Korean J Pediatr

Metformin displays *in vitro* and *in vivo* anti-tumor effect against osteosarcoma

Yunmi Ko[1], Aery Choi, MD[2], Minyoung Lee, PhD[3], Jun Ah Lee, MD, PhD[1,2]

[1]Division of Clinical Translational Research, Korea Cancer Center Hospital, Seoul, [2]Department of Pediatrics, Korea Cancer Center Hospital, Seoul, [3]Division of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Seoul, Korea

Purpose: Patients with unresectable, relapsed, or refractory osteosarcoma need a novel therapeutic agent. Metformin is a biguanide derivative used in the treatment of type II diabetes, and is recently gaining attention in cancer research.

Methods: We evaluated the effect of metformin against human osteosarcoma. Four osteosarcoma cell lines (KHOS/NP, HOS, MG-63, U-2 OS) were treated with metformin and cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell cycle progression and apoptosis were evaluated using flow cytometric analysis, and migration and wound healing assay were performed. Fourteen female Balb/c-nude mice received KHOS/NP cell grafts in their thigh, and were allowed access to metformin containing water (2 mg/mL) *ad libitum*. Tumor volume was measured every 3–4 days for a period of 4 weeks.

Results: Metformin had a significant antiproliferative effect on human osteosarcoma cells. In particular, metformin inhibited the proliferation and migration of KHOS/NP cells by activation of AMP-activated protein kinase and consequent inhibition of the mammalian target of rapamycin pathway. It also inhibited the proliferation of cisplatin-resistant KHOS/NP clone cells. Analysis of KHOS/NP xenograft Balb/c-nude models indicated that metformin displayed potent *in vivo* antitumor effects.

Conclusion: Further studies are necessary to explore metformin's therapeutic potential and the possibilities for its use as an adjuvant agent for osteosarcoma.

Key words: Metformin, Osteosarcoma, Antitumor effect

Corresponding author: Jun Ah Lee, MD, PhD
Division of Clinical Translational Research, Korea Cancer Center Hospital, 75 Nowon-ro, Nowon-gu, Seoul 01812, Korea
Tel: +82-2-970-1248
Fax: +82-2-970-1970
E-mail: junahlee@kcch.re.kr

Introduction

Osteosarcoma is the most common primary bone cancer, affecting children and adolescents during periods of rapid growth[1]. With multidisciplinary treatment, the survival of patients with localized extremity tumor is now approaching 70%[1]. However, the survival has reached a plateau[2,3]. Intensification of chemotherapy by adding second-line chemotherapeutic agents or dose-dense scheduling has failed to further improve the survival rate[2,3]. Patients with metastatic disease or relapsed disease still have dismal prognosis[1,4,5], and new therapeutic agents based on biologic characteristics are required to improve the survival of this group of patients. Metformin is an oral hypoglycemic agent used in the treatment of type II diabetes mellitus (DM), and recently gaining attention in cancer research[6,7]. Epidemiologic studies report that metformin treatment was associated with a decreased incidence of breast, prostate, colon, and pancreatic cancers[8,9]. In retrospective studies, the use of metformin was associated with improved cancer-related mortality[10,11]. It is suggested that metformin lowers blood glucose levels in insulin-resistant individuals, thus reducing the plasma concentration of insulin and/or insulin-like growth factor-1

