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Metformin enhances the chemosensitivity of hepatocarcinoma cells to cisplatin through AMPK pathway

HAO DONG[1*], JUNGANG HUANG[2*], KANG ZHENG[3], DONG TAN[3], QI CHANG[4], GENQIANG GONG[5], QING ZHANG[6], HANQIU TANG[7], JIANGUO SUN[8] and SHAOYU ZHANG[9]

[1]Department of Hepatobiliary Surgery, The Central Hospital of Xianyang, Xianyang; [2]Department of General Surgery, Traditional Chinese Medicine Hospital of Hanzhong, Hanzhong; [3]Department of General Surgery, 215 Hospital of Shaanxi Nuclear Industry, Xianyang; [4]Department of General Surgery, Affiliated Hospital of Yan'an University, Yan'an; [5]Department of General Surgery, The Central Hospital of Baoji, Baoji; [6]Department of Infectious Diseases, The Central Hospital of Hanzhong; [7]Department of Hepatobiliary Surgery, The Central Hospital of Hanzhong, Hanzhong; [8]Xingyuan Hospital of Yulin City, Yulin; [9]Department of Gastroenterology, Traditional Chinese Medicine Hospital of Baoji, Baoji, Shaanxi, P.R. China

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Abstract. This study investigated the effect of metformin on chemosensitivity of hepatocarcinoma cells to cisplatin and the possible mechanism. HepG2 and Huh-7 hepatoma cells were treated with cisplatin at concentrations of 0, 2, 4, 6, 8 and 10 μM for 48 h. Proliferation of HepG2 and Huh-7 hepatoma cells were detected by MTT assay. Apoptosis of hepatocellular carcinoma cells was detected by flow cytometry. Western blot analysis was used to detect the expression of 5-monophosphate-activated protein kinase (AMPK) and p-AMPK protein. Proliferative activity of HepG2 and Huh-7 cells decreased with the increase of cisplatin concentration. After adding metformin, proliferation ability of hepatocarcinoma cells was significantly reduced. Apoptosis rate of the metformin was significantly higher than that of the control group, and apoptosis rate of the cisplatin + metformin was significantly higher than that of the cisplatin group. There was no significant difference in expression level of AMPK protein found between control, metformin, cisplatin and cisplatin + metformin group. Compared with the control, ratio of p-AMPK/AMPK in metformin group was increased, and ratio of p-AMPK/AMPK in cisplatin + metformin was significantly higher than that in cisplatin group. Activity of cells in cisplatin + metformin + compound C (AMPK pathway blocker) group was significantly higher than that of cisplatin + metformin, while apoptosis of cells in cisplatin + metformin + compound C (AMPK pathway blocker) was significantly lower than that of cisplatin + metformin group. In conclusion, metformin can inhibit the proliferation, promote apoptosis and enhance the chemosensitivity of hepatocarcinoma cells to cisplatin through AMPK pathway.

Correspondence to: Dr Shaoyu Zhang, Department of Gastroenterology, Traditional Chinese Medicine Hospital of Baoji, 43 Baofu Road, Baoji, Shaanxi 721001, P.R. China
E-mail: tb24sy@163.com

*Contributed equally

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Introduction

As one of the common malignant tumors in China, hepatocarcinoma shows an increasing incidence rate and the age of onset is also becoming increasingly younger. Surgical resection is the most important treatment of early non-metastatic hepatocarcinoma, but the 5-year recurrent rate is still higher than 60% after surgery (1). Resistance to chemotherapy drugs is the main reason for poor prognosis of hepatocarcinoma. As the first-line drug used in the treatment of hepatocarcinoma, cisplatin has broad spectrum anticancer activity. Cisplatin has been widely used in the treatment of various malignant tumors including hepatocarcinoma, ovarian, prostate, lung, esophageal, head and neck squamous cell carcinoma and thyroid cancer. However, application of high doses of cisplatin can bring adverse side effects on the nervous system, kidney and gastrointestinal tract, treatment with cisplatin usually failed to provide satisfactory outcomes in treatment of hepatocarcinoma (2,3). Therefore, it will be of significant clinical value for treatment of hepatocarcinoma to identify drugs that can enhance the chemosensitivity of hepatocarcinoma cells to cisplatin.

