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Original Article

Inhibition of proliferation and migration of osteosarcoma cells U2OS by phenformin

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Abstract: Purpose: This study aims to detect the effects of phenformin on proliferation and migration of osteosarcoma cells U2OS *in vitro* and *in vivo*. Methods: Osteosarcoma cells U2OS were treated with phenformin. Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and cell cycle distributions were evaluated using flow cytometric analysis. Wound healing assay and transwell migration assay were performed to detect cell migration ability. In addition, U2OS were injected in thigh of nude mice, and the mice were treated with phenformin (50 mg/kg/day) by intraperitoneal injection when tumor volume reached about 600 mm^3. Results: We found that phenformin could significantly inhibit cell proliferation of U2OS in a dose-dependent manner, through repressing cell cycle in G1 and S stage. In addition, phenformin inhibited colony formation of U2OS in two-dimensional clonogenic survival assay and soft agar colony formation assay Phenformin also suppress cell migration ability in U2OS. Meanwhile, the results of xenograft nude models also indicated that phenformin displayed powerful antitumor effects *in vivo,* and phenformin promoted Ki67 expression and inhibited caspase3 expression in U2OS xenograft tissues. Conclusion: Phenformin displayed powerful antitumor effects of U2OS *in vitro* and *in vivo*, and phenformin maybe a potential adjuvant antitumor drug for osteosarcoma.

Keywords: Phenformin, osteosarcoma, U2OS, proliferation, migration

Introduction

Osteosarcoma, the most common highly malignant childhood bone cancer, comprises about 20% of all primary bone cancers [1]. It originates more frequently in the metaphyseal region of tubular long bones, with 42% occurring in the femur, 19% in the tibia, and 10% in the humerus [2]. With multidisciplinary treatment, the survival of osteosarcoma patients has been improved dramatically during the late 20th century [3-5]. Despite the success of treatment for osteosarcoma, it still has one of the lowest survival rates for pediatric cancer. New therapeutic agents based on biologic characteristics and signal pathways are required to improve the survival of osteosarcoma patients.

As we all know, cancer cells, including osteosarcoma, always characteristics of active proliferation and vigorous growth, so these cells often perform metabolic abnormalities and metabolic reprogramming to adapt the proliferation [6-8]. Active glycolysis is very common in cancer cells, so enhanced glucose metabolism always become a target for cancer therapy [9-11]. Biguanides, a class of medication used to treat type II diabetes, exert ananti-tumor effect on many cancer cells. Many retrospective studies have reported an association between utilized biguanides and improved prognosis of cancer patients, and the antitumor activity of biguanides in animal models and cell lines has also been reported by many other authors [12-15].

Because of phenformin-associated lactic acidosis in elderly patients with renal failure compared with metformin, phenformin use has been limited to relatively few countries [16]. However, some studies found that phenformin was more active against tumor cells than metformin [15, 17, 18]. So far, two studies have showed that metformin displayed antitumor effect against osteosarcoma in animal models and cell lines experiments [19, 20]. Never-

theless, the effect of phenformin on osteosarcoma has not yet reported. Therefore, in this study the effect of phenformin will be examined as a potential agent for osteosarcoma *in vitro* and *in vivo*.

Materials and methods

Cell culture and agents

Cell line U2OS, obtained from ATCC, was cultured in Dulbecco's modifid Eagle's medium (DMEM) supplemented with 10% FBS (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) inside an incubator containing 5% CO_2 at 37°C. Phenformin was purchased from sigma.

MTT assay and IC_{50} detection

About 5 × 10^3 U2OS cells were seeded in 96 well plates and incubated overnight for inside an incubator containing 5% CO_2 at 37°C. Then different concentration of phenformin was added to 96 well plates on the following day, and incubated for 24, 48 and 72 h respectively. At the end of incubation, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) were added to each well. The plates were incubated in a humidified incubator at 37°C, under 5% CO_2 for 4 h, following which 150 μL dimethyl sulfoxide was added. The plates were gently agitated until the formazan was completely dissolved, and the absorbance was measured at 490 nm wavelength. The IC_{50} of 24, 48, 72 h values were determined from dose-response curves.

