Int J Clin Exp Med 2017;10(4):6604-6612
www.ijcem.com /ISSN:1940-5901/IJCEM0045252

Original Article

Metformin induces apoptosis of osteosarcoma U2OS and 143B cells through the mitochondria-dependent pathway and potentiates the anti-neoplastic activity of cisplatin in vivo

Yanguo Wang[1,2], Zheng Wang[2], Dalei Shi[2], Hongxin Zhu[2], Nan Feng[2], Xinyu Liu[1], Yanping Zheng[1]

[1]Department of Orthopaedics, Qilu Hospital of Shandong University, Jinan, Shangdong, China; [2]Department of Orthopedics, Binzhou Central Hospital, Binzhou Medical College, Binzhou, Shangdong, China

Received November 28, 2016; Accepted February 14, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Metformin is an oral biguanide commonly used for treating type II diabetes and has recently been demonstrated to reduce cancer risk and improve cancer prognosis. The anti-tumor mechanisms mainly include activation of the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) pathway and direct inhibition of Insulin growth factor (IGF)-mediated cellular metabolism. However, the antineoplastic mechanisms of metformin in Osteosarcoma (OS) have not been fully verified. The purpose of this project was to detect whether metformin in combination with or without cisplatin has an anticancer effect on OS cell lines, and to further explore the possible mechanisms. The U2OS and 143B OS cells were treated with metformin, cisplatin or a combination of both drugs respectively, and cell viabilities were performed in triplicates by using Methylthiazolyldiphenyl-tetrazolium-bromide (MTT) assay. The cell cycle and the cell apoptosis were detected by flow cytometry. Levels of the Bcl-extra large (Bcl-xl), B-cell lymphoma (Bcl)-2, PARP and Bax proteins were evaluated by western blot analyses. Finally, nude mice were transplanted with 143B cells and the tumor growth inhibition rate was detected. Metformin could inhibit the proliferation of OS cells in a time- and dose-dependent manner, and induce cell apoptosis and cell cycle arrest. For example, once the U2OS cells had been treated with 20 mM metformin for 48 hrs, the cell cycle was arrested in the S phase and the apoptosis rate was 30.31±2.25%. Western blotting results indicated that the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl were down-regulated, while the expression of pro-apoptotic protein Bax was significantly up-regulated following the treatment with metformin. *In vivo* studies, metformin alone did not significantly affect the tumor growth in nude mice. However, metformin could remarkably potentiate the anti-tumor effect of cisplatin *in vivo*, tumor size in the cisplatin plus metformin group was significantly smaller then the cisplatin alone group or the control group. The inhibition rates in cisplatin group and cisplatin plus metformin group were 46.8 and 67.1%, respectively, compared with the control group. Collectively, these data provide evidence on the role of metformin as an anti-neoplastic therapeutic that can act through the intrinsic mitochondria-dependent pathway, and metformin could potentially be used for the treatment of Osteosarcoma especially in combination with cisplatin.

Keywords: Metformin, osteosarcoma, cisplatin, apoptosis, mitochondria-dependent, tumor xenograft

Introduction

Osteosarcoma is the main malignant primary bone tumor found in children and young adults, and accounts for over 50% of all bone sarcomas [1, 2]. With the highest incidence at around 18 years, the male/female sex ratio is 1:4. A second peak of incidence is described in the elderly following radiotherapy, or suffering from Paget disease at the same time [3, 4]. Traditional therapeutic methods are based on early diagnosis, surgical resection and postoperative chemotherapy. However, Osteosarcomas are particularly tend to pulmonary metastases, which occur within three years of diagnosis and have a negative effect on survival rate. The 5-year survival rate is evaluated at around 65% for patients without metastases and decreases to 30% when lung metastases were discovered at the time of diagnosis (around 20% of patients) [5]. Unfortunately, this therapeutic approach has met a plateau of efficacy

and the mortality of OS patients has elevated in the last ten years. Thus, new therapeutic treatments are urgently required to improve the prognosis of OS patients.

