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Inhibition of the JNK signaling pathway increases sensitivity of hepatocellular carcinoma cells to cisplatin by down-regulating expression of P-glycoprotein

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Abstract. – OBJECTIVE: The resistance of hepatocellular carcinoma (HCC) to chemotherapy may be mediated by the c-Jun N-terminal kinase (JNK) pathway. We wished to verify the involvement of this pathway in resistance of HCC cells to cisplatin.

MATERIALS AND METHODS: We used HepG2 cell line and cisplatin-resistant clone (HepG2/DDP). Expressions of drug resistance and apoptosis-related genes were analyzed by qPCR. Protein expressions were assessed by Western blot. The JNK pathway was assessed as total JNK1/2 and JNK1/2 phosphorylation. Cell growth kinetics was quantified by the CCK-8 assay, and cell apoptosis (Annexin V / propidium iodide) by flow cytometry.

RESULTS: HepG2/DDP cells were more resistant and less apoptotic on cisplatin. Expression of drug-resistance genes MDR1, MRP1 and MPR2 was significantly up-regulated in HepG2/DDP cells ($p < 0.05$), with up-regulation of MDR1 being the highest. This was confirmed by Western blot analysis of P-glycoprotein (P-gp), MRP1 and MRP2 proteins, the proteins encoded by the above genes. Expression of anti-apoptotic genes Bcl-2 and Bcl-XL was significantly up-regulated, and expression of pro-apoptotic genes Bak and Bad was significantly reduced, in HepG2/DDP cells ($p < 0.05$). Cisplatin treatment of HepG2 led to increased phosphorylation of JNK1/2; the trend reversed by the inhibitor SP600125. Furthermore, cisplatin increased expression of P-gp, which was also attenuated by SP600125. Cell growth was inhibited more substantially, and cell apoptosis promoted, when HepG2 cells were exposed to both cisplatin and SP600125.

CONCLUSIONS: Inhibition of the JNK signaling pathway enhances the sensitivity of HCC cells to cisplatin by down-regulating the expression of P-gp.

Key Words:
Cisplatin, Drug resistance, P-gp, JNK, SP600125.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant cancer and is the third cause of malignant cancer-related deaths in developing countries[1]. Most patients are diagnosed at progressed stages of cancer, with a little window of opportunity for surgery, which leaves chemotherapy as the principal treatment option[2]. Cisplatin is the most commonly used chemotherapeutic agent for the treatment of HCC[3]. It is efficacious but toxic, and drug resistance develops easily, limiting its overall application[4]. It is, therefore, important to uncover the mechanisms of drug resistance to improve or restore the sensitivity of HCC cells to chemotherapy, and prolong the survival of the patients.

Molecular mechanisms of cancer resistance to cisplatin are quite complicated and include abnormal gene expression, reduced intracellular drug accumulation, enhanced DNA damage repair, development of more efficient cell detoxification, diminished cell apoptosis, abnormal formation of blood vessels, cytoskeletal abnormalities, and extracellular matrix density abnormalities[5,6].

In HCC, reduction of intracellular drug accumulation may be one of the molecular mechanisms of cisplatin resistance development[5]. It was shown that multi-drug resistance gene (*MDR1*) is involved in cisplatin resistance, and P-glycoprotein (P-gp), encoded by *MDR1*, acts as the first known drug resistant membrane protein in cancer cells[7]. P-gp can shuffle anti-cancer drugs outside of the cells[8], such as was documented with regard to dasatinib, erlotinib, gefitinib, imatinib and lapatinib[9].

Another potential mechanism of cisplatin resistance development in HCC is abnormal resistance to apoptosis. The c-Jun N-terminal kinases (JNK)

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pathway is an important component of cellular response to anticancer drugs and may play a critical role in drug resistance[10]. It was shown that platinum drugs can up-regulate expression of *c-jun*, the downstream target of the JNK pathway, thereby reducing drug-induced cell apoptosis and increasing HCC drug resistance[11]. It was further found that cisplatin can cause sustained activation of the JNK pathway in ovarian cancer cell lines, with phosphorylation of c-jun and formation of the transcription factor Activator Protein 1, with subsequent termination of cell apoptosis[12].

However, the role of the JNK signaling pathway in chemo-resistance of cancer cells is far from being established. Controversially to previous studies, one report[13] demonstrated that overexpression of the *JNK1* gene reverses drug resistance of the ovarian cancer cell line to cisplatin. It is possible that this controversy is due to the differential role of the JNK signaling pathway in chemotherapy resistance in different cancer types. To further verify the involvement of this pathway in resistance of HCC cells to cisplatin, we conducted the present study.

Materials and Methods

Cell Line and Reagents

The HCC cell line HepG2 and its cisplatin-resistant counterpart (HepG2/DDP cell line) were provided by Shanghai Cell Bank (Shanghai Life Sciences Research Institute, Shanghai, China). Supplies for cell culture, such as DMEM, fetal bovine serum, penicillin and streptomycin, and 0.25% trypsin containing EDTA were purchased from, respectively, Gibco (Grand Island, NY, USA), Sijiqing (Zhejiang Tianhang Biotechnology Company, China), Hyclone (Logan, UT, USA), and Shanghai Biyuntian Biotechnology Company (Binyuntian, Shanghai, China). Cell cytotoxicity was determined using cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Japan), whereas cisplatin (diamminedichloroplatinum, DDP) and SP600125 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The BCA kit was from Thermo Scientific (Waltham, MA, USA). The cell cycle and cell apoptosis detection kits were purchased from Shanghai Biyuntian Biotechnology Company (Binyuntian, Shanghai, China). For Western blot analysis, we utilized the following primary antibodies: rabbit anti-JNK1/2, rabbit anti-phospho-JNK1/2, rabbit anti-MRP1, mouse anti-MRP2 (all from Abcam; Cambridge, UK), mouse anti-P-gp, mouse anti-Bcl-2, rabbit anti-Bcl-XL, mouse Bak rabbit anti-Bad (all from Santa Cruz; Santa Cruz, CA, USA), and rabbit anti-GAPDH (Beijing Kangwei Century Biotech Company; Beijing, China). The secondary HRP-labeled goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Shanghai Biyuntian Biotechnology Company.