Cell cycle analysis

U2OS cells were plated in 6 well plates (400,000 cells/well) and incubated overnight following treated with IC_{50} doses of phenformin. After 24 h, the cells were harvested and washed with PBS, then fixed with 70% ethanol and stored at -20°C overnight. The fixed cells were washed twice in PBS, resuspended in PBS containing 200 μg RNase A (Sigma) and incubated at 37°C for 30 min. The samples were stained with 20 μg propidine iodide protected from light for 30 min and the distribution of cells in the cell cycle was analyzed using a flow cytometer (BD Company, Franklin Lakes, NJ, USA). All experiments were performed at least three times.

Two-dimensional clonogenic survival assay

About 500 U2OS cells were seeded to one well of on 6 well plates and incubated for 10 days in medium added phenformin or not. Then colonies were washed with PBS, fixed with methanol and stained with gentian violet. Take pictures and colonies containing more than 50 cells were scored as surviving cells.

Soft agar colony formation assay

According to the protocol, about 1 × 10^4 U2OS cells were plated in 0.4% agarose on top of a 1% agarose base supplemented with complete medium added phenformin or not. Cells in agarose were allowed to grow for 4 weeks inside an incubator containing 5% CO_2 at 37°C and total colonies were counted. The pictures were taken and the number of colonies was counted by microscope.

Wound-healing assay

About 5 × 10^5 U2OS cells were seeded in one well of 6 well plates and grown overnight cell monolayer was wounded by scratching with a 20 μL pipette tip, followed by washing three times with PBS. Then cells were incubated in serum-free culture medium added phenformin or not. For each well, images of the scratch were taken and the distances between the lesion edges were calculated using an inverted microscope in oh and 24 h. The relative migrating distance of cells is measured by the distance of cell migration/the distance measured at 0 h.

Cell migration assay

First transwell filters (pore size, 8 μm; Falcon; BD Biosciences) were placed on a 24-well plate containing 500 ml DMEM added phenformin or not, about 1 × 10^5 U2OS cells were added to the upper compartment of a transwell chamber and allowed to migrate for 24 h at 37°C. Then the cells were harvested and cells that had migrated to the bottom surface of the filter membrane were stained with 0.5% crystal violet solution and photographed in five preset fields per insert.