Metformin, an oral antidiabetic drug globally, is widely used for the treatment of type 2 diabetics with insulin resistance, obesity, and serious hyperinsulinemia. At the cellular and molecular level, metformin exerts an anti-mitogenic effect by activation of the AMPK and subsequent inhibition of the mTOR pathway, which play an important role in regulating cell growth and metabolism [6-8]. These effects were the theoretical basis to consider metformin as a prospective drug for adjuvant anticancer therapy. Up to the present, several other anti-cancer mechanisms have also been demonstrated, including the suppression of tumor stem cells, the reversal of chemotherapeutics resistance and inducing cell cycle arrest [9-12]. In the present study, we evaluated the anti-neoplastic activity of metformin in OS cells, and its relationship to mitochondria-dependent signal pathway.

Materials and methods

Chemicals and reagents

Metformin and MTT were obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in phosphate-buffered saline (PBS). Cisplatin was purchased from Mayne Pharmaceuticals (Montreal, Quebec, Canada) as a stock solution of 1 mg/ml. The antibodies against B-cell lymphoma (Bcl)-2, Bax, caspase-3, PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). Enhanced chemiluminescence (ECL) reagents, horseradish peroxidase-conjugated anti-rabbit immnoglobulin (Ig)G and anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and treatments

The human OS cell lines U2OS and 143B were purchased from American Type Culture Collection (Manassas, VA, USA). The cells grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS: Hyclone, Logan, UT, USA), 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 humidified incubator. The cells were routinely passaged every 2~3 days when they reached ~80% confluence.

Cell proliferation assay

The MTT assay was used to detect the effect of metformin on the OS cells. In Brief, a number of 2000-2500 living cells were plated into 96-well flat-bottom cell culture plates (Corning Incorporated, NY, USA) in medium containing 10% FBS, Controls included 0.2% DMSO. Metformin was used at different concentrations (10, 20, and 50 mM). For time course, cells were incubated under similar conditions for variable times ranging from 24 h to 72 h. In the cisplatin experiments, cells were incubated for 4 h at 37°C with cisplatin of 0, 5 or 10 μg/ml, respectively. Then the medium was changed and the cells were further treated with DMSO as control or metformin at different concentrations. Absorbance was finally detected in an automated microplate reader (BioRad, Hercules, CA, USA) at 450 nm. All experiments were performed at least 3 times.

Flow cytometry

The U2OS and 143B OS cells were starved of serum for 24 h and then treated with different doses (10 or 20 mM) of metformin for 48 h respectively. The cells were then washed with PBS (pH = 7.4) and fixed with 70% ice-cold ethanol at 4°C overnight. After fixation, the cells were stained with propidium iodide (PI) at 1 mg/ml for 30 min at room temperature. The cell cycle was analyzed by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ, USA). For cell apoptosis detection, apoptotic rate of the two OS cells was analyzed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). A total of 1×10^5 OS cells/well were seeded into six-well plates and cultured in DMEM at 37°C overnight. Subsequent to starvation for 12 h, the OS cells were treated with different doses of metformin (0, 10 and 20 mmol/l) in complete medium for 48 h, digested with 2.5 mg/ml trypsin, washed twice with PBS and suspended with 300 μl binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The cells were then incubated with 2 μl Annexin V and 5 μl PI for 15 min at room temperature, and the distribution of viable, early apoptotic, late apop-

totic and necrotic cells was detected using a FACS Caliber flow cytometer (BD Biosciences). Cells that were negative for the Annexin V-FITC and PI were considered to be viable cells, and the sum of the early and late apoptotic cells constituted the total number of apoptotic cells, which was presented as the percentage of the total cells.

Mitochondrial and cytosolic fractionation

We carried out the isolation of the mitochondria and cytosol on the basis of the specifications of the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). Specimen of cytosol and mitochondria were dissolved in lysis buffer, and the related proteins were analyzed by western blotting, respectively.