Virus Propagation and Purification

The adenoviral vector containing *JNK* gene and driven by CMV promoter were constructed in-house. The vector was propagated, purified, and titred as described previously[14,15].

Cell Culture

HepG2 were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. To lift and passage cells, we utilized 0.25% trypsin-EDTA. Logarithmically growing cells were seeded for experiments. HepG2/DDP cells were cultured as above with the addition of 2 μM DDP to maintain their resistant phenotype.

Cell Viability Assay

The CCK-8 kit was used to quantify viability of HCC and HepG2/DDP cells. The cells were seeded onto 96-well plates at the concentration of 5×10^3 per well and treated with different concentration of DDP (0, 2, 4, 8, 16 and 32 μM) or control vehicle. In time kinetics experiments, HepG2 and HepG2/DDP cells were treated with 8 μM DDP or control vehicle at different time point. After treatment, CCK-8 solution (10 μl/well) was added, and cells were incubated at 37°C, 5% CO_2 for 2-4 hours. Afterwards, absorbance was read at 450 nm using a microplate reader. Cell viability and cell inhibition rates were, then, calculated.

Cell Apoptosis Quantification

Cells were grown on a 6-well culture plate. When the cells reached about 70% confluency, they were divided into 4 groups: control group, DDP group (treated with 8 μM DDP), SP600125 group (treated with 2 μM SP600125, the chemical JNK inhibitor), and DDP + SP600125 group (treated with, respectively, 8 and 2 μM) group. The cells in two last two groups were pre-treated with SP600125 for 2 hours, following which DDP was added. After treatments, cells were lifted with trypsin, and 1×10^6 cells were double-stained with fluorescein APC-labeled Annexin-V and propidium iodide (Becton Dickinson,

Franklin Lakes, NJ, USA). The percentage of apoptotic cells was quantified by flow cytometry (Becton Dickinson).

Cell Cycle Analysis

Cell cycle distribution was examined by flow cytometry using propidium iodide staining. The cells were collected at 48 hours after treatment. Cells were exposed to propidium iodide (final concentration of 50 μg/ml) and RNase A (20 μg/ml). Proportions of cells in G0/G1, S and G2/M phases were analyzed by flow cytometry by counting 20,000 cells.

qRT-PCR Assay

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. The NanoDrop-1000 was used to determine the concentration and purity of total RNA. One μg of total RNA was reverse transcribed using the reverse transcription kit (Promega, Madison, WI, USA). qPCR assay was used to quantify mRNA expression of *MDR1*, *MRP*, *LRP*, *BCRP* and *BCL-2* family members *Bcl-2*, *Bcl-XL*, *Bcl-W*, *Bax*, *Bak*, *Bad*, *Bid* and *Bim*, and expression of *GAPDH* mRNA served as an internal control.

Western Blot Assay

Total proteins were isolated from HepG2 and HepG2/DDP cells, mixed with 5× loading buffer, and boiled for 10 min at 95-100ºC. Subsequently, 20 μg of total protein lysate were loaded onto a 10% polyacrylamide gel. Proteins were then transferred onto a PVDF membrane. The membrane was blocked with 5% milk/PBST and incubated with respective primary antibodies. Primary antibodies were diluted as follows: P-gp at 1:500, MRP1 at 1:500, MRP2 at 1:100, Bcl-2 at 1:1000, Bcl-XL at 1:500, Bad at 1:300, Bak at 1:500, phospho JNK1/2 at 1:1000, total JNK1/2 at 1:1000, and GAPDH at 1:1000. The protein band density was analyzed in the QuantityOne software. GAPDH expression was used as a loading control.

Statistical Analysis

Experiments were repeated three times. The SPSS 18.0 statistic software (SPSS Inc., Chicago, IL, USA) was used for data processing. The results are presented as mean ± SD. The analysis of variance (ANOVA) test was used to compare differences among groups. The difference with the p-value of < 0.05 was considered statistically significant.

Results

Higher Resistance to DDP in HepG2/DDP Cells

We first tested the effects of DDP (cisplatin) on cell morphology, cell proliferation ability, and cell viability in HepG2 and HepG2/DDP cells, i.e. in DDP-sensitive and -resistant counterparts. HepG2 cells markedly changed morphology when treated with different concentrations of DDP, but this phenomenon was not observed in HepG2/DDP cells (Figure 1A). We next used the CCK-8 assay to quantify the proliferating ability of both cell lines. Interestingly, we observed that HepG2/ DDP cells were proliferating slower than HepG2 cells (Figure 1B).