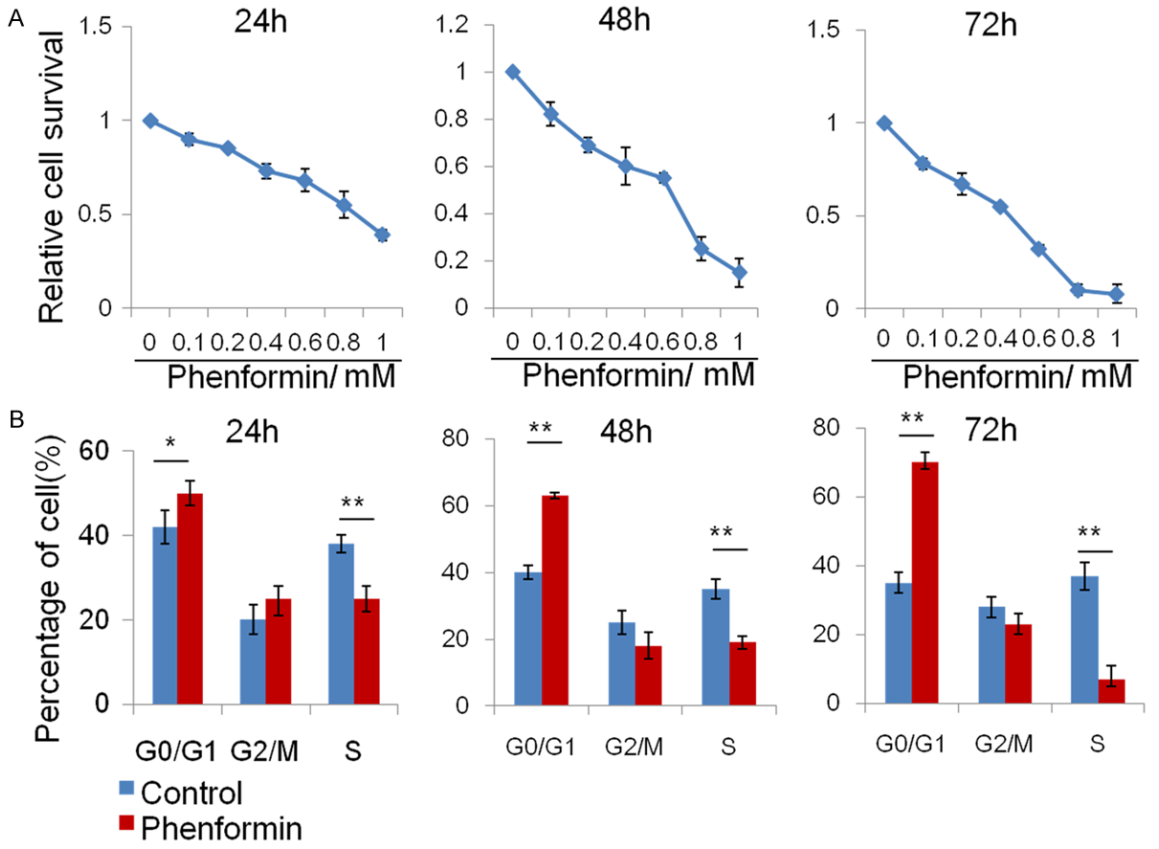

Figure 1. Phenformin inhibited cell proliferation and arrested cell cycle of U2OS. A: The cell survival of U2OS treated with different concentration of phenformin (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1 mM) in 24 h, 48 h, and 72 h respectively using MTT assay. B: The cell cycle fraction of U2OS after treatment with IC_{50} dose of phenformin in 24 h (0.6 mM phenformin), 48 h (0.4 mM phenformin) and 72 h (0.3 mM phenformin) respectively. (*P < 0.05, **P < 0.01).

Animal experiments

4 to 6 weeks male BALB/c-nude mice were purchased from the Experimental Animal Center of Nanjing Medical. Mice were administered with a standard, housed and maintained in pathogen-free house in a 12:12 h light-dark cycle. Temperature and humidity were maintained at 24±2°C and 50±5%, respectively. All mice were injected subcutaneously in the flanks region with 100 μL suspension (2×10^6) of U2OS cells. Once tumors reached approximately 0.5 to 0.6 cm^3, all the mice were divided into two groups, one group was treated with phenformin by intraperitoneal injection, and another groups as control. The size of the tumor was measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (length × $width^2$ × 0.5).

Statistical methods

SPSS 16.0 was used to evaluate the data, and the data were given as means ± SD. The standard two-tailed independent samples t-test was used to compare the differences in two groups. The significance level was defined as P < 0.05, and the significance level was defined as P < 0.05 (*means P < 0.05, **means P < 0.01). Each assay was performed in triplicate in at least two independent experiments.

Results

Phenformin inhibited cell proliferation of U2OS

To evaluate the antitumor effects of phenformin, cell survival of U2OS was first detected in medium added with different concentrations of phenformin (0-1 mM). The results of MTT assay showed that phenformin could significantly inhibit cell proliferation of U2OS in a dose-dependent manner, and the IC_{50} value was 0.6-0.7 mM in 24 h, 0.4-0.5 mM in 48 h, and 0.3-0.4 mM in 72 h respectively (**Figure 1A**). In addition, to further explore the mechanism of phenformin in inhibiting cell proliferation of U2OS, cell cycle analysis by flow cytometry was