In previous years, combined chemotherapy has been proved to be an import way in increasing the efficacy of the treatment of hepatocarcinoma (4) and cisplatin treatment combined with specific molecular targeted drug has attracted increased attention. As an oral antidiabetic drug, metformin is the first-line drug for the treatment of type 2 diabetes. Studies have shown that metformin has anticancer function. Therefore,

in this study, cisplatin was used to treat hepatocarcinoma cells HepG2 and Huh-7 and effects of metformin on chemosensitivity of hepatocarcinoma cells to cisplatin and the specific molecular mechanism were studied. Our study provided new ideas and theoretical basis for improving the chemosensitivity of hepatocarcinoma cells to cisplatin.

Materials and methods

Main reagents. RPMI-1640 cell culture medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Metformin, cisplatin, adenosine 5-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway blocker compound C and methyl thiazolyl tetrazolium assay (MTT) cell proliferation assay kit were purchased from Sigma (Merck & Co., Inc., Whitehouse Station, NJ, USA). Annexin V-FITC apoptosis detection kit was from Bogu Biological Science and Technology Co., Ltd. (Shanghai, China). Rabbit anti-human AMPK and p-AMPK polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) (dilution, 1:1,000; cat. nos. 2532 and 4184). Rabbit anti-human β-actin polyclonal antibody and goat anti-rabbit horseradish peroxidase-labeled secondary polyclonal antibody were purchased from Boster Biological Technology Co., Ltd. (Pleasanton, CA, USA) (dilution, 1:2,000; cat. nos. BM0627 and BA1054). RIPA protein lysate was purchased from Beijing Kangwei Century Biotechnology Co., Ltd. (Beijing, China).

Cell culture. HepG2 and Huh-7 cell lines were purchased from Guangzhou Cellcook Biotech Co., Ltd. (Guangzhou, China). HepG2 and Huh-7 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were kept in a humidified incubator (37˚C, 5% CO_2). Trypsin 0.25% was added for digestion for 2 min and complete medium was added to terminate the digestion before subculture. After centrifugation for 3 min at 1,000 rpm, cells were resuspended and transferred to a new culture bottle. Culture medium was replaced every day and subculture was performed every three days. Cells were collected at logarithmic growth phase for follow-up experiments.

MTT assay to detect the proliferative activity of hepatocarcinoma cells. HepG2 and Huh-7 hepatocarcinoma cells in good growth conditions were inoculated into 96-well plates with 100 μl ($2x10^5$/ml) per well, and were cultured in an incubator. Twenty four hours later, culture medium was replaced with complete culture medium containing different concentrations of cisplatin or metformin. Three wells were set in each group and cells were incubated for 48 h in the cell incubator. MTT reagent was added and incubated for another 4 h. Optical density value of each well was read at a wavelength of 480 nm using a multi-function microplate reader.

Flow cytometry to detect cell apoptosis. Cells treated with cisplatin (0, 2, 4, 6, 8 and 10 μM), metformin (10 mmol/l) and AMPK pathway blocker compound C (10 μmol/l) were digested to make single cell suspension. Hepatocarcinoma cells ($5x10^5$ cells) were mixed with 100 μl of 1X binding buffer, and 5 μl of Annexin V-FITC and 5 μl of PI reagent were also added, followed by incubation at room temperature in the dark for 15 min. After that, 400 μl 1X binding buffer was added to resuspend the cells and cell apoptosis rate was detected by flow cytometry.

Western blot analysis to detect the expression levels of AMPK and p-AMPK protein. Cells treated with cisplatin (0, 2, 4, 6, 8 and 10 μM), metformin (10 mmol/l) and AMPK pathway blocker compound C (10 μmol/l) were digested and havested. Cells were lysed to extract total protein. Protein samples were mixed with loading buffer and denatured at 98˚C for 5 min. A total of 10 μg of protein from each sample was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by transmembrane to polyvinylidene difluoride membrane. After blocking with 5% skimmed milk at room temperature for 45 min, membranes were incubated with primary antibody overnight at 4˚C. Membranes were then rinsed with washing solution 3 times, 10 min each time. Horseradish peroxidase labeled secondary antibody (1:2,000) was used to incubate the membranes at room temperature for 1 h. Membranes were then rinsed with washing solution 3 times, 15 min each time. Color development was performed using ECL chemiluminescence kit (Beijing Kangwei Century Biotech Co. Ltd., Beijing, China). ImageJ software (NIH Image, Bethesda, MD, USA) was used to quantitatively analyze the western blot analysis.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the experimental data. Experimental data were expressed as mean ± standard deviation. Comparisons between groups were performed by t-test. A $P<0.05$ was considered to indicate a statistically significant analysis.

