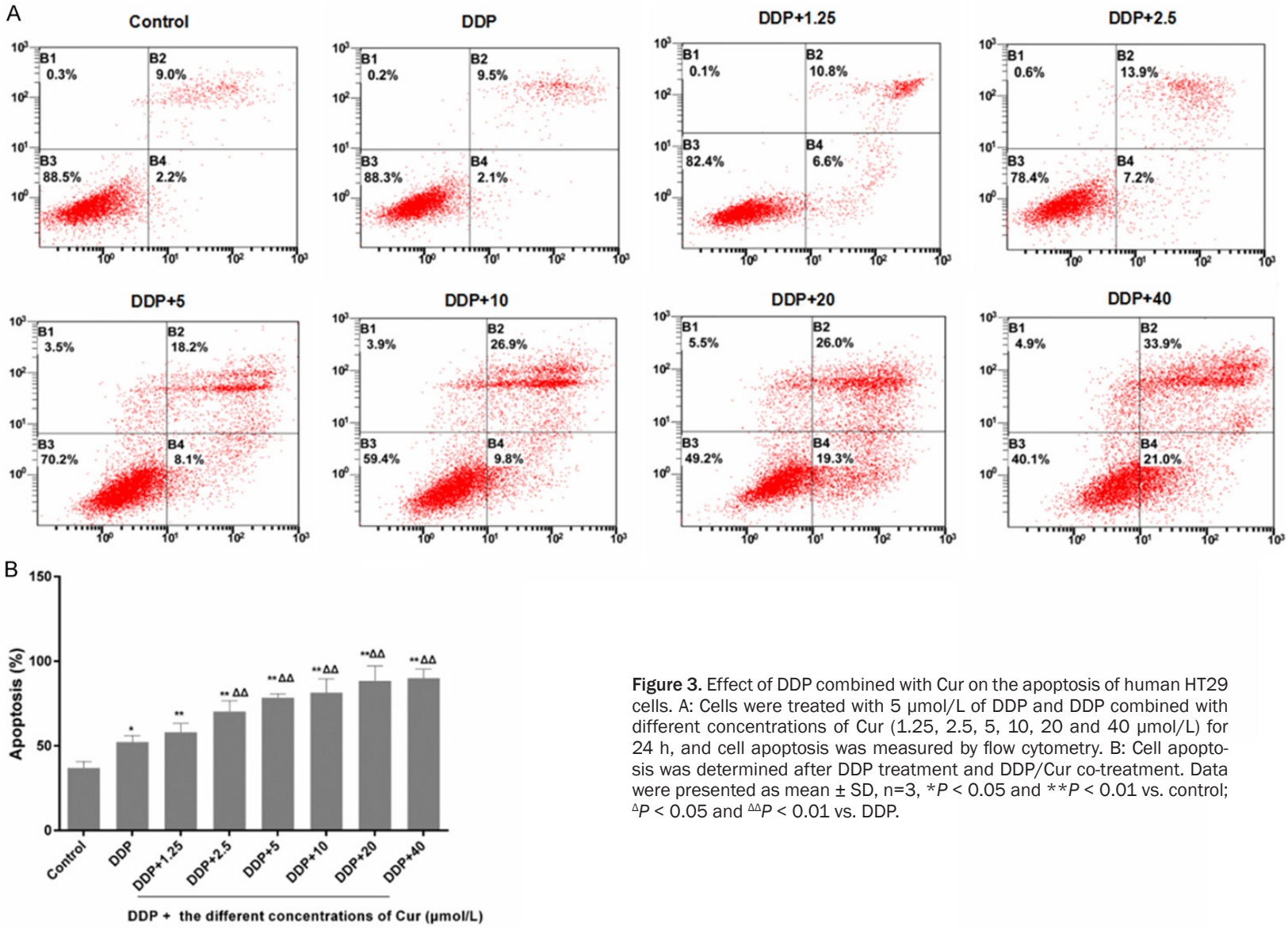

Figure 3. Effect of DDP combined with Cur on the apoptosis of human HT29 cells. A: Cells were treated with 5 μmol/L of DDP and DDP combined with different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) for 24 h, and cell apoptosis was measured by flow cytometry. B: Cell apoptosis was determined after DDP treatment and DDP/Cur co-treatment. Data were presented as mean ± SD, n=3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ vs. DDP.