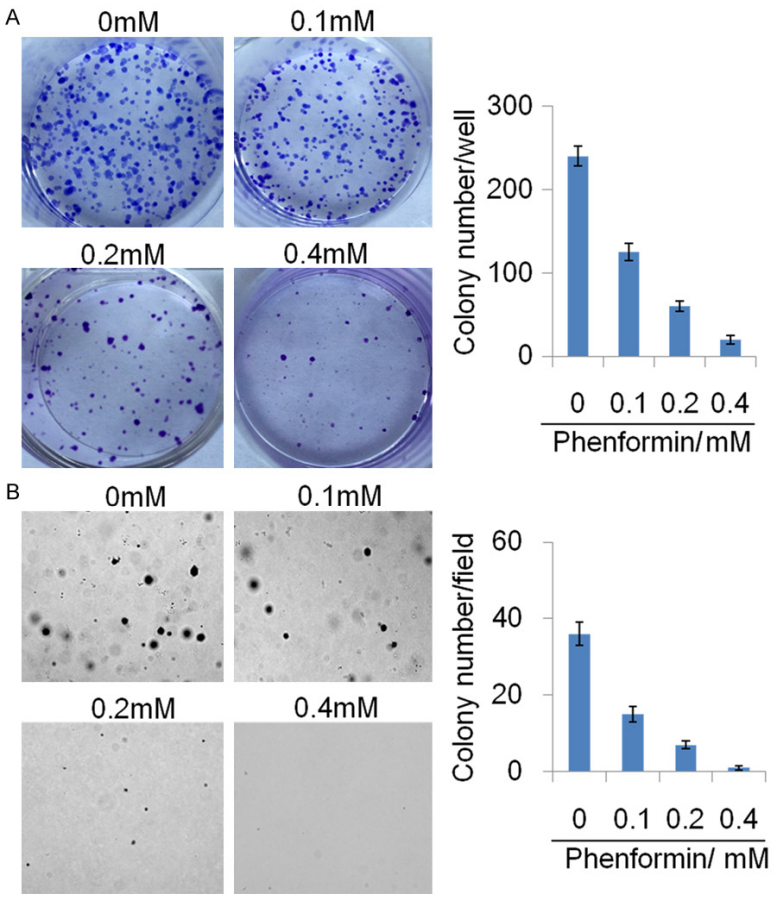

Figure 2. Phenformin inhibited cell colony formation of U2OS. A: The affection of different dose of phenformin (0.1 mM, 0.2 mM, and 0.4 mM) on adherent colony formation of U2OS detected by two-dimensional clonogenic survival assay. B: The affection of different dose of phenformin (0.1 mM, 0.2 mM, and 0.4 mM) on suspending colony formation of U2OS detected by soft agar colony formation assay.

performed following exposure of U2OS to IC_{50} dose of phenformin in 24 h (0.6 mM phenformin), 48 h (0.4 mM phenformin) and 72 h (0.3 mM phenformin) respectively. Then the results of cell cycle analysis revealed that G1 phase arrest was found in every concentration of phenformin, and more cells were accumulated in S phase in longer exposure time 72 h (**Figure 1B**).

Phenformin inhibited cell colony formation of U2OS

Clonogenic assay is a cell biology technique for studying the effectiveness of specific agents on the survival and proliferation of cells, which determines the ability of a cell to proliferate indefinitely. In this study, we utilized two-dimensional clonogenic survival assay and soft agar colony formation assay to detect the cells ability of colony formation with phenformin treatment. The lower concentrations of phenformin (0.1 mM, 0.2 mM, and 0.4 mM) were chosen, and the results showed that all concentrations of phenformin could inhibit colony formation significantly than control cells not just in two-dimensional clonogenic survival assay (**Figure 2A**), but also in soft agar colony formation assay (**Figure 2B**).

Phenformin inhibited cell migration ability of U2OS

Phenformin has been reported that it could inhibit cell metastasis in many kinds of cancers, so in this study we examined if phenformin also could affect the migration ability of U2OS cells using wound-healing assay and transwell migration assay. In these experiments, we used 0.2 mM phenformin to treat U2OS cells, and we found that lower concentration of phenformin was sufficient to significantly inhibit the cell migration in both wound-healing assay (**Figure 3A**) and transwell migration assay (**Figure 3B**).