Afterwards, HepG2 and HepG2/DDP cells were treated with 8 μM of DDP for 0, 24, 48, 72, and 96 hours. Starting from the 24-hour time point, the viability of HepG2 cells was significantly ($p < 0.05$ for all observations) lower than that of its DDP-resistant counterpart (Figure 1C). A subsequent experiment was conducted to define whether differences would be observed if HepG2 and HepG2/DDP cells were exposed to different concentrations of DDP (0, 2, 4, 8, 16 or 32 μM for 48 hours). As expected, HepG2 cells decreased viability at much lower concentrations of DDP, compared with HepG2/DDP cells, confirming that the latter cells are more resistant to increasing concentrations of DDP (Figure 1D).

HepG2/DDP Cells Exhibit Higher Colony Formation Efficiency and Resistance to DDP-Induced Apoptosis

We next assessed the resistance to DDP in HepG2 and HepG2/DDP cells using the colony formation assay. As expected, HepG2/DDP cells formed more drug resistant clones than HepG2 cells ($p < 0.05$; Figure 2A).

Subsequently, HepG2/DDP and HepG2 cells were treated with DDP and analyzed for cell apoptosis by flow cytometry. As shown in Figure 2B, there were markedly higher percentages of apoptotic cells in HepG2 cells exposed to DDP than in HepG2/DDP cells.

Following the above experiments, we analyzed the cell cycle distribution in both cell lines by flow cytometry. Interestingly, we did not observe significant differences in cell cycle distribution between HepG2/DDP and HepG2 cells treated with 8 μM of DDP (Figure 2C).

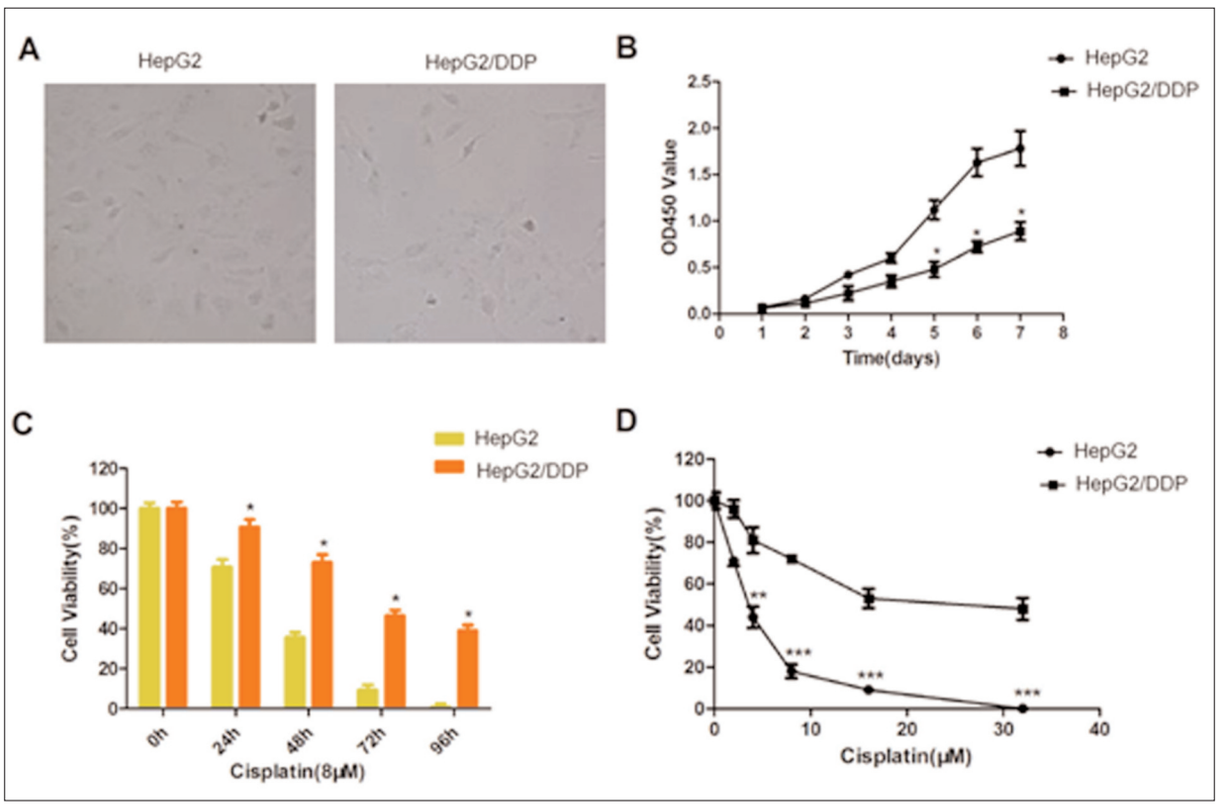

Figure 1. HepG2 and HepG2/DDP cells with or without treatment with DDP (cisplatin). ***A,*** Cell morphology of HepG2 and HepG2/DDP cells. ***B,*** CCK-8 assay was used to analyze proliferation of HepG2 and HepG2/DDP cells. *$p < 0.05$. ***C,*** HepG2 and HepG2/DDP cells were treated with DDP (8 μM) for 0-96 hours. Cell viability was quantified by CCK-8 assay. *$p < 0.05$. ***D,*** HepG2 and HepG2/DDP cells were treated with different concentrations of DDP (0, 2, 4, 8, 16 and 32 μM) for 48 hours, and cell viability was measured by CCK-8 assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Overexpression of Drug-Resistance Gene MDR1 in HepG2/DDP Cells

Overexpression of MDR1 is often associated with substantially higher resistance to chemotherapy[8]. Thus, we quantified in both cell lines the expression of *MDR1*, as well as other multi-drug resistance genes *MRP1*, *MRP2*, *LRP* and *BCRP*. As observed, expressions of *MDR1*, *MRP1* and *MPR2* genes were significantly up-regulated in HepG2/DDP cells ($p < 0.05$; Figure 3A), whereas *LRP* and *BCRP* expressions did not differ between both cell lines (Figure 3A). Notably, the magnitude of up-regulation of *MDR1* was the highest (Figure 3A). We further validated the above observations by conducting a Western blot analysis of expression of P-gp, MRP1 and MRP2 proteins, the proteins encoded by the above three genes. In line with mRNA observations, P-gp protein expression was most significantly up-regulated, compared with MRP1 and MRP2 (Figure 3B).