Results

Metformin enhances the inhibitory effect of cisplatin on proliferation of hepatocarcinoma cells. HepG2 and Huh-7 cells were treated with cisplatin at 0, 2, 4, 6, 8 and 10 μM for 48 h and proliferation activity of HepG2 and Huh-7 cells was detected by MTT assay. Results showed that proliferation activity of HepG2 and Huh-7 cells decreased with the increase of cisplatin concentration. After treatment with metformin (10 mmol/l) for 4 h, proliferation activity of HepG2 and Huh-7 cells were significantly reduced compared with control group. Results of this experiment suggest that metformin can enhance the inhibitory effect of cisplatin on proliferation of HepG2 and Huh-7 cells (Fig. 1).

Metformin enhances cisplatin-induced apoptosis of hepatocarcinoma cells. Flow cytometry experiment was carried out to investigate whether metformin can enhance apoptosis of hepatocarcinoma cells induced by cisplatin. Apoptosis rate of HepG2 hepatocarcinoma cells in metformin (10 mmol/l) group was significantly higher than that in control group ($P<0.05$). After incubation for 48 h, apoptosis rate of cisplatin (8 μM) + metformin (10 mmol/l) was significantly higher than that of cisplatin group ($P<0.05$) (Fig. 2A). Similar result was found in Huh-7 hepatocarcinoma cells (Fig. 2B). These results indicate that metformin can promote apoptosis of HepG2

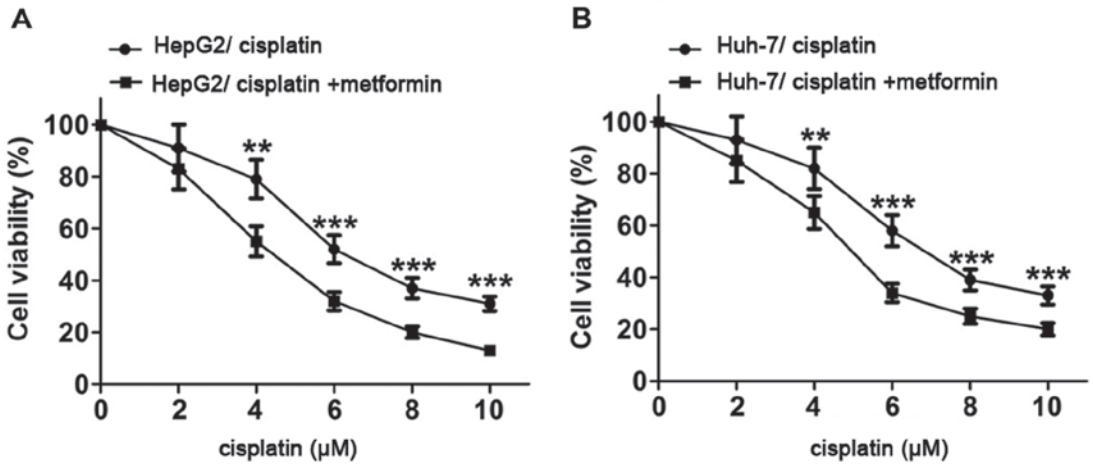

Figure 1. Role of metformin in the inhibition of hepatocarcinoma cell proliferation induced by cisplatin. MTT assay showed that metformin could enhance the inhibitory effect of cisplatin on proliferation of HepG2 (A) and Huh-7 (B) hepatocarcinoma cells. **Compared with the cells treated with the same concentration of cisplatin, P<0.01; ***compared with the cells treated with the same concentration of cisplatin, P<0.001; MTT, methyl thiazolyl tetrazolium assay.