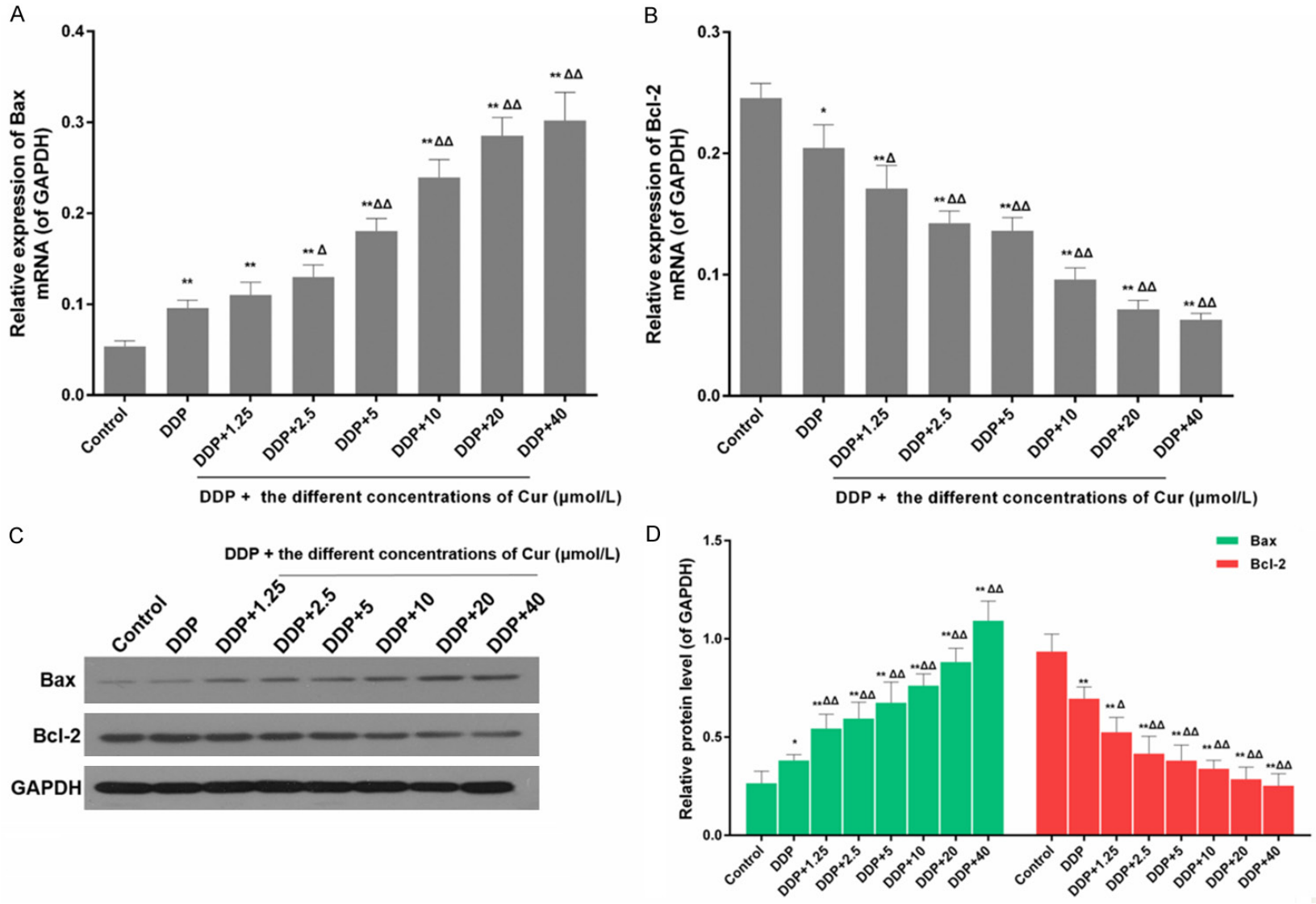

Figure 4. Effect of DDP combined with Cur on Bax and Bcl-2 expressions. A, B: Cells were treated with 5 μmol/L of DDP and DDP combined with different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) for 24 h, mRNA expression of Bax and Bcl-2 were detected by RT-PCR. C, D: Bax and Bcl-2 protein expression was determined after DDP treatment and DDP/Cur co-treatment for 24 h by Western blot. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n=6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ vs. DDP.