Phenformin inhibited tumor growth of U2OS in vivo

To determine whether phenformin could suppress tumor growth *in vivo*, BALB/c nude mice were injected subcutaneously in the flanks with U2OS cells and treated with phenformin (20 mg/kg/day for 18 days, intraperitoneal injection). We found that phenformin significantly inhibited the growth of tumors in BALB/c nude mice (**Figure 4B**), and tumor volume of the phenformin-treated mice was significantly smaller than that of control group (**Figure 4A**). Meanwhile, the levels of Ki67 expression in tumor tissues of phenformin-treated group were lower than that of control group (**Figure**

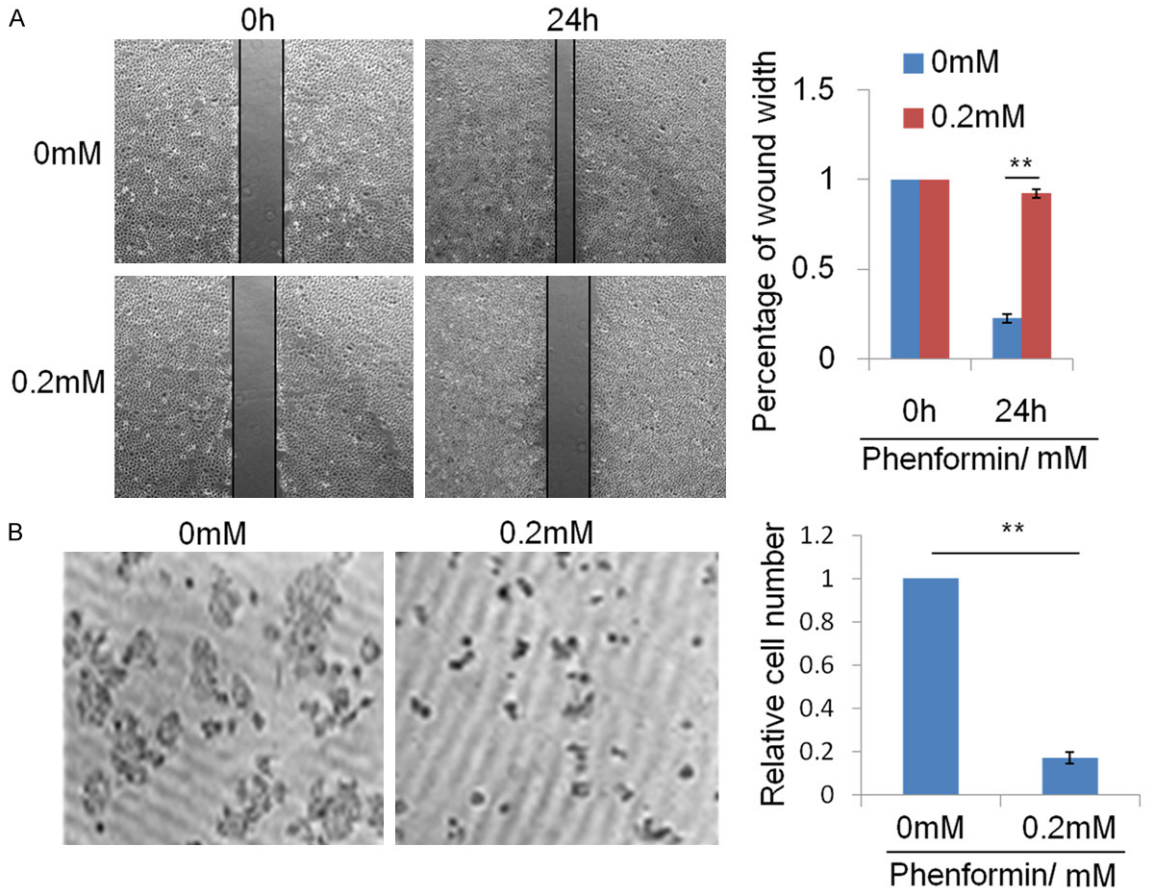

Figure 3. Phenformin inhibited cell migration of U2OS. A: The affection of 0.2 mM phenformin on cell migration of U2OS detected by wound-healing assay in 24 h. B: The affection of 0.2 mM phenformin on cell migration of U2OS detected by transwell assay in 24 h. (*P < 0.05, **P < 0.01).