Overexpression of Anti-Apoptotic Genes Bcl-2 and Bcl-X, and Down-Regulation of Pro-Apoptotic Genes Bak and Bad, in HepG2/DDP Cells

We next tested whether resistance to DPP would be associated with abnormal expression of apoptosis-related genes. Thus, we quantified the expression of anti-apoptotic genes *Bcl-2*, *Bcl-XL*, *Bcl-w*, *Mcl-1*, as well as pro-apoptotic genes *Bax*, *Bak*, *Bad*, *Bid*, *Bim* in HepG2 and HepG2/DDP cells. Expression of anti-apoptotic genes *Bcl-2* and *Bcl-XL* was significantly up-regulated in HepG2/DDP cells ($p < 0.05$; Figure 4A), whereas *Bcl-XL* and *Bcl-w* expression was comparable between both cell lines (Figure 4A). Furthermore, expression of pro-apoptotic genes *Bak* and *Bad* was significantly reduced in HepG2/DDP cells compared with HepG2 cells ($p < 0.05$; Figure 4A).

We validated the observation of gene expressions by studying Bcl-2, Bcl-XL, Bak and Bad protein levels by Western blot. In line with qPCR

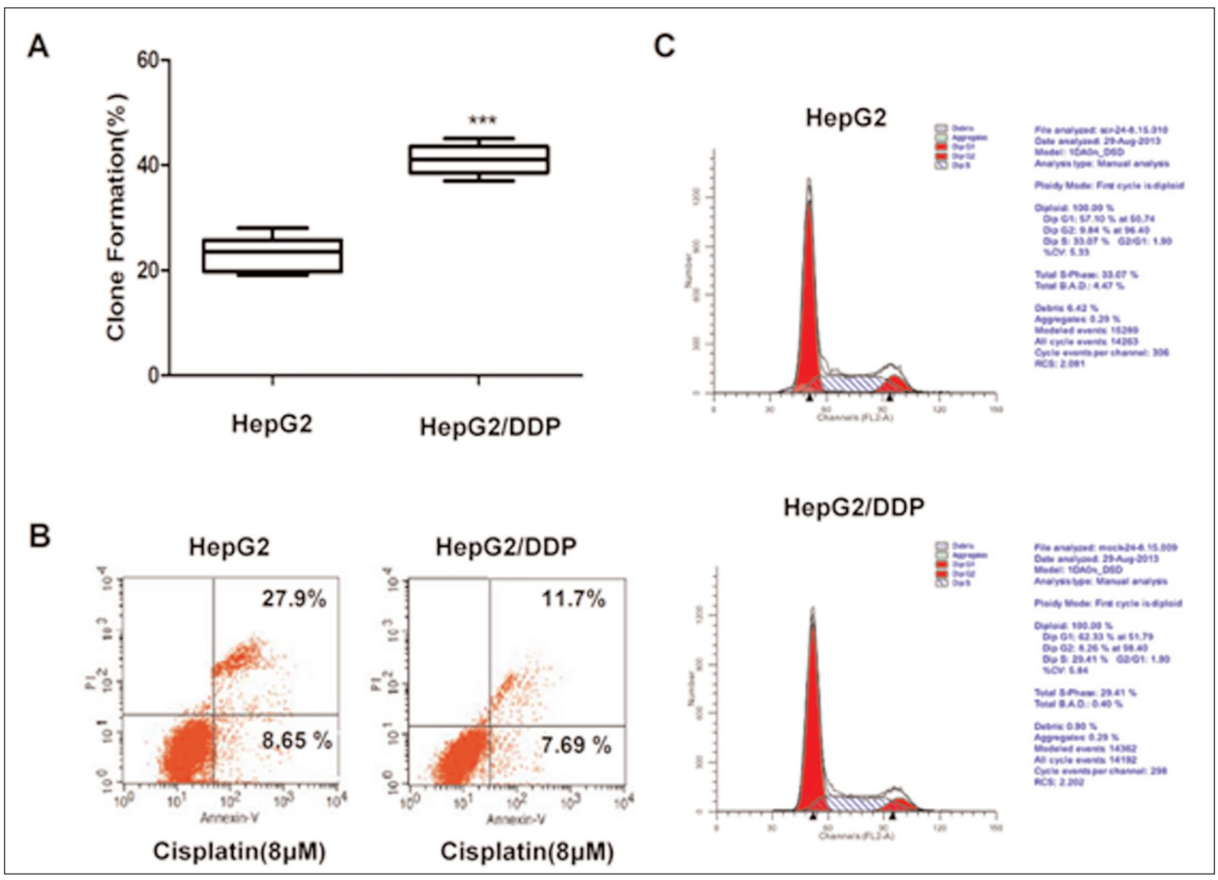

Figure 2. Colony formation efficiency, resistance to DDP-induced apoptosis, and cell cycle in HepG2 and HepG2/DDP cells. ***A,*** Colony formation efficiency in HepG2 and HepG2/DDP cells. ***$p < 0.001$. ***B,*** HepG2 and HepG2/DDP cells were treated with 8 μM DDP for 48 hours, and cell apoptosis was quantified by flow cytometry using the Annexin V and propidium iodide (PI) assay. ***C,*** HepG2 and HepG2/DDP cells were treated with 8 μM DDP for 48 hours, and cell cycle phases were assessed by flow cytometry.

findings, Bcl-2 and Bcl-XL protein expression levels were markedly higher in HePG2/DDP cells (Figure 4B). This was accompanied by lower levels of Bak and Bad (Figure 4B).