Western blot analysis

The two OS cell lines were treated with metformin at different concentrations (0, 5, 10, 20 and 50 mM, respectively), and the protein collections from the above treated cells were harvested subsequently in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium vanadate and 0.2 U/ml aprotinin) containing protease inhibitor cocktail (Santa Cruz Biotechnology; Santa Cruz, CA). Western blotting was used to detect the effect of metformin on Bcl-2 (1:1000), Bcl-xl (1:1000), PARP and Bax (1:1000) proteins. And protein concentrations were determined using the bicinchoninic acid protein assay kit (KenGen Biotechnology Co. Ltd, Nanjing, China). Clarified protein lysates (50 g) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (10%), and transferred to nitrocellulose membranes. The membranes were then blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with the indicated specific primary antibodies for 3 h. Proteins were visualized with Horseradish peroxidase (HRP)-conjugated secondary antibodies. To corroborate equal loading, membranes were stripped and reprobed using an antibody specific for β-actin (1:1000; Abcam, Cambridge, MA). Finally, antigen-antibody complexes were detected using the ECL system, and digital images were captured with a FlourChem HD2 Imager system (Alpha Innotech, San Leandro, CA).

143B cells tumor xenograft

A total of 0.2 mL (1×10^{11} L^{-1}) 143B cell suspension were injected into the right flank of 40 BALB/c nude mice (Four-week-oldmale, supplied by the Experimental Animal Department of QiLu Hospital, Shandong University, Shandong, China). Six days later, 24 mice with tumors ~100 mm^3 in size were randomly distributed into the following four groups (six mice/group): Control group (PBS), metformin (200 mg/kg/day) group, cisplatin (10 mg/kg/day) group and cisplatin (10 mg/kg/day) plus metformin (200 mg/kg/day) group. Mice received daily intraperitoneal (i.p.) injections with PBS, metformn, cisplatin or the combined drugs, respectively. After the initial injection, the tumor volume was calculated every third day by the following formula: Tumor volume (mm^3) = $A/2\times B^2$, where A and B represent the longest and the shortest diameters, respectively. After being treated with above drugs for 21 days, all the mice were euthanized and the tumors were collected for western blot analysis. This study was approved by the ethics committee of Qi Lu Hospital Shandong University (Shandong, China).

Statistical analysis

Data are presented as mean ± standard deviation. All experiments, except that of the nude mice study, were repeated at least three times. Statistical significance was determined by Student's t-test, with $P<0.05$ defined as statistically significant.

Results

The effects of metformin on cell proliferation

To detect the effect of metformin on the proliferation of OS cells *in vitro*, Metformin at a variety of concentrations (0, 5, 10, 20 and 50 mM) was used to treat the U2OS and 143B cells for different time periods (0, 24, 48 and 72 h), respectively. The cell viabilities was detected with MTT assay. Finally, our research indicated that metformin could inhibit the OS cell proliferation in a dose- and time-dependent manner (**Figure 1A**, **1B**). Cisplatin (CDDP) is one of the most effective anti-tumor drugs for Osteosarcoma. However, many cancer patients have drug resistance to cisplatin. As shown in **Figure 1C**, **1D**, our research indicated that metformin could potentiate the anticancer activity of cis-

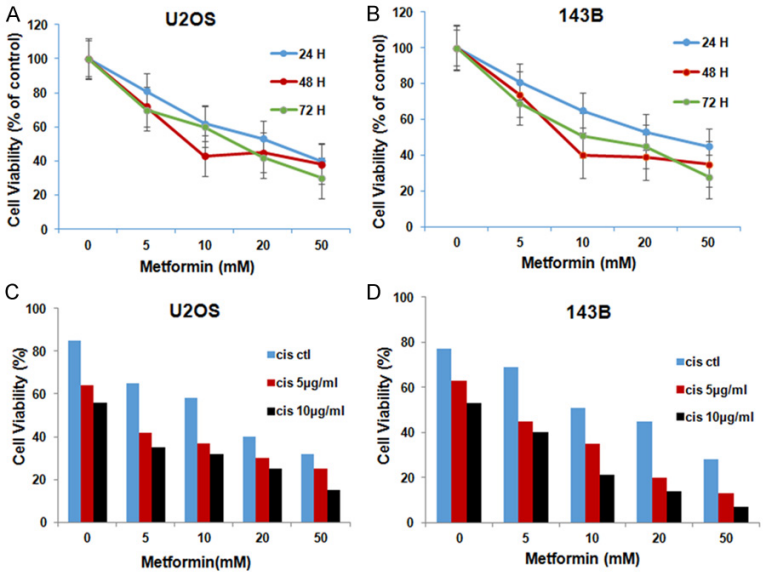

Figure 1. Metformin in combination with or without cisplatin inhibit the proliferation of OS cells in vitro. A, B. OS cells were treated with metformin for 24, 48 or 72 hrs. Cell viabilities was detected via the MTT assay. The results revealed proliferation of OS cells in a dose-and time-dependent manner. C, D. The addition of metformin potentiated the cytotoxicity induced by cisplatin. OS cells were cultured for 48 h in the presence of increasing amounts of cisplatin with and without metformin, and cell proliferation was measured using the MTT assay. The results are shown as the mean ± standard deviation of triplicate experiments. Ctl, control; cis, cisplatin.