(IGF1), which can act as growth factors for tumor cells[12]. Meanwhile, metformin exerts direct effects against cancer cells. For example, it significantly reduces the Ki67 index in primary operable breast cancer of nondiabetics[13]. Metformin activates AMP-activated protein kinase (AMPK), and subsequently inhibits the mammalian target of rapamycin (mTOR) pathway[14]. AMPK stimulates catabolic pathways to generate more energy and inhibits anabolic pathways such as protein and lipid synthesis, and mTOR stimulates energy-consuming pathways and regulates cell growth[15]. The features of metformin are particularly interesting for pediatric sarcomas, which have been shown to be dependent on IGF and the insulin system for their pathogenesis and progression[16,17]. Metformin exerts antiproliferative and chemosensitizing effect both on sensitive and chemoresistant pediatric sarcoma cells[16]. Limited data exist about the efficacy of metformin against osteosarcoma cells[16,18-20]. Metformin synergistically enhances antitumor activity of the histone deacetylase inhibitor trichostatin A in osteosarcoma cells, inhibits cell growth, and sensitizes osteosarcoma cells to cisplatin via cell cycle modulation[18]. Chen et al.[20] reported that metformin inhibits metastasis and cancer stem-like sphere formation in osteosarcoma MG-63 cells. In this study, we evaluated the antitumor effects of metformin on human osteosarcoma cells. Metformin had a significant antiproliferative effect on HOS, KHOS/NP, MG-63, and U-2 OS human osteosarcoma cells. In particular, metformin inhibited the migration of KHOS/NP cells. Metformin also inhibited the proliferation of both parent and cisplatin-resistant KHOS/NP clone cells by activation of AMPK and consequent inhibition of the mTOR pathway. Finally, analysis of tumor xenograft models indicated that metformin displayed potent *in vivo* antitumor effects. Collectively, our data suggest that metformin deserves further investigation as an anticancer agent, implying that inhibition of both cell proliferation and metastatic potentials may significantly contribute to the treatment of human osteosarcoma.

Materials and methods

1. Cell cultures and reagents

The human osteosarcoma cell lines KHOS/NP, MG-63, and U-2 OS were obtained from the American Type Culture Collection (Manassas, VA, USA) and HOS was obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were maintained in α-MEM medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cisplatin-resistant osteosarcoma cells were derived from original parent cells and established by repeated subcultures in the presence of increasing concentrations (up to 50 μM) of cisplatin (Sigma-Aldrich, Gillingham, UK). For *in vitro* experiments,

metformin (Selleck Chemicals, Houston, TX, USA) was dissolved in water to make a 100 mM stock solution.

2. Cell proliferation, cell cycle, apoptosis, and wound healing assay

Osteosarcoma cells (5×10^3/50 μL of media) were seeded in each well of 96-well plates and incubated overnight at 37°C in a humidified 5% CO_2 incubator. On the following day, 50 μL metformin was added to each well. After incubation for an additional 72 hours, cell proliferation was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The half maximal inhibitory concentration (IC_{50}) values were determined from dose-response curves.

For flowcytometric analysis, osteosarcoma cells were plated in complete culture medium and treated with metformin (2.5 mM). Cells were harvested 24 hours later, stained with propidium iodide (1 mg/mL, Sigma, St Louis, MO, USA), and analyzed using a BD FACSCanto II Flow cytometer. Apoptosis was analyzed with an FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The fluorescent intensity was measured by BD FACSCanto Flow Cytometer (BD Biosciences).

For wound healing assay, KHOS/NP cells were seeded in 6-well plates and grown until 80% confluence. The cells were scratched with a pipette tip across the monolayer, simulating a wound. Cells were washed with phosphate-buffered saline (PBS) and cultured in α-MEM supplemented with 2% FBS, and were treated with either 1.5 mM metformin or culture medium (control). Microscopic appearance was captured 24 hours after wounding and the area of the gap region was quantified.

3. Western blotting

Cell lysates were prepared using a kit from Cell Signaling (Beverly, MA, USA). Equal amounts of protein (determined using the Bio-Rad Protein assay) were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes, which were washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), blocked with TBST and 5% wt/vol nonfat dry milk overnight at 4°C, and incubated with the following primary antibodies: CDK2, β-actin, CyclinD1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), phospho-p70S6K (Thr389), phospho-4E BP1 (Thr37/46), phospho-AMPKα (Thr172), phospho-Acetyl-CoA carboxylase (Ser79), phospho-extracellular signal–regulated kinase (Thr202/Tyr204), phospho-AKT (Ser473) (Cell Signaling). Membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000). Signals were detected using an Amersham enhanced chemiluminescence (GE Healthcare Life Sciences, Munich, Germany).