DDP Up-Regulates JNK1/2 Phosphorylation, and This is Reversed by the JNK Inhibitor SP600125

The next set of experiments studied the effects of DDP on activation of the JNK pathway. JNK is involved in many cellular processes, such as cell survival, cancerogenesis, cell growth/division, and cell death[16]. It is a subject of controversy whether the JNK pathway is involved in resistance of cancer cells to chemotherapy drugs.

We treated HepG2 with 8 μM DDP for 72 hours. Then, cellular proteins were extracted, and JNK1/2 total protein expression and phosphorylation was analyzed by Western blot. We observed that expression of phospho-JNK1/2 increased when HepG2 cells were treated with DDP (Figure 5A), whereas the levels of total protein were not affected (Figure 5A).

SP600125 is a specific inhibitor of the JNK signaling pathway. In our preliminary experiments, we verified that SP600125 at the concentration of 2 μM exerts no significant inhibitory effect on cell growth. In the next experiment, HepG2 cells were pre-treated with 2 μM of SP600125 for 2 hours and then exposed to 8 μM of DDP for 72 hours. Then, we quantified expression of total and phospho-JNK1/2 by Westen blot. We observed that JNK1/2 phosphorylation was significantly decreased when HepG2 cells were treated with both SP600125 and DDP (Figure 5B). Interestingly, SP600125 did not affect JNK1/2 phosphorylation when used in the absence of DDP (Figure 5B).

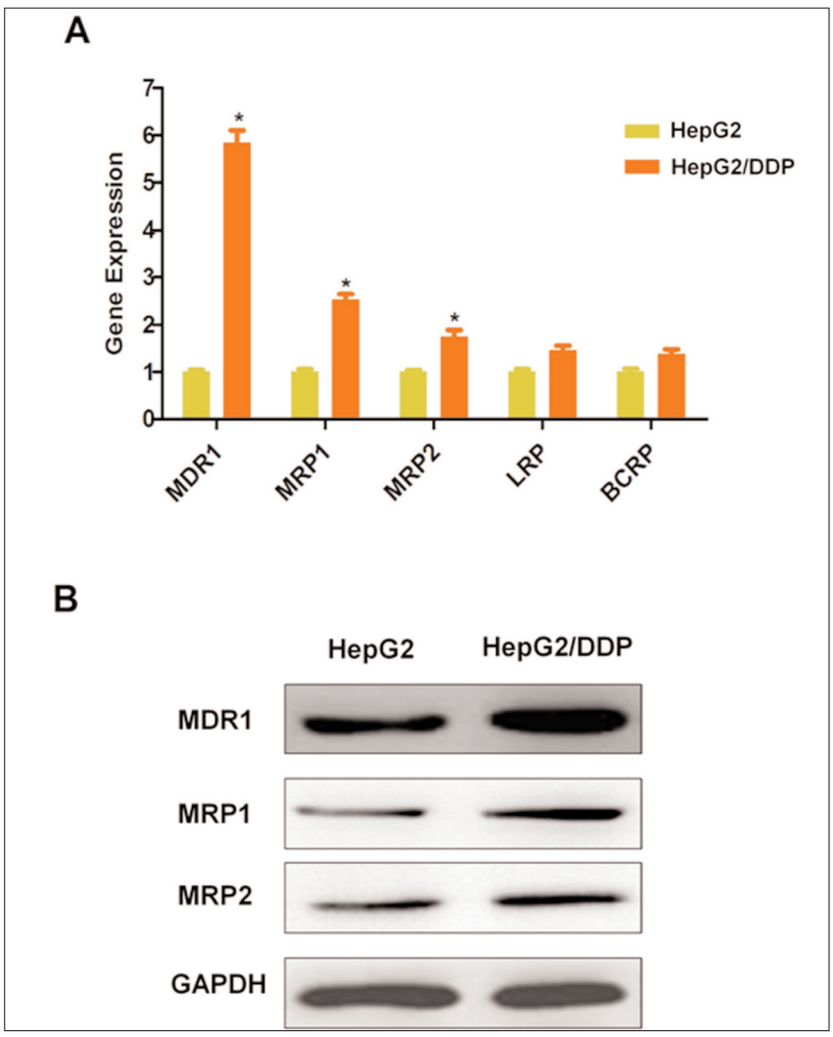

Figure 3. Expression of MDR1, MRP1, MRP2, LPR, and BCRP genes in HepG2 and HepG2/DDP cells. ***A,*** Expressions of MDR1, MRP1, MRP2, LRP, and BCRP genes were quantified in HepG2 cells and HepG2/DDP cells by qRT-PCR. $*p < 0.05$. ***B,*** Western blot analysis of expression of P-gp, MRP1, MRP2, and GAPDH (gel-loading control) in HepG2 and HepG2/DDP cells.