4C), in contrast, the levels of caspase3 expression in tumor tissues of phenformin-treated group were higher than that of control group (**Figure 4D**), which suggested that phenformin could successfully inhibit cell proliferation and promote cell apoptosis *in vivo*.

Discussion

In this study, we first found that phenformin could significantly inhibit growth and migration of U2OS osteosarcoma cells *in vitro* and in vivo. Phenformin inhibited the ability of proliferation of osteosarcoma U2OS cells, with a dose-dependent manner. Meanwhile, phenformin could successfully suppress cell migration of osteosarcoma U2OS cells. In addition, phenformin inhibited the growth of U2OS in a tumor xenograft model by inhibiting cell proliferation and promoting cell apoptosis. Our findings powerfully suggest the possibility of phenformin being used as an adjuvant agent in the treatment of osteosarcoma patients.

Osteosarcoma is one of the most common highly malignant bone tumors in childhood and adolescence [2]. At present, surgical resection and combination chemotherapy are standard treatment of osteosarcoma. In spite of advances in treatment of osteosarcoma over the past decade, the cure rate improved modestly [3]. Osteosarcoma is still characterized by frequent relapse and metastatic disease, which may be explained by resistance to chemotherapeutic treatment and multidrug resistance (MDR) [21-23]. To overcome MDR, new drugs for osteosarcoma treatment must been developed. However, new drug development is a high-risk business with the potential for high reward but also with the possibility of significant loss. On