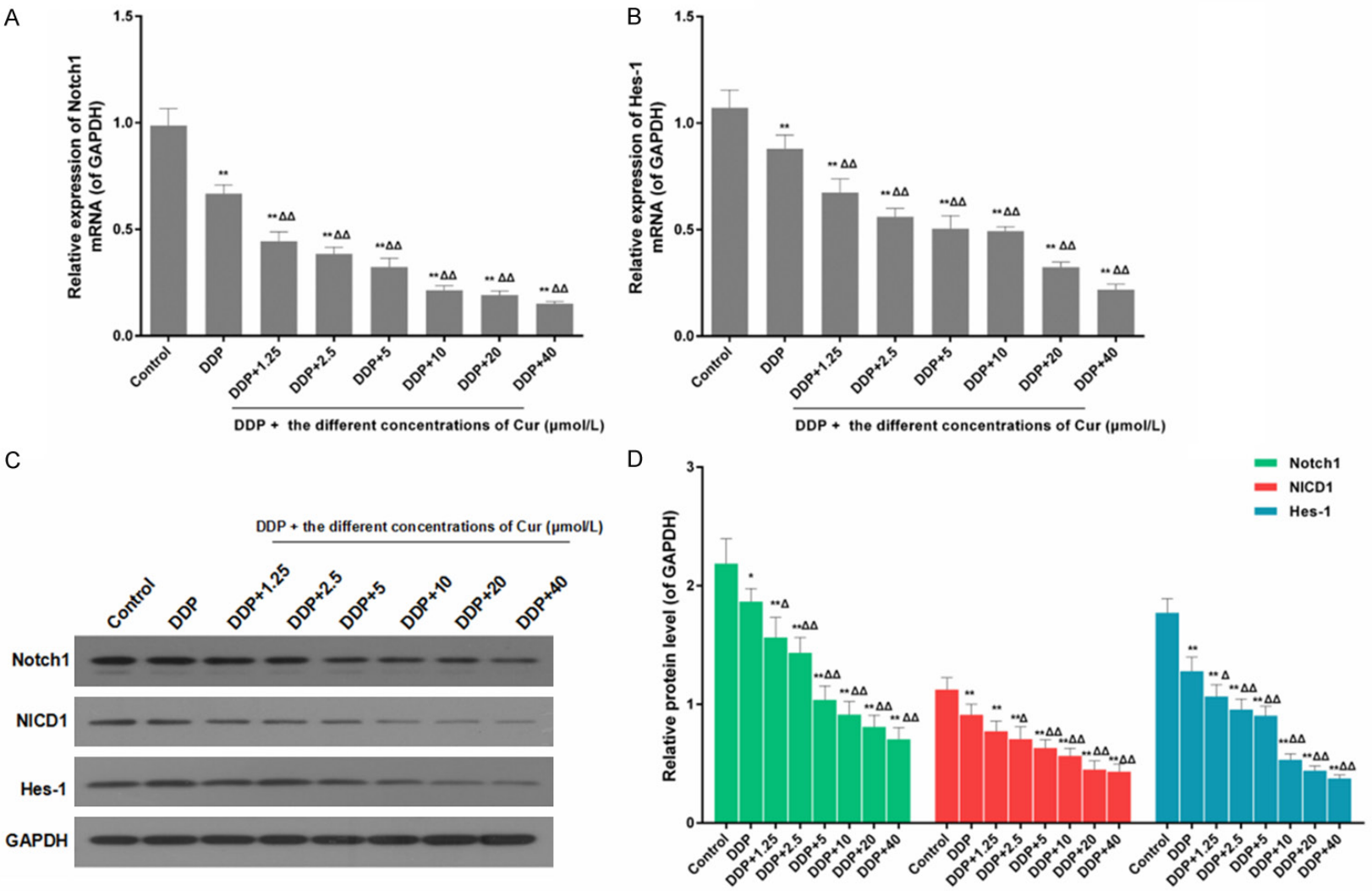

Figure 5. Effect of DDP combined with Cur on Notch1 signaling pathway. A and B: Cells were treated with 5 μmol/L of DDP and DDP combined with different concentrations of Cur (1.25, 2.5, 5, 10, 20 and 40 μmol/L) for 24 h, mRNA expression levels of Notch1 and Hes-1 were detected by RT-PCR. C and D: The expression levels of Notch1, NICD1 and Hes-1 were determined after DDP treatment and DDP/Cur co-treatment for 24 h by Western blot. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n=6, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ vs. DDP.

10, 20 and 40 μmol/L) treatment for 24 h, the expression levels of Notch1 and Hes-1 were determined using RT-PCR and Western blot, Hes-1 expression was detected by Western blot. The results are shown in **Figure 5**, the mRNA expression levels of Notch1 and Hes-1 were up-regulated after DDP treatment and DDP/Cur co-treatment (**Figure 5A** and **5B**), the protein expression levels of Notch1, NICD1 and Hes-1 were significantly down-regulated in DDP treatment and DDP combined with Cur at a dose dependent manner (**Figure 5C** and **5D**), Notch1, NICD1 and Hes-1 expressions of group DDP combined with Cur were lower than that of group control and group DDP, there was statistical difference between group control and group DDP.