platin in U2OS and 143B cells. What's more, a significant decrease in the proliferation of the OS cells were observed in the combination group compared with those of the metformin or cisplatin alone treatment groups for 48 h ($P<0.05$). So we concluded that metformin could increase the cytotoxicity of cisplatin on OS cells.

The effects of metformin on cell cycle arrest and cell apoptosis in OS cells

To detect the effect of metformin on the cell cycle of U2OS and 143B cells, the cell cycle distribution of OS cells treated with different concentrations of metformin (0, 10 and 20 mM) for 48 h was determined using a PI staining assay. As shown in **Figure 2**, metformin induced cell cycle arrest at the S phase in the U2OS cells, while arrest at the G0/G1 phase in the 143B cells. For example, treatment with different doses of metformin (10 or 20 mM) for 48 h resulted in an increase in U2OS cells in the S population, to 38.9% and 47.6%, respectively, compared with 25.5% in the control group (**Figure 2A**). On the contrary, metformin treatment lead to G0/G1 phase arrest in 143B cells (from 38.6% in non-treated cells to 52.4% and 69.7%, respectively), as is shown in **Figure 2B**. To detect the effect of metformin on the cell apoptosis, Annexin-V/PI staining technique was carried out after the two cell lines were treated with metformin. The percentage of apoptotic cells was determined by cell flow cytometric analysis following PI staining. Compared to the control group, metformin treatment leads to a moderate induction of apoptosis in both cell lines (1.20% for untreated cells, 21.86% for 10 mM group and 30.31% for 20 mM group in U2OS cells, while 1.10% for untreated cells, 17.50% for 10 mM group and 26.18 % for 20 mM group in 143B cells, **Figure 2C**, **2D**). Furthermore, Metformin induce the OS cells apoptosis in a dose-dependent manner.

Metformin induces apoptosis mainly through the mitochondria-mediated pathway

The mitochondrial pathway is confirmed to be involved in the induction of intrinsic apoptosis [13, 14]. Here, to further explore the mechanism of metformin-induced apoptosis, we detected the levels of anti- and pro-apoptotic proteins in the mitochondria-mediated signal pathway. Firstly, U2OS and 143B cells were incubated with or without metformin and then harvested for western blotting analyses. Incubation of cells with metformin downregulated the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl, while upregulated the expression of pro-apoptotic protein Bax in a dose-dependent manner in U2OS and 143B cells (**Figure 3A**, **3B**). What's more, in the downstream of the apoptosis signaling pathways, there is a significant cleavage activation of poly (ADP-ribose) polymerase (PARP). An obvious release of cytochrome C from the mitochondria to the cytosol was observed in the metformin-treated group (**Figure 3C**). According to the above results, we demonstrate that metformin could induce apoptosis mainly through the mitochondria-mediated internal pathway in U2OS and 143B cells.