In the same experiment, we documented expressions of P-gp, Bcl-2 and Bak. P-gp and Bcl-2 expressions were increased, and Bak expression decreased, by DDP treatment (Figure 5B). These DDP-induced changes were reverted by SP600125 (Figure 5B).

Finally, we transduced HepG2 cells to constitutively overexpress JNK1/2. In transduced cells (infection with adenoviral vector at the Multiplicity of Infection of 10), phospho-JNK1/2 levels were dramatically increased when HepG2 cells were treated with 8 μM DDP (Figure 5C), confirming that DDP activates JNK1/2.

JNK Inhibition Enhances Sensitivity to DDP and Increases Cell Apoptosis

In this experiment, HepG2 cells were exposed to DDP at the concentration of 8 μM for 72 hours, with or without pre-treatment with SP600125 (2 μM for 2 hours). Experimental outcomes were growth inhibition and cell apoptosis.

We observed that cell growth was inhibited more substantially when HepG2 cells were exposed to both DDP and SP600125 (Figure 6A). Following this experiment, we quantified cell apoptosis and observed that the highest number of apoptotic cells was present in the wells exposed to both DDP and SP600125 (Figure 6B).

Discussion

We demonstrate here that expression of *MDR1* gene, the gene that encodes the drug efflux protein P-gp[17,18], is higher in the HepG2/DDP cell line, resistant to cisplatin. Moreover, this acquired resistance is associated with hyperactivation of JNK, whereas JNK inhibition increases

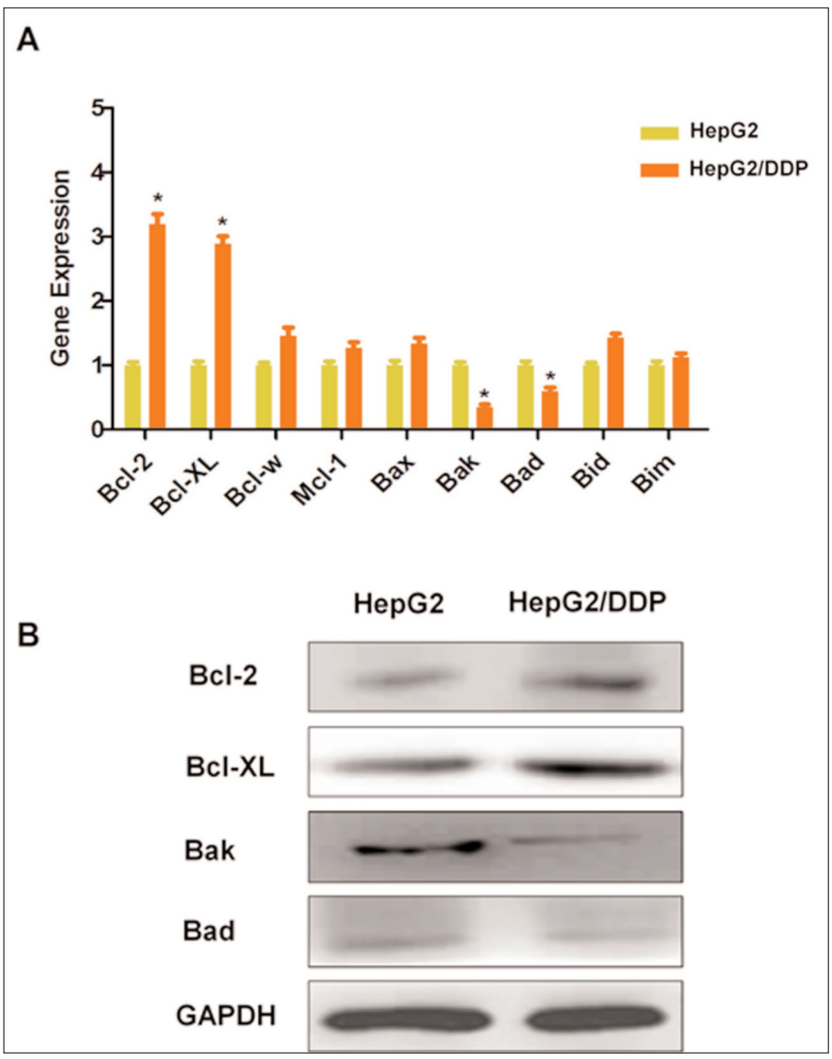

Figure 4. Expressions of pro- and anti-apoptotic gens in HepG2 and HepG2/DDP cells. ***A,*** Expressions of Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bax, Bad, Bid, and Bim genes were quantified in HepG2 cells and HepG2/DDP cells by qPCR. *$p < 0.05$. ***B,*** Western blot analysis of expression of Bcl-2, Bcl-XL, Bak, Bad, and GAPDH (gel-loading control) in HepG2 and HepG2/DDP cells.

sensitivity to cisplatin. These results underscore a close association between resistance to cisplatin in HCC cells, *MDR1* overexpression, and the JNK signaling pathway.