4. Tumor xenografts and metformin treatments

KHOS/NP cells (2×10^6) were suspended in 100-μL PBS and injected subcutaneously in the flanks of 6-week-old BALB/c nude mice (Orient Bio Inc., Seongnam, Korea). When tumors reached a volume of 50–100 mm³, mice were randomized into control and metformin-treatment groups. Metformin was dissolved in drinking water to a final concentration of 2 mg/mL. Mice were allowed to drink water, and the water was replenished every 4 days. The estimated metformin dose per mouse was 12 mg/day. Tumor volume was measured with precision calipers every 3 to 4 days for a period of 4 weeks. The tumor volume (V) was calculated using the standard formula: V (mm³)=(width)²×length/2. Experiments were conducted according to the guidelines for ethical use of animals of our Institution under an approved protocol (KIRAMS 2014-0039).

5. Statistical analysis

All data were plotted as the mean±standard error of the mean. All calculations were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) and SPSS ver. 13.0
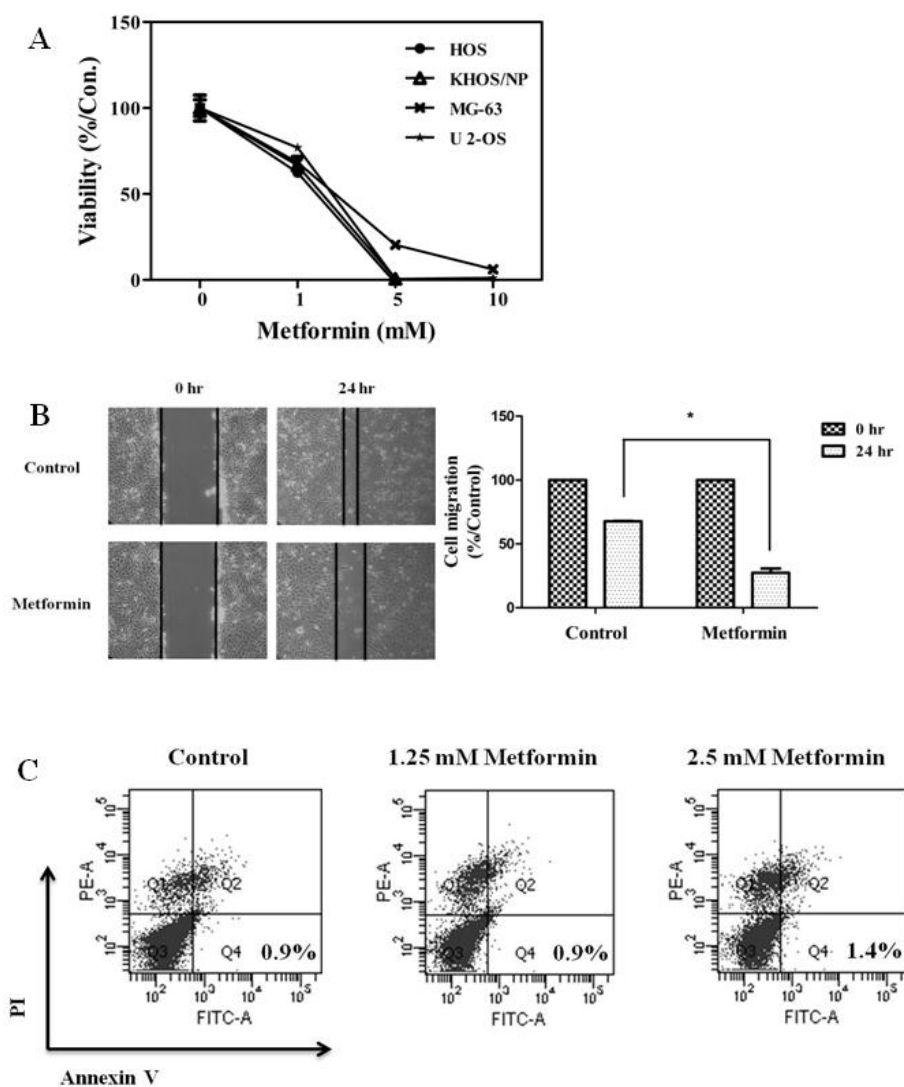

Fig. 1. (A) Metformin decreased the proliferation and migration of osteosarcoma cells. Four osteosarcoma cells were treated with metformin (0–10 mM) and cell viability assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was performed 72 hours later. (B) KHOS/NP cells were plated in 6-well plates and grown to 80% confluence. After scratching, cells were incubated with or without metformin (1.5 mM). Microscopic appearance was captured 24 hours after wounding. The area of the gap region was quantified by ImageJ and data are shown in the bar graph (*P<0.05). (C) KHOS/NP cells were treated with metformin (1.25 or 2.5 mM) for 24 hours. Apoptotic cells were detected by Annexin V-propidium iodide (PI) dual staining.

(SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered statistically significant.

Results

1. Effects of metformin on proliferation and migration of human osteosarcoma cells

To evaluate the antitumor effects of metformin, the *in vitro* antiproliferative effects of metformin (0–10 mM) were first examined against four native osteosarcoma cell lines. We found that metformin significantly inhibited the proliferation of the 4 native osteosarcoma cell lines in a dose-dependent manner and the IC_{50} value was 2.2–2.9 mM (Fig. 1A). To simulate the clinical setting of recurrent or refractory osteosarcoma, we established cisplatin-resistant cells by exposing KHOS/NP and U-2 OS cells to cisplatin (Fig. 2A) and investigated the effect of metformin on cell proliferation. Metformin inhibited the proliferation of both native and cisplatin-resistant KHOS/NP and U-2 OS cells. The IC_{50} values of metformin were higher in cisplatin-resistant cells than in native cells, 4.2–4.5 mM vs. 2.2–2.9 mM, respectively (Fig. 2B). Therefore, we concluded that metformin inhibited the proliferation of both native and cisplatin-resistant osteosarcoma cells. Since metformin has been demonstrated to have antimetastatic effects, we examined the effect of metformin on the migration of osteosarcoma cells. We found that metformin treatment significantly inhibited the migration of KHOS/NP cells via analysis of wound-healing (Fig. 1B). From these results, we suggest that metformin could potentially inhibit proliferation and migration of human osteosarcoma cells. Although metformin inhibited cell proliferation, it did not induce apoptosis of KHOS/NP cells (Fig. 1C). To determine the effect on cell cycle, KHOS/NP cells were treated with 2.5 mM metformin for 24 hours, stained with propidium iodide, and analyzed using flow cytometry. As shown in Fig. 3A, compared with control, the fraction of G0/G1 cells increased after metformin treatment (from 43% to 51.3%). Moreover, we found that the levels of cyclin D1 and CDK2, key regulators for the G1/S transition, were decreased (Fig. 3B). This result indicated that metformin induced cell cycle arrest at IC_{50} (2.5 mM) in KHOS/NP cells.
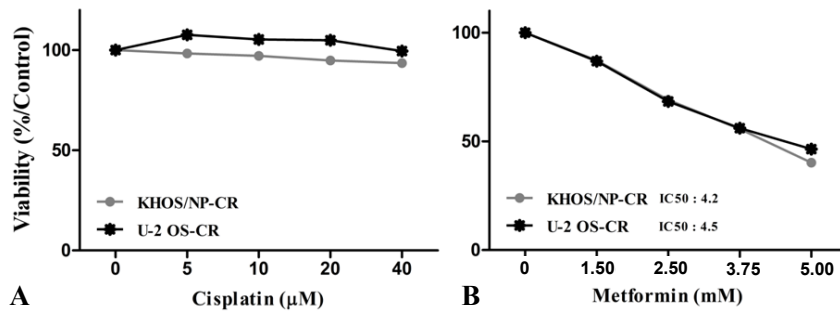

Fig. 2. Metformin decreased the proliferation of cisplatin-resistant osteosarcoma cells. Cisplatin-resistant osteosarcoma cells were established (A) and treated with metformin (B) and cell viability assay was performed 72 hours later. The half-maximal inhibitory concentration (IC_{50}) values were determined from dose-response curves.
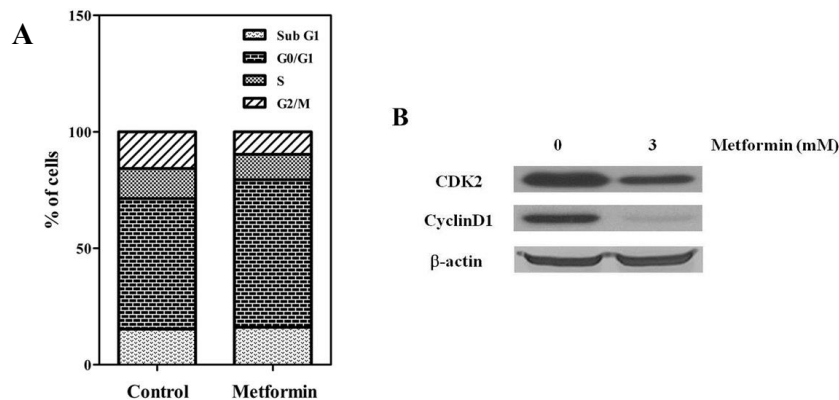