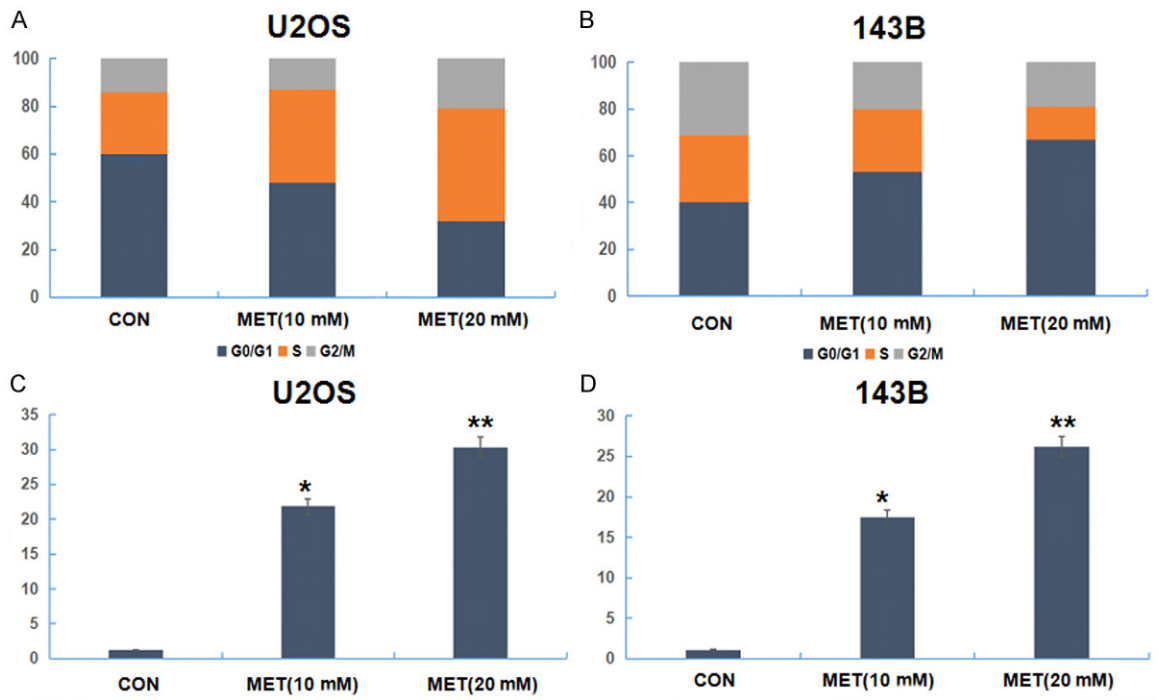

Figure 2. Metformin alters cell cycle distribution and induces the cell apoptosis in U2OS cells and 143B cells. A, B. Flow cytometry analysis of proliferating OS cells 48 h after the treatment with metformin (10 and 20 mM). Distributions of cells in the G0/G1, S and G2/M phases of the cell cycle are detected. C, D. OS cells were treated with metformin at different concentrations for 48 hrs and stained with Annexin V-propidium iodide. The percentage of apoptotic cells was measured by FACS can analysis. Untreated cells were used as controls. MET, metformin; CON, control. *P<0.05 and **P<0.01 compared with control.

Metformin significantly potentiate the anti-neoplastic effect of cisplatin in vivo

In order to determine whether metformin affect tumor growth *in vivo*, the effect of metformin on tumor growth inhibition was studied through intraperitoneal injection. Compared with the control group, we found that metformin alone did not affect the tumor growth conspicuously (*P*>0.05). However, OS tumor growth was markedly inhibited in the metformin plus cisplatin groups (**Figure 4A**). Compared with the other groups, there was a significant decrease of tumor volume in the metformin (200 mg/kg/day) plus cisplatin group (**Figure 4B**). The tumor volume in the control group, metformin group and metformin plus cisplatin groups were 795.6±27.3 mm^3, 743.6±41.7 mm^3 and 261.9±30.7 mm^3, respectively. The tumor growth inhibition rate was 67.1% in the metformin plus cisplatin group (P<0.01), compared with the control group. To determine whether metformin affects tumor growth via the mitochondria-dependent pathway *in vivo*, we detected Bcl-2, Bcl-xl and Bax expression in xenografts in the above four groups by western-blotting. Consistent with the tumor growth data *in vitro*, metformin significantly decreased Bcl-2 and Bcl-xl expression, and increase Bax expression in tumor xenografts (**Figure 4C**). Thus, we conclude that metformin alone affect the tumor growth unconspicuouslyin nude mice. However, we demonstrate that metformin significantly potentiate the anti-neoplastic effect of cisplatin in vivo.