Drug resistance in cancer cells involves complicated intracellular mechanisms, including decreased drug uptake and increased drug efflux. This reduces intracellular drug concentration[19]. Furthermore, cancer cell resistance to chemotherapeutic drug may also involve abnormal resistance to cell apoptosis[20,21]. Cell apoptosis is regulated by multiple intracellular signaling pathways[22]. JNK is one of the Mitogen-Activated Protein Kinases (MAPK). It was confirmed in multiple studies that the JNK signaling pathway is involved in cell differentiation, cell apoptosis and stress response[23]. The precise role of this pathway in chemotherapy-induced apoptosis in cancer cells is still controversial. Our results are in line with previous reports that demonstrate that activation of the JNK signaling pathway promotes cell proliferation and chemotherapy resistance[24,25]. Also, silencing of the *c-jun* gene that encodes c-Jun, the transcriptional factor downstream of JNK, or inhibition of c-Jun, makes intestinal cancer smaller and prolong the life in a mouse model of intestinal cancer[26]. Furthermore, cell apoptosis could be enhanced, and cell cycle arrested, when the JNK pathway was inhibited by SP600125 or siRNA[27]. Sustained activation and overexpression of JNK was shown to correlate with drug resistance in the doxorubicin-resistant leukemia cell line HL-60/ADR, whereas inhibition of the JNK pathway reduced expression of multidrug resistance associated protein 1 and simultaneously increased sensitivity to chemotherapeutic drugs[28,29]. Similar observations were made in gastric cancer cell lines SGC7901 and SGC7901/DDP[30].

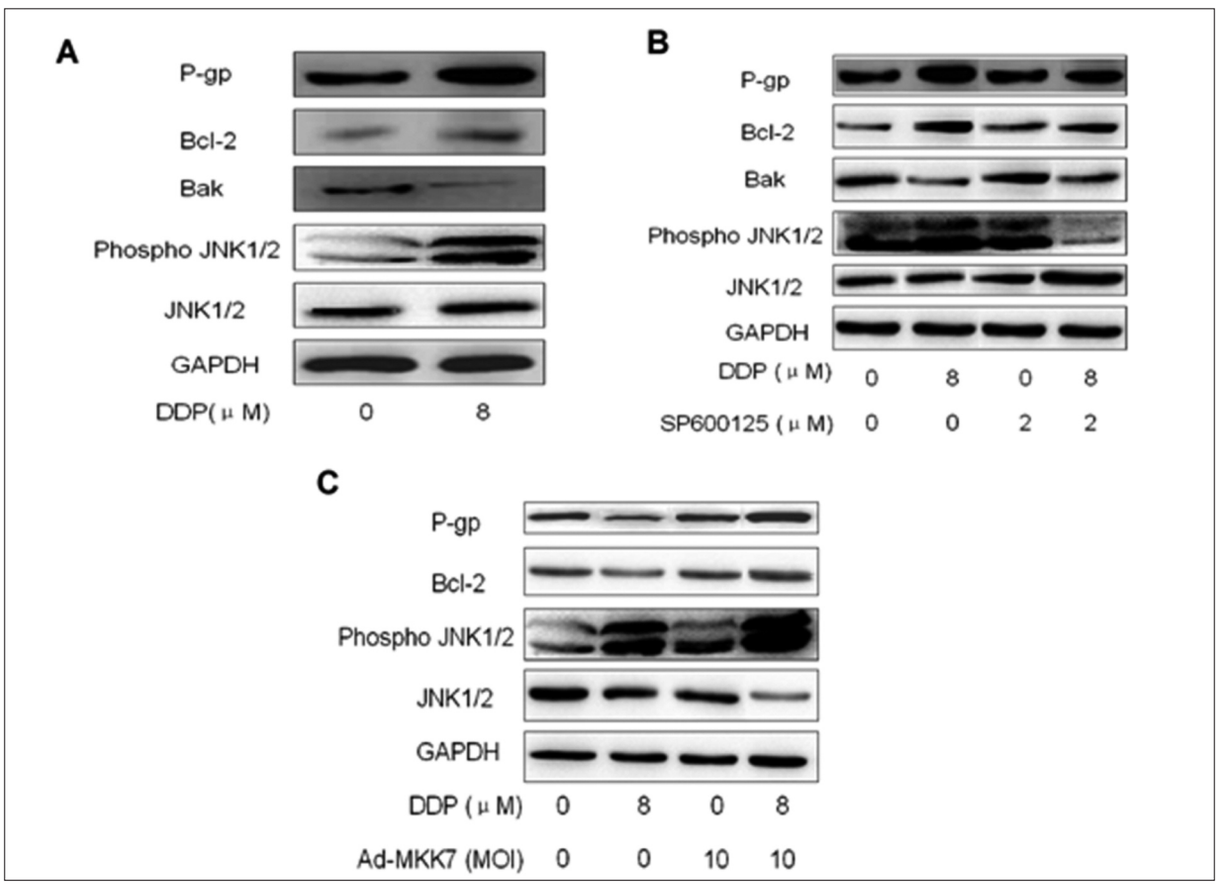

Figure 5. JNK signaling pathway in HepG2 cells treated with DDP and JNK inhibitor SP600125. ***A,*** Western blot analysis of P-gp, Bcl-2, Bak, and JNK1/2 expression, and JNK1/2 phosphorylation, in HepG2 cells treated for 72 hours with 8 μM DDP. GAPDH expression served as gel-loading control. ***B,*** Western blot analysis of P-gp, Bcl-2, Bak, and JNK1/2 expression, and JNK1/2 phosphorylation, in HepG2 cells treated for 72 hours with 8 μM DDP, with or without pre-treatment for 2 hours with SP600125 (2 μM). GAPDH expression served as gel-loading control. ***C,*** Western blot analysis of P-gp, Bcl-2, and JNK1/2 expression, and JNK1/2 phosphorylation, in HepG2 cells treated for 72 hours with 8 μM DDP. GAPDH expression served as gel-loading control. In some cells, JNK was overexpression by adenoviral transduction (Ad-MKK7) at the Multiplicity of Infection (MOI) of 10.