Fig. 3. Metformin induced cell-cycle arrest of KHOS/NP cells. KHOS/NP cells were treated with 2.5–3 mM metformin for 24 hours. (A) Cell cycle was analyzed by flow cytometry; (B) the CDK2 and cyclin D1 protein expression of KHOS/NP cells after metformin treatment.

2. Metformin activates the AMPK pathway and inhibits mTOR and its downstream pathways

Metformin activity has been responsible of activation of AMPK and its inhibitory function on mTOR. To test the functional activation of AMPK by treatment with metformin, we examined the phosphorylation level of both AMPK and acetyl CoA carboxylase, a target of AMPK known to be one of the substrates of AMPK activity, in KHOS/NP cells. Twenty-four hours after treatment with 3 mM metformin, p-AMPK (Thr172) and p-ACC (Ser79) were visible in KHOS/NP cells (Fig. 4A, B). To further elucidate the
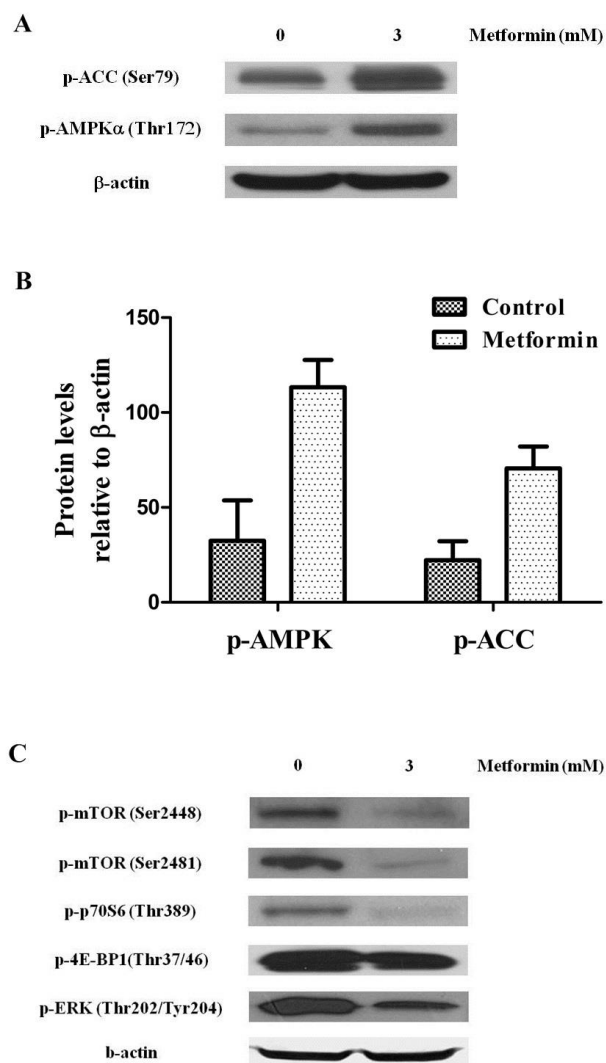
underlying mechanism, levels of mTOR and its substrates were measured. We found that metformin inhibited the phosphorylation of mTOR (Ser2481), mTOR (Ser2448), P70S6K, 4E-BP1, and ERK (an indicator for mTOR activity) (Fig. 4C).

3. Metformin inhibits the growth of KHOS/NP tumor xenograft

To determine whether metformin could curtail tumor growth *in vivo*, BALB/c nude mice were injected with KHOS/NP cells and treated with metformin (approximately 12 mg/day/mouse for 4 weeks). Metformin significantly inhibited the growth of KHOS/NP tumors in BALB/c nude mice (Fig. 5). From the 14th day of study entry, tumor volume of the metformin-treated mice was significantly smaller than that of the control mice ($P<0.05$). When the mean tumor volume was compared using fold increase at the 27th day, fold increase of the metformin-treated mice was 48% that of the control mice.