Discussion

Metformin has been an important drug for treatment of type 2 diabetes (T2D) for decades. It is one of the most widely used oral antihyperglycemicagent and is currently recommended as first line therapy for all new-onset diabetic patients. Interestingly, pharmaco-epidemiologic studies demonstrate that metformin has great utility in neoplasm prevention and therapy. Tseng [15] reported recently that metformin use among Taiwanese patients with T2DM could significantly reduce the risk of gastric cancer, particularly when metformin was used

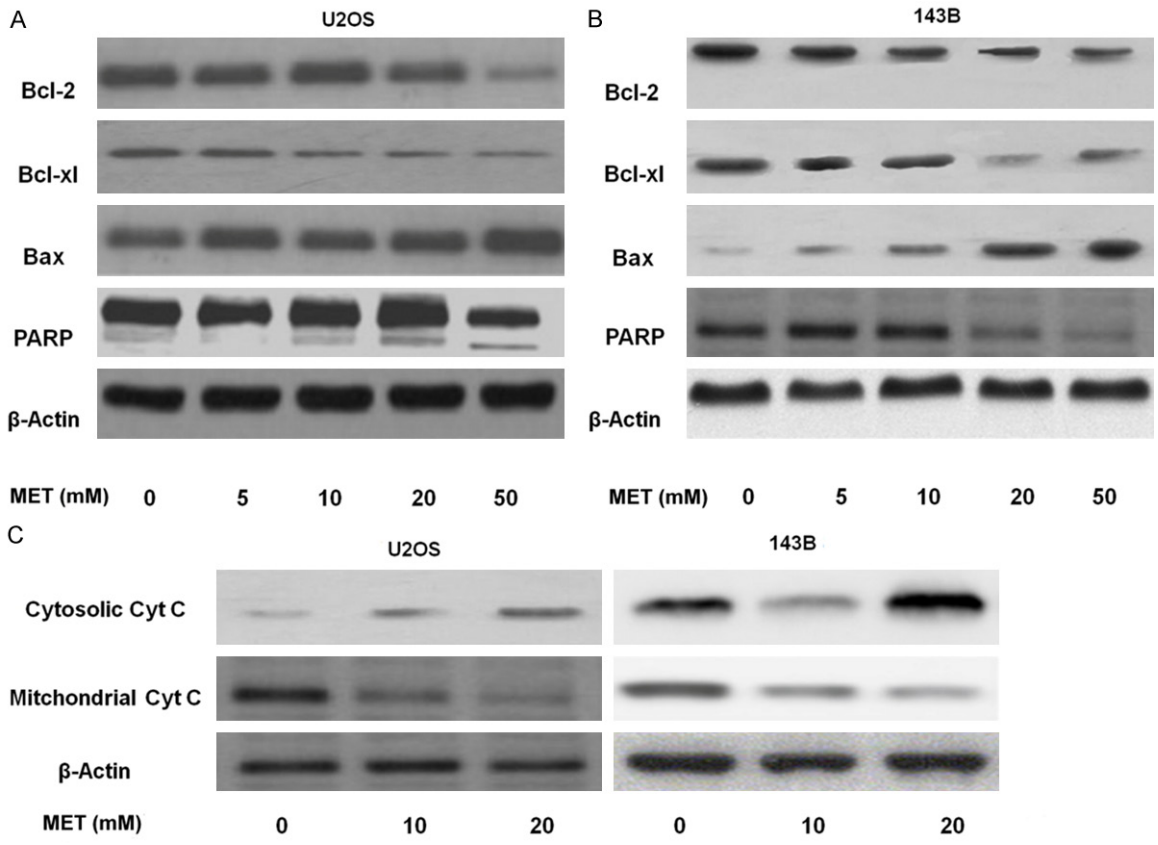

Figure 3. Metformin-induced apoptosis occurs via the mitochondria-mediated apoptotic pathway. A, B. Effect of metformin on the expression of apoptosis-regulating proteins, as assessed by western blotting. Protein fractions of total cells were assessed for expression of Bcl-2, Bcl-xl, Bax and PARP. C. Effect of metformin on Cytochrome C distribution in the cytosol and mitchondria. An obvious release of Cytochrome C from the mitochondria to the cytosol was observed in the metformin-treated group. Data represent one of three independent experiments. β-actin was used as a loading control. MET, metformin; Bcl, B-cell lymphoma; Bcl-xl, Bcl-extra large; PARP, poly (ADP-ribose) polymerase.