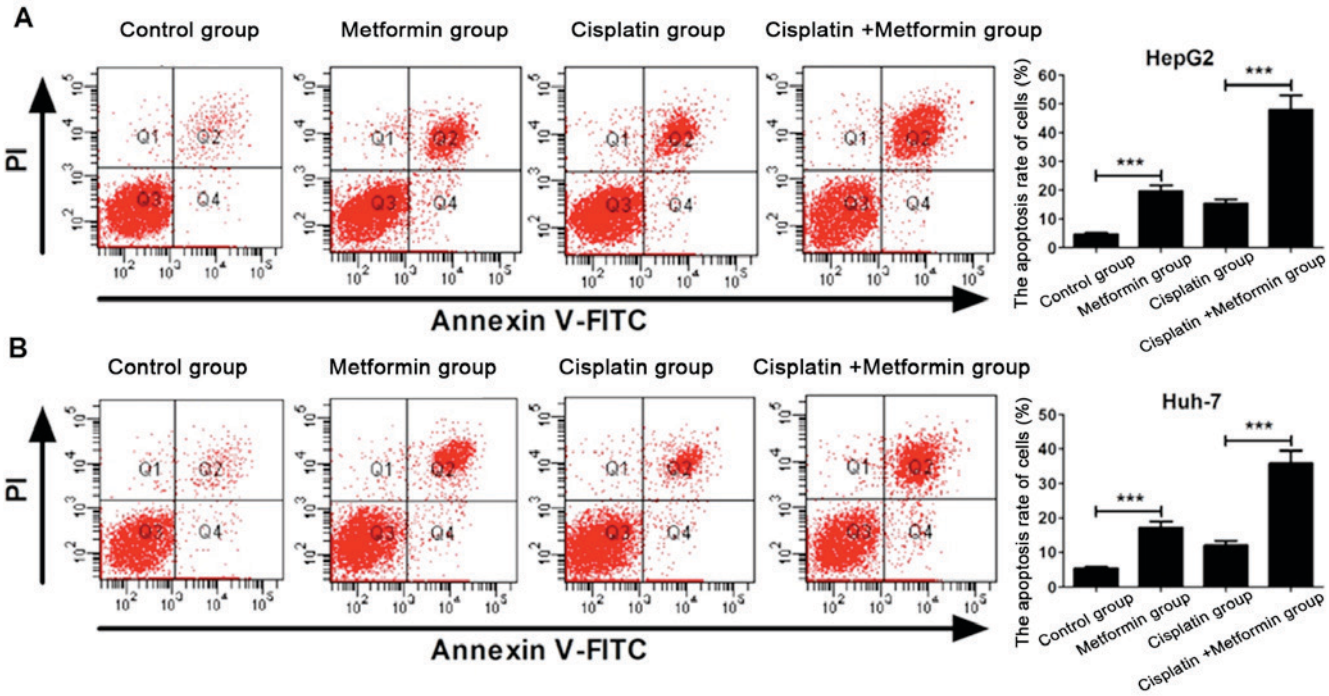

Figure 2. Role of metformin in cisplatin-induced apoptosis of hepatocarcinoma cells. Flow cytometry showed that metformin can enhance apoptosis of HepG2 (A) and Huh-7 (B) hepatocarcinoma cells induced by cisplatin. ***Compared with the cells treated with same concentration of cisplatin, P<0.001.

and Huh-7 hepatocarcinoma cells and enhance the ability of cisplatin in inducing apoptosis of HepG2 and Huh-7 hepatocarcinoma cells.

AMPK signaling pathway is activated in hepatocarcinoma cells treated with metformin. In order to study the molecular mechanism of metformin in enhancing the sensitivity of hepatocarcinoma cells to cisplatin, the status of AMPK signaling pathway was detected by western blot analysis. HepG2 and Huh-7 hepatocarcinoma cells were treated with cisplatin (8 μM) and metformin (10 mmol/l) for 48 h. Western blot analysis are shown in Fig. 3A. No significant difference in expression level of AMPK protein was found among control, metformin, cisplatin and cisplatin + metformin group (Fig. 3B). Compared with control, ratio of p-AMPK/AMPK was increased in metformin group. Ratio of p-AMPK/AMPK in cisplatin + metformin was significantly higher than that in cisplatin group (Fig. 3C). These results suggest that metformin can activate the AMPK signaling pathway in hepatocarcinoma cells.