Fig. 4. Metformin controlled the AMP-activated protein kinase (AMPK)/ mammalian target of rapamycin (mTOR) signaling pathway. KHOS/ NP cells were treated with 3 mM metformin for 24 hours and Western blot analysis was performed to examine the levels of phospho-AMPK (p-AMPK) (T172), phospho-Acetyl-CoA carboxylase (p-ACC) (S79) (indicator for AMPK activity) (A, B) and mTOR and its substrates (C) in KHOS/NP cells.

Discussion

In the present study, we have observed the *in vitro* and *in vivo* effects of metformin against osteosarcoma. Metformin decreased the proliferation and migration of osteosarcoma cells, and inhibited the growth of KHOS/NP osteosarcoma in a tumor xenograft model. In addition, metformin showed antiproliferative effects on cisplatin-resistant osteosarcoma cells, which were established as an *in vitro* model of recurrent or refractory osteosarcomas. Our findings suggest the possibility of metformin being used as an adjuvant agent in the treatment of recurrent or refractory osteosarcoma patients.

As in previous reports, we observed that metformin activated the AMPK pathway and downregulated the mTOR pathway of osteosarcoma cells. Recent genomic study suggested the PI3K/ mTOR pathway as a common vulnerability in osteosarcoma[21], and we assume that our findings suggest the constitutive over-activation of the PI3K-mTOR pathway in osteosarcoma cells. It is
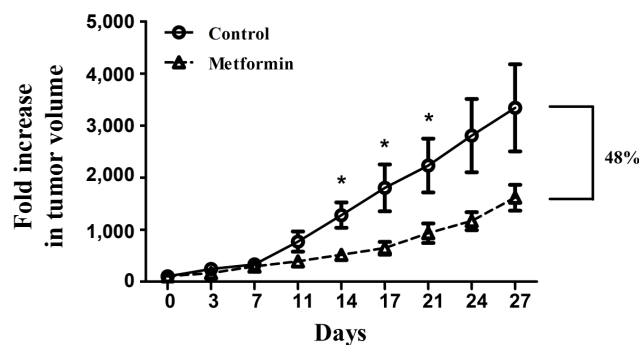

Fig. 5. Metformin inhibited the growth of KHOS/NP xenograft tumor of Balb/c-nude mice. Graphs represent mean±standard error of the mean of five tumors per condition (*$P<0.05$).

reported that metformin inhibits the migration and epidermal-mesenchymal transition of thyroid cancer cells[22]. Similarly, we observed that metformin decreased the migration of osteosarcoma cells. The critical factors regulating the growth, migration, and invasion of tumor cells are cyclin D1 and c-Myc[19,22,23]. Although it is presumed that metformin inhibits these factors through AMPK activation, another mechanism suggested to be involved in this phenomenon is the Akt-MDM2-Foxo3a signaling axis[24]. Our observation, together with other reports, indicated that either the AMPK-dependent or independent mechanism of metformin might be implicated in inhibition of proliferation and migration of human osteosarcoma.

Controversy exists about the association between the effect of metformint and TP53 or RB mutation status. We observed that metformin induces cell cycle arrest of both KHOS/NP (mutated TP53 and wild type RB) and U-2 OS cells (wild type TP53 and RB, data not shown). For U-2 OS cells, metformin (2.5 mM) treatment increased the expression of TP53 and RB protein (data not shown). For KHOS/NP cells, metformin treatment did not induce any changes of RB and TP53 protein expression, but decreased the expression of cyclin D1 protein (Fig. 3B). It is reported that metformin treatment phosphorylates the Chk1 of TP53 wild-type osteosarcoma cells, but not that of TP53 null MG-63 cells[19]. AMPK activation induces a p53-dependent metabolic G1/S checkpoint in response to low glucose conditions[25]. On the contrary, other studies suggest that TP53 loss increased the growth inhibitory and toxic effect of metformin on cancer cells[26]. In the present study, effects of metformin did not appear to be influenced by TP53 mutation status of osteosarcoma cells.