for nearly 2 years. Dozens of preclinical medicine researches have depicted the antineoplastic effects of metformin and the possible mechanisms [16-18]. For instance, Xiong Yu et al demonstrated that metformin played an antitumor activity against Hepatocellular carcinoma through inhibition of mTOR translational pathway in an AMPK-independent manner, their further studies show that metformin-treatment leads to G1 cell cycle arrest and subsequent cells apoptosis via the mitochondria-dependent signal pathway [19]. Gao's group [20] indicate that metformin-induced apoptosis is mediated by the accumulation of reactive oxygen species (ROS) via the mitochondria-mediated signal pathway in human breast cancer cells. Besides, there are also some researcers who confirm that metformin synergistically enhances cisplatin cytotoxicity in the esophageal cancer cells, especially under the condition of glucose free, the potential mechanisms may include enhancement of metformin-associated cytotoxicity, marked reduction in the cellular adenosine triphosphate expression and the imbalance of the AMPK/mTOR signaling pathways [21]. In terms of osteosarcoma, Researchers also found that metformin could effectively inhibit the proliferation and migration of human OS cells by activation of AMPK and consequent down-regulation of the mTOR signal pathway. In addition, the proliferation of cisplatin-resistant OS cells were also suppressed by metformin [22, 23]. Nevertheless, the molecular mechanisms of metformin-induced apoptosis in human OS cells have never been clearly clarified. Consistent with the above studies, we here indicate that the anticancer effect of metformin in combination with