Discussion

Chemotherapy resistance is an inevitable phenomenon in long-term chemotherapy for the most colorectal cancer patients, which leads to reduce chemosensitivity and have strong side effects to the affect treatment effect. The multidrug resistance of tumor can be attributable to concomitance of multiple intricate mechanisms, which were induced by the following three major reasons: cyto-dynamics, biochemical and pharmacology reasons [23, 24]. If the two drugs can represent synergism, the dosage of two drugs is reduced without affecting the curative effect, which further reduce side effect and overcome drug resistance caused by the low production and high inactivation of active metabolite in clinically [6, 14]. Traditional Chinese medicine has plentiful resource and multi target, and it has obvious curative of antitumor and has a good development foreground, therefore, many researchers pay attention to natural drugs [25]. The cell viability of HT29 cells was significantly inhibited after HT29 cells treated with different concentrations of DDP (1, 2, 5, 10, 20, 40 and 60 μmol/L) for 12, 24 and 48 h by MTT analysis (**Figure 1**), we found the inhibitory effect of 5 μmol/L DDP coincided with experimental demands [6, 14], there was no significantly statistical difference between 24 h and 48 h, the cellular sensitivity to DDP was decreased with culture time, therefore, 24 h was the appropriate time for cell culture. It showed that DDP suppressed the cell viability with a dose- and time-dependent manners, HT29 cells were not sensitive to DDP with culture time.

The anti-tumor drugs can prohibit the propagation of the cancer cells and promote the cell apoptosis. MTT and CCK8 both are the common testing methods for cell proliferation and cell viability. MTT assay is used for evaluate the drug sensitivity to tumor cells in vitro, therefore, at first, MTT assay is used to detect cell viability after DDP treatment. For further research the anti-tumor effect of DDP combined with Cur, cell proliferation was determined by CCK8 analysis. CCK8 mainly used for suspension cells with many times centrifugal [26]. The cell proliferation ability was significantly inhibited after DDP treatment and DDP/Cur co-treatment, the inhibitory rate of DDP in combination with Cur treatment is higher than that of DDP treatment with dose-dependent manner (**Figure 2**). Flow cytometry analysis revealed that the cell apoptosis was dramatically increased after DDP/Cur co-treatment, and the cell apoptosis of group DDP combined with Cur was higher than group DDP (**Figure 3**). It showed that the anti-tumor effect of combined therapy is better than single-drug treatment. Apoptosis is the destroy process of cell self on condition that physiology or pathology under the control of polygenes. Both Bcl-2 as anti-apoptotic protein and Bax as apoptotic protein belong to Bcl-2 gene family, which are related to apoptosis process [27]. The Bcl-2 and Bax expression levels can determined the state of cell survival and apoptosis [17, 28]. In our results, mRNA and protein of Bax expression was up-regulated and mRNA and protein of Bcl-2 expression was down-regulated after DDP treatment and DDP/Cur co-treatment, DDP combined with Cur treatment affected the expressions of Bax and Bcl-2 much more than DDP treatment (**Figure 4**). Synthesize the above results, we suggest that DDP/Cur co-treatment could enhance the killing effect of DDP on HT29 cellsss in *vitro*.

Notch1 signaling pathway is one of the Notch signaling pathway, previous research showed that over-expression of Notch1 protein was found in colorectal cancer tissues, and which has carcinogenesis in the initiation and progression of colorectal cancer [29]. Overexpression of Notch1 protein is correlated with decreased survival and poor prognosis of colorectal cancer patients [30, 31]. Therefore, Notch1 expression as a biomarker estimated prognosis and therapeutic [32]. The cleavage of Notch1 protein NICD1 formed intracellular

active domain after the activation of Notch1 signaling pathway, which called NICD1, and it as the middle link of the activation of Notch1 signaling pathway played a pivotal role [33]. NICD1 expression would more reflect the activated state of Notch1 signaling pathway [33, 34]. Hes-1 is Notch1 downstream/target genes [34, 35], Notch1, NICD1 and Hes-1 expressions were detected by RT-PCR and Western blot, DDP combined with Cur suppressed the expressions of Notch1, NICD1 and Hes-1, and the effect was better than DDP (**Figure 5**). It showed that the Notch1 signaling pathway was suppressed after DDP treatment and DDP/Cur co-treatment, the inhibition effect of combined therapy is better than single-drug treatment. Cur could enhance the inhibition ability of DDP to Notch1 signaling pathway.

In conclusion, Cur enhances the chemo-sensitivity of DDP, and DDP combined with Cur further promote the apoptosis of HT29 cells via regulating the expression of related apoptotic gene and inhibiting the activation of Notch1 signaling pathway.

Disclosure of conflict of interest

None.

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