However, there are also reports that activation of the JNK signaling pathway can promote cell apoptosis[31,32]. Thus, in ovarian cancer cell lines sensitive to cisplatin, cisplatin activates JNK, leading to hyper-phosphorylation of its downstream factor c-jun and formation of the transcription factor Activator Protein 1, up-regulation of the death ligand FasL, and eventual cell apoptosis[12].

Thereby, it can be noted that the JNK signaling pathway may play a dual role in the chemoresistance of cancer cells, and the final pro- or anti-resistance role of this pathway may be related to cancer type, characteristics of apoptosis stimuli, duration of JNK activation, and involvement of other signaling pathways[33,34]. In HCC cells, tested in this study, inhibition of JNK decreased resistance to cisplatin.

Conclusions

The JNK signaling pathway promotes resistance to cisplatin in HCC cells, and inhibition of this pathway enhances the sensitivity of HCC cells to cisplatin by down-regulating the expression of P-gp.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

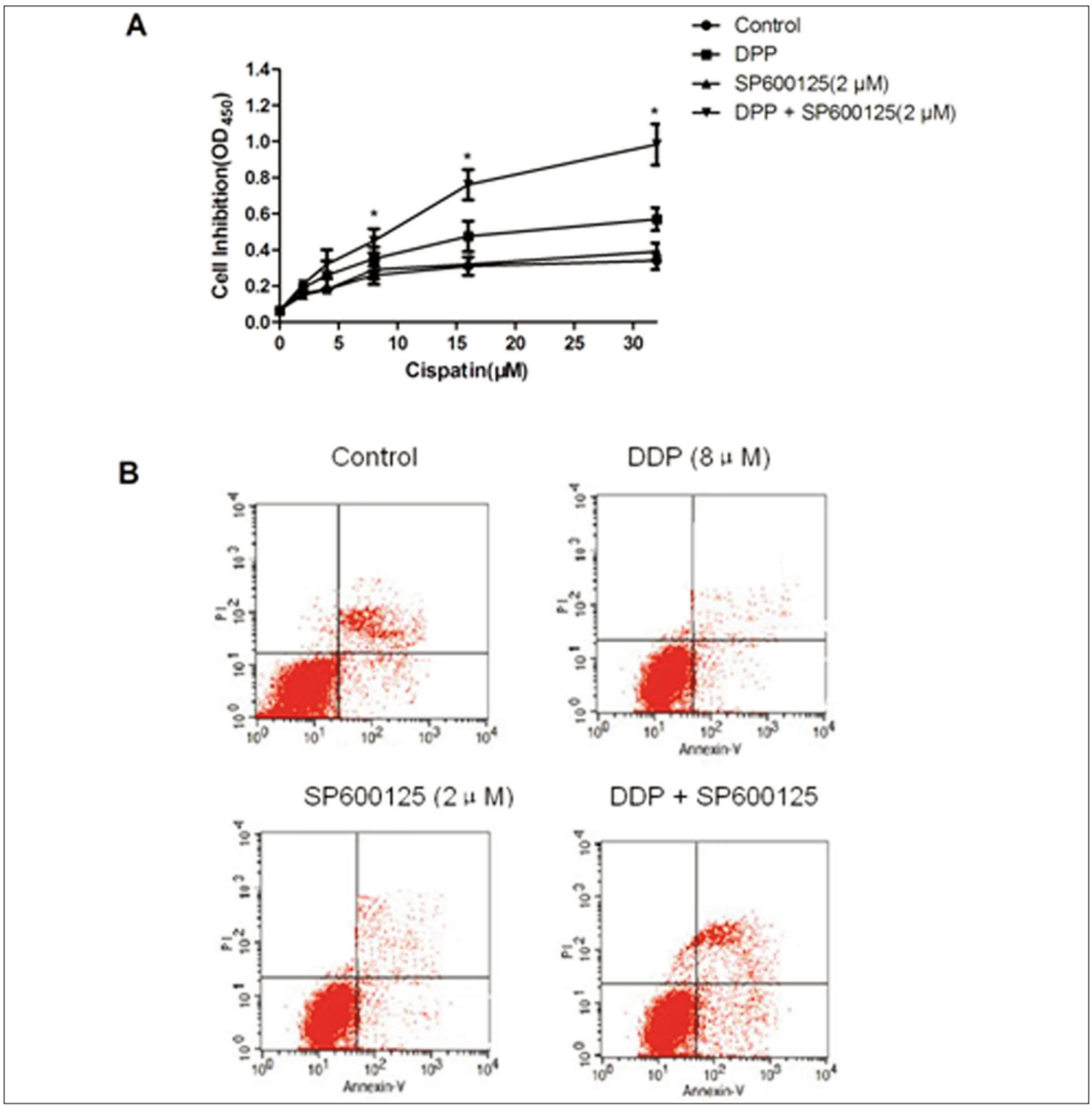

Figure 6. Cell growth inhibition and cell apoptosis in HepG2 cells treated with DDP and JNK inhibitor SP600125. ***A,*** HepG2 cells were treated for 72 hours with 8 μM DDP, with or without pre-treatment for 2 hours with SP600125 (2 μM). CCK-8 assay was used to quantify the cell growth inhibition. *$p < 0.05$. ***B,*** HepG2 cells were treated as above, and cell apoptosis was quantified by flow cytometry using the Annexin V and propidium iodide (PI) assay.

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