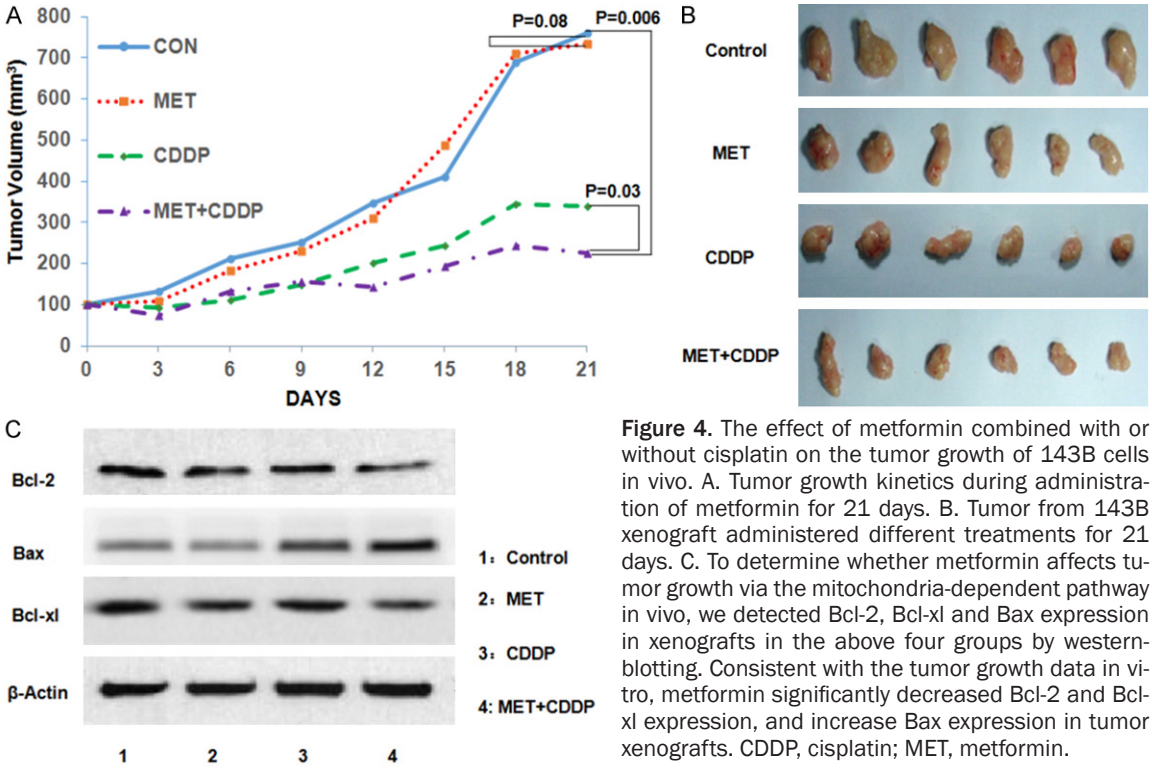

Figure 4. The effect of metformin combined with or without cisplatin on the tumor growth of 143B cells in vivo. A. Tumor growth kinetics during administration of metformin for 21 days. B. Tumor from 143B xenograft administered different treatments for 21 days. C. To determine whether metformin affects tumor growth via the mitochondria-dependent pathway in vivo, we detected Bcl-2, Bcl-xl and Bax expression in xenografts in the above four groups by western-blotting. Consistent with the tumor growth data in vitro, metformin significantly decreased Bcl-2 and Bcl-xl expression, and increase Bax expression in tumor xenografts. CDDP, cisplatin; MET, metformin.

cisplatin is better than that of metformin or cisplatin treatment alone. In the present study, we focus on the metformin-induced apoptosis and its relationship with the mitochondria-mediated signal pathway, one of two important apoptotic pathways in mammalian cells (the other pathway is death receptor mediated apoptotic pathway). Finally, we observed that metformin potently suppress the viabilities of human OS cells in a dose- and time-dependent manner. Cell cycle redistribution usually occurs in metformin-induced apoptosis. Some studies reported that metformin-induced tumour cell arrest in G0/G1 phase [24-26]. However, we found that metformin-induced cell cycle arrest in S phase in U2OS cells and arrest in G0/G1 phase in 143B cells, which indicates that metformin might induce different modles of cell cycle arrest in different cell lines. However, cell cycle regulation is not only extremely complex, but also cell specific. Further studies are necessary to reveal the mechanisms of metformin-induced cycle arrest in OS cells. To further clarify the molecular mechanisms of metformin-induced apoptosis in human OS cells, we analysed total proteins of U2OS and 143B cells treated with different concentrations of metformin (0, 5, 10, 20 and 50 mM) for 48 hrs by Western blotting, we demonstrated that the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl were both down-regulated, while the pro-apoptotic protein Bax was up-regulated. What's more, there is also a cleavage activation of PARP, and cytochrome C release to cytoplasm induced by metformin was also detected. The results show that metformin induces apoptosis in U2OS and 143B cells mainly through mitochondria-mediated internal pathway. In vivo studies, we found that metformin alone affect the tumor growth inconspicuously in nude mice. However, we demonstrate that metformin significantly potentiate the anti-neoplastic effect of cisplatin in vivo, as the tumor growth in the metformin plus cisplatin group were significantly suppressed compared with cisplatin group or the control group. By using western blotting assay, we found that expression levels of Bcl-2, Bcl-xl were down-regulated, while the expression of Bax was up-regulated in tumor xenografts after the metformin treatment. These results are of particular important because it is the first time that metformin has been revealed to potentiate the anti-neoplastic effect of cisplatin in a xenograft model. Consist with our research, Rattana et al also indicated that metformin in combination with cisplatin

exerted a better anti-cancer effect in ovarian cancer cells via suppressing tumor invasion and reversing the cisplatin resistance [27].

In conclusion, by using several *in vitro* and *in vivo* studies, we demonstrated that metformin could significantly induce U2OS and 143B cells apoptosis and attenuated tumor xenografts via mitochondria-mediated signal pathway. The elucidation of the mechanism of metformin-induced cell apoptosis suggests a potential therapeutic way of metformin and its potential of combination with cisplatin targeting mitochondria-mediated signal pathway to treat osteosarcoma. However, the optimal dose of metformin required to elicit antitumor effect remains elusive. The antitumor concentrations that usually used *in vitro* studies were in the millimolar range [28-30], while the serum levels of metformin achieved in DM patients and in many *in vivo* models were in the micromolar ranges [31]. Furthermore, our conclusions are based on preliminary experimental researches and a large-scale clinical trials are required to validate our preclinical findings.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation (NO.26-010105131233).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanping Zheng, Department of Orthopaedics, Qilu Hospital of Shandong University, 107 Wenhua Road, Jinan 250012, Shangdong, China. E-mail: lidymed@126.com

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