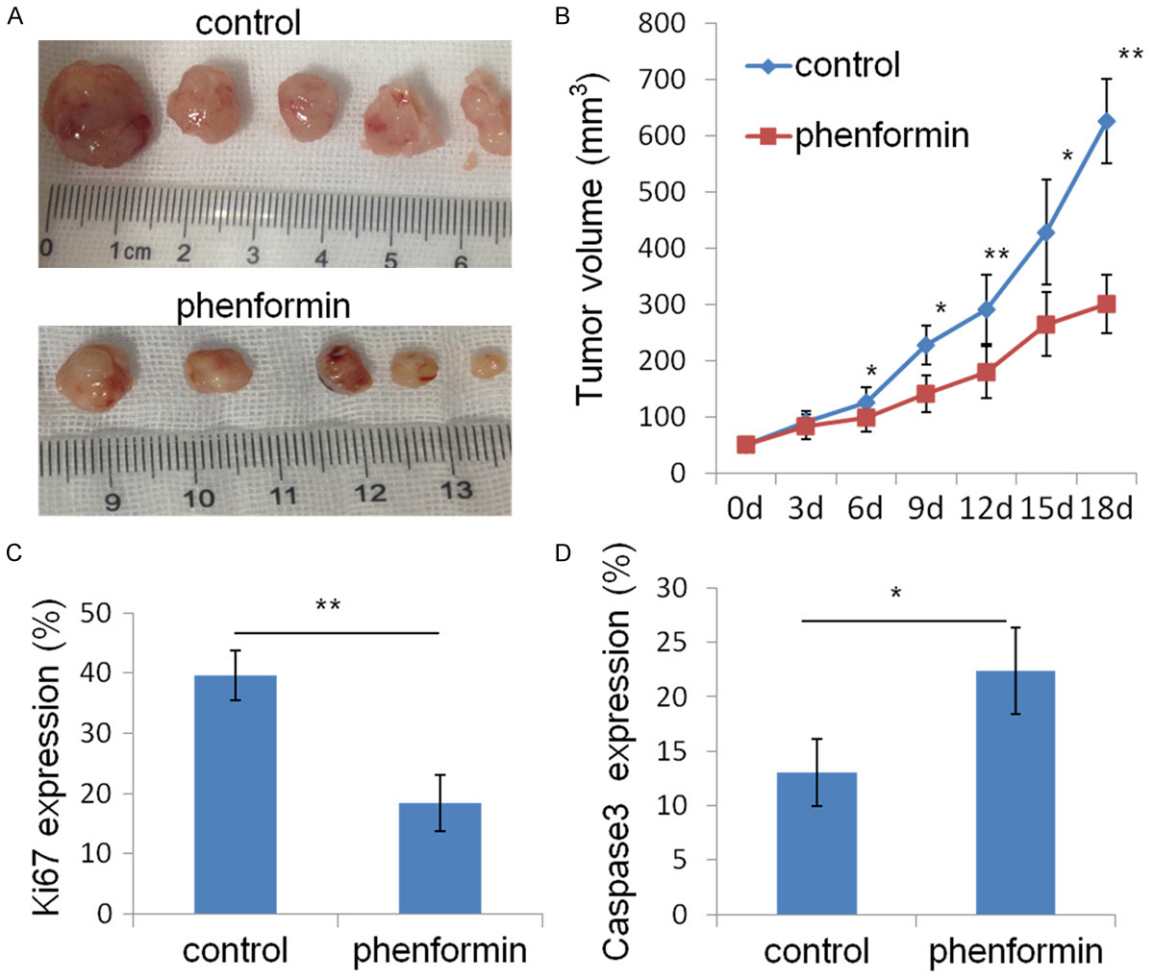

Figure 4. Phenformin inhibited tumor growth of U2OS in vivo xenograft. A: The U2OS xenograft tumor of nude mice treated with phenformin or not. B: Phenformin inhibited the growth of U2OS xenograft tumor of nude mice. C: Ki67 expression in U2OS xenograft tumor treated with phenformin or not. D: Caspase3 expression in U2OS xenograft tumor treated with phenformin or not. (*P < 0.05, **P < 0.01).

average, bringing a new drug to market will takes 10 to 15 years, and will costs of approximately $800 million to $1.7 billion [24].

Old drugs are new again. At present, many drugs used in other diseases were found to have functions in antitumor. Artemisinin and its derivatives, which are commonly used in malaria therapy, have also potent anticancer activity in the nano- to-micromolar range in both sensitive and drug- or radiation-resistant cell lines [25, 26]. Thalidomide, preventing nausea in pregnant women early, found a new use as a treatment for multiple myeloma [27]. Aspirin and other agents characterized as nonsteroidal anti-inflammatory drugs are designed primarily to decrease pain and inflammation, now many experiments and clinical data assay showed that aspirin had ability of antitumor [28].

Biguanides, commonly used to treat type II diabetes, exert an antitumor effect on many types of cancer [17]. Lots retrospective studies have reported an association between utilized biguanides and improved prognosis of cancer patients, and the antitumor activity of biguanides in animal models and cell lines has also been reported [29, 30]. Both metformin and phenformin are belongs to biguanides, always used to treat type II diabetes. But phenformin use has been limited to relatively few countries, because of phenformin-associated lactic acidosis in elderly patients with renal failure compared with metformin [16]. However, some

studies found that phenformin was more active against tumor cells than metformin [17, 18]. Phenformin could greatly inhibit MDA-MB-231x enografts tumor growth than metformin [15]. In this study, we found that phenformin also could significantly inhibit proliferation and migration of U2OS osteosarcoma cells *in vitro* and *in vivo*. So far, just two studies have showed that metformin displayed antitumor effect against osteosarcoma in animal models and cell lines experiments.

Because osteosarcoma is one of the most common malignant tumors in childhood and adolescence, while phenformin-associated lactic acidosis often occurred in elderly patients with renal failure, and phenformin was more active than metformin, so we think that phenformin maybe more useful for osteosarcoma treatment than metformin. Further studies should be performed to value the effect of phenformin on clinical osteosarcoma.

Conclusion

Taken together, our data showed that phenformin displayed powerful antitumor effects of U2OS *in vitro* and *in vivo*, and phenformin maybe a potential adjuvant antitumor drug for osteosarcoma.

Disclosure of conflict of interest

None.

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