Compound C reverses the effect of metformin in increasing the chemosensitivity of hepatocarcinoma cells to cisplatin. In order to further confirm that metformin can increase the chemosensitivity of hepatocarcinoma cells to cisplatin by activating AMPK pathway, cells were treated with AMPK signal

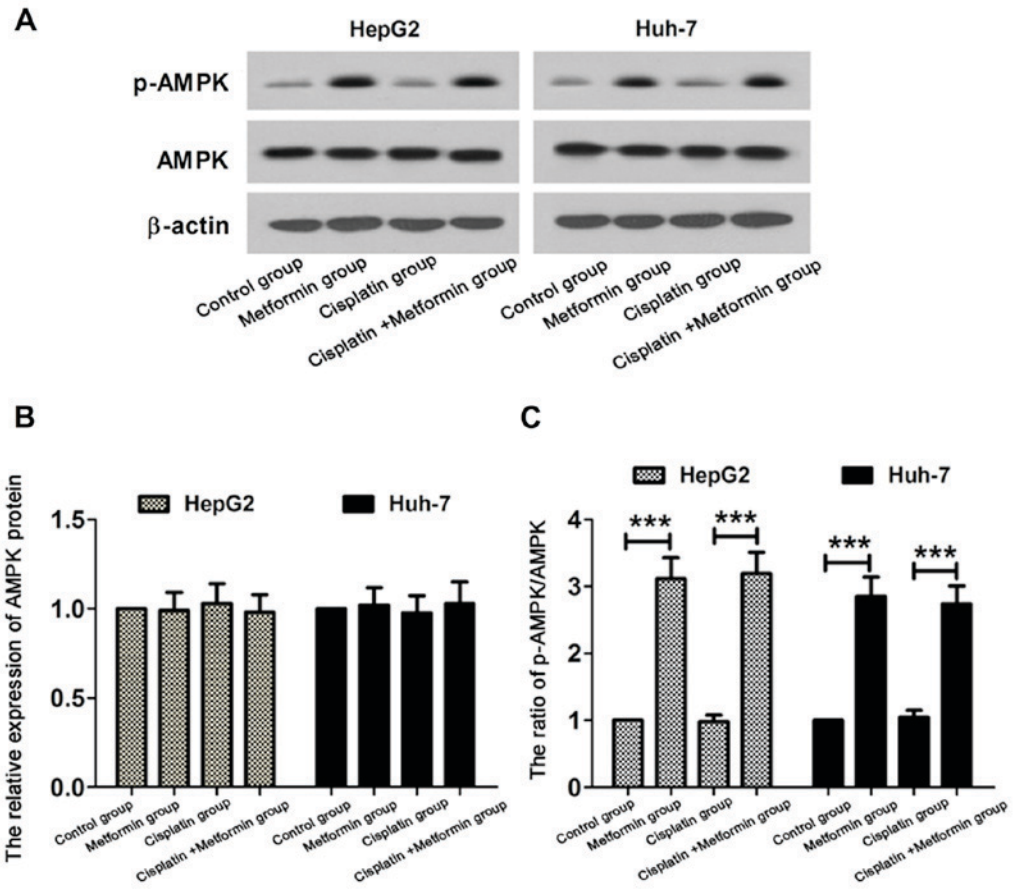

Figure 3. Effect of metformin on AMPK signaling pathway in hepatocarcinoma cells. Western blot analysis showed that metformin was able to activate AMPK signaling pathway in hepatocarcinoma cells (A and B). Ratio of p-AMPK/AMPK in cisplatin + metformin was significantly higher in metformin group and cisplatin + metformin group (C). ***Compared with cells in control group or cells treated with the same concentration of cisplatin, P<0.001; AMPK, 5-monophosphate-activated protein kinase.

blocking agent compound C (10 μmol/l) for 2 h. Changes of the effect of metformin in inhibiting proliferation of hepatocarcinoma cells and promoting apoptosis of hepatocarcinoma cells were observed after the inhibition of AMPK signaling pathway. Flow cytometry showed that, compared with cisplatin group, cell viability of cisplatin + compound C group was increased and apoptosis was decreased. Cell viability of cisplatin + metformin + compound C was significantly higher than that of cisplatin + metformin group. Apoptosis rate of cisplatin + metformin + compound C was significantly lower than that of cisplatin + metformin group (Fig. 4A and B). Similar results were found in Huh-7 hepatocarcinoma cells (Fig. 4C and D). The results suggested that effect of metformin in increasing the chemosensitivity of hepatocarcinoma cells to cisplatin was reversed by compound C. Therefore, we speculate that metformin may be an effective activator of AMPK, and combined metformin treatment can enhance the cytotoxic effect of cisplatin on tumor cells. This study showed that AMPK signaling pathway was activated in metformin-treated hepatocarcinoma cells, and compound C treatment reversed the effect of metformin in increasing the chemosensitivity of hepatocarcinoma cells to cisplatin, further confirming that activation of AMPK signaling pathway is the key for metformin to improve the chemosensitivity of hepatocarcinoma cells to cisplatin.

Discussion

As a non-specific cell cycle toxic drug, cisplatin is one of the main drugs used in the clinical treatment of hepatocarcinoma. Cisplatin can be hydrolyzed into platinum diaminodichloride after entering cells to damage cell membrane structure. Cisplatin can also bind to purine and pyrimidine bases of DNA in nucleus to form cisplatin and DNA complex to cause DNA breakage and error code, which in turn inhibit DNA replication and transcription, thereby inducing tumor cell apoptosis to play a role in treating tumors (5). However, patients with hepatocarcinoma usually show varying degrees of resistance to cisplatin, which in turn lead to the insensitivity to cisplatin and unsatisfactory treatment outcomes, and this is the main cause of failure of the treatment of hepatocarcinoma (6). Previous studies have shown that the automatic DNA damage repair function is one of the main mechanisms of cisplatin resistance (7). The emergence of resistance to cisplatin can greatly reduce treatment efficacy of hepatocarcinoma. Most researchers believe that optimizing the combined drugs to enhance the antitumor effect of cisplatin, and reduce the concentration of cisplatin to reduce its side effects is the preferred strategy in treatment of hepatocarcinoma (8). Therefore, the identification of effective cisplatin resistance reversal agents is the most effective method in increasing

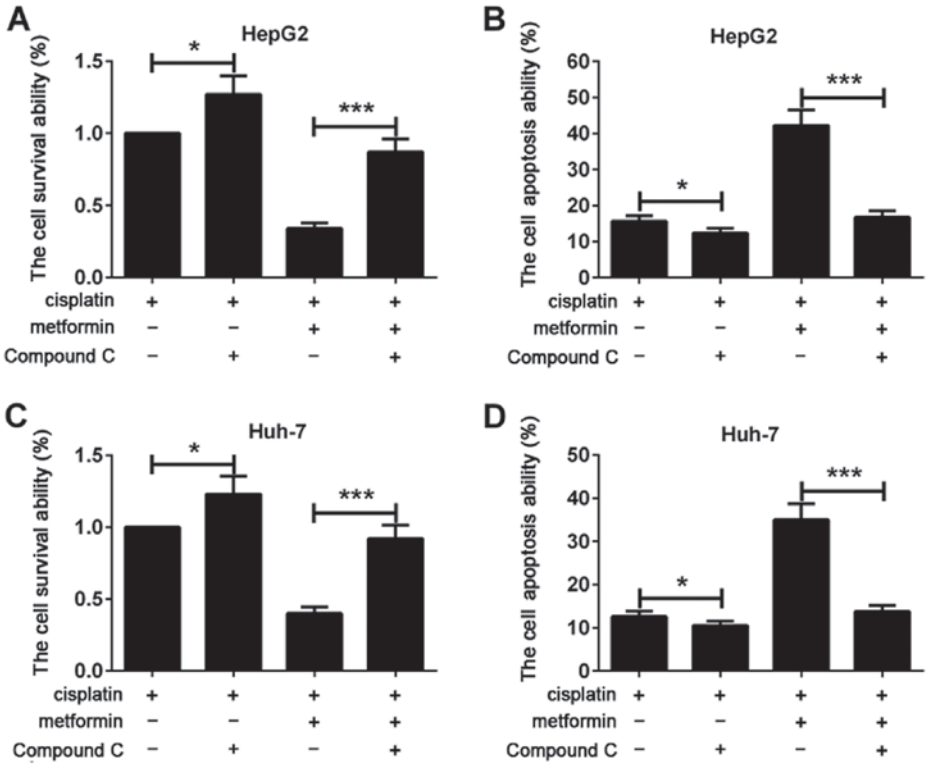

Figure 4. Effects of compound C on increased chemosensitivity of hepatocarcinoma cells to cisplatin induced by metformin. Apoptosis and survival ability experiments showed that compound C reversed the effect of metformin in increasing the chemosensitivity of hepatocarcinoma cell HepG2 (A and B) and Huh-7 (C and D) to cisplatin. *Compared with the cells treated with the same concentration of cisplatin, P<0.05; ***compared with cells treated with the same concentration of cisplatin + metformin, P<0.001.

treatment efficacy of hepatocarcinoma, it is also an active research topic and difficult problem in chemotherapy of hepatocarcinoma.

Many studies have shown that metformin can reduce the occurrence of oxidative phosphorylation and regulate energy metabolism processes mainly through the inhibition of mitochondrial respiratory chain complex I activity to reduce the energy state of cells. Clinical study carried out by Evans *et al* showed that, compared with type 2 diabetes patients treated with other drugs, patients treated with metformin showed significantly reduced incidence of cancer (9). Meta-analysis showed that risk of hepatocarcinoma was reduced by 62% in type 2 diabetes patients treated with metformin (10). A prospective study on patients with endometrial cancer showed a significant correlation between metformin intake and increased recurrence-free survival and overall survival (11). Wheaton *et al* found that metformin could inhibit the proliferation and promote apoptosis of A549 human lung adenocarcinoma cells by reducing the activity of complex I in mitochondria of cancer cells (12). Overexpression of IL-6 can induce tyrosine kinase inhibitor (TKI) resistance in TKI sensitive cells. Metformin can enhance the sensitivity of human lung cancer cells to epidermal growth factor (EGF) receptor TKI (EGF receptor TKI) by inhibiting IL-6 signaling pathway. EGFR-TKI kinase inhibitors combined with metformin therapy effectively prevented tumor growth in nude mice transplanted with TKI-resistant tumor cells (13). Compared with the chemotherapy using cisplatin alone, combination of cisplatin and metformin treatment significantly increased the inhibitory effect on tumor growth (14). This study found that metformin can enhance the chemosensitivity of drug-resistant hepatocarcinoma cell lines to chemotherapeutic drugs cisplatin, and the possible molecular mechanism of the function of metformin was also explored. Results showed that cisplatin could inhibit the proliferation and promote apoptosis of HepG2 and Huh-7 hepatocarcinoma cells. HepG2 and Huh-7 hepatocarcinoma cells were more sensitive to cisplatin after adding metformin.

AMPK is a serine/threonine protein kinase in eukaryotic cells. As an important energy receptor kinase in the cell, AMPK regulates intracellular production of adenosine triphosphate (ATP) in a poor nutrient environment (15). Abnormal AMP/ATP ratio in the body can be caused by various factors including ischemia, hypoxia, lack of energy, and heat shock. Under such conditions, AMPK is activated, resulting in a decrease in AMP/ATP ratio. Downstream substrate of intracellular energy metabolism cycle will also be activated to participate in the regulation of the synthesis of energy metabolism related protein (16). Studies have shown that metformin can activate AMPK, thereby inhibiting signal transduction of mitogen-activated protein kinase and phosphatidylinositol 3-kinase/protein kinase B and inhibiting the growth of breast cancer, lymphoma and other cancers (17,18). Honjo *et al* have found that metformin can inhibit oncogene PI3K/Mammalian target of rapamycin (mTOR) signal pathway to reduce survival rate of esophageal cancer cells and promote esophageal cancer cell apoptosis, thereby enhancing the sensitivity of esophageal cancer to chemotherapy (19). Metformin can inhibit phosphorylation of mTOR downstream target gene p70-S6 and ribosomal S6 protein kinase, and DEPTOR-related mTOR

inhibition has been shown to be one of the mechanisms of anti-hepatocarcinoma function of metformin (20). These studies suggest that AMPK can serve as a potential target for tumor therapy.

In conclusion, metformin enhanced the cytotoxicity of cisplatin to hepatocarcinoma cells. This study revealed the mechanism of metformin in increasing the chemosensitivity of hepatocarcinoma cells to cisplatin. This study also proved that metformin may potentially serve as an effective adjuvant drug of cisplatin in the treatment of hepatocarcinoma. Our study provided experimental basis for the development of chemotherapy in the treatment of hepatocarcinoma.

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