The optimal dose of metformin required to elicit antitumor effect remains elusive. The *in vitro* concentration of metformin showing antitumor effect was in the millimolar range, as the IC_{50} value 3 mM of our study[14,16,18]. However, the serum levels of metformin achieved in DM patients and in many *in vivo* models were in the micromolar ranges[27]. The epidemiologic data suggest that usual recommended doses of metformin might have antitumor effect[8,9]. It is uncertain what metformin concentrations are achieved in tumor tissues of patients receiving conventional antidiabetic doses of metformin. We assume that the high concentrations of glucose and growth factors in cell culture media, along with the presence of oncogenic mutations and unknown expression of the membrane transporter OCT1, which is essential for the transport of metformin across cell membrane[28], may account for the higher concentrations of metformin required to elicit *in vitro* antitumor effect[29].

At present, clinical trials are underway to evaluate the effect of metformin as a single agent, or in combination with cytotoxic chemotherapy, and/or radiotherapy for the treatment of various cancers[30]. The preliminary results from patients with breast and endometrial cancer have shown that metformin can significantly impact markers of tumor proliferation[31-33]. Currently ongoing clinical trials are designed to assess the survival benefit of metformin in patients with breast, pancreas, lung, endometrial, brain, prostate, and gynecological cancers[30].

Summarizing, we observed that metformin has some efficacy against osteosarcoma and suggest the possibility of using it as an adjuvant agent in the treatment of osteosarcoma. Our findings are based on preclinical data and a large-scale prospective study is required to validate our preclinical findings.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

References

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 2009;115:1531-43.
2. Bacci G, Forni C, Ferrari S, Longhi A, Bertoni F, Mercuri M, et al. Neoadjuvant chemotherapy for osteosarcoma of the extremity: intensification of preoperative treatment does not increase the rate of good histologic response to the primary tumor or improve the final outcome. J Pediatr Hematol Oncol 2003;25:845-53.
3. Eselgrim M, Grunert H, Kühne T, Zoubek A, Kevric M, Bürger H, et al. Dose intensity of chemotherapy for osteosarcoma and outcome in the Cooperative Osteosarcoma Study Group (COSS) trials. Pediatr Blood Cancer 2006;47:42-50.
4. Kempf-Bielack B, Bielack SS, Jurgens H, Branscheid D, Berdel WE, Exner GU, et al. Osteosarcoma relapse after combined modality therapy: an analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). J Clin Oncol 2005;23:559-68.
5. Kager L, Zoubek A, Pötschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol 2003;21:2011-8.
6. Leone A, Di Gennaro E, Bruzzese F, Avallone A, Budillon A. New perspective for an old antidiabetic drug: metformin as anticancer agent. Cancer Treat Res 2014;159:355-76.
7. Pierotti MA, Berrino F, Gariboldi M, Melani C, Mogavero A, Negri T, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multi-faceted effects. Oncogene 2013;32:1475-87.
8. Franciosi M, Lucisano G, Lapice E, Strippoli GF, Pellegrini F, Nicolucci A. Metformin therapy and risk of cancer in patients with

type 2 diabetes: systematic review. PLoS One 2013;8:e71583.

9. Hernández-Díaz S, Adami HO. Diabetes therapy and cancer risk: causal effects and other plausible explanations. Diabetologia 2010;53:802-8.

10. Currie CJ, Poole CD, Jenkins-Jones S, Gale EA, Johnson JA, Morgan CL. Mortality after incident cancer in people with and without type 2 diabetes: impact of metformin on survival. Diabetes Care 2012;35:299-304.

11. Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, Bilo HJ. Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. Diabetes Care 2010;33:322-6.

12. Anisimov VN, Bartke A. The key role of growth hormone-insulin-IGF-1 signaling in aging and cancer. Crit Rev Oncol Hematol 2013; 87:201-23.

13. Hadad S, Iwamoto T, Jordan L, Purdie C, Bray S, Baker L, et al. Evidence for biological effects of metformin in operable breast cancer: a pre-operative, window-of-opportunity, randomized trial. Breast Cancer Res Treat 2011;128:783-94.

14. Choi YK, Park KG. Metabolic roles of AMPK and metformin in cancer cells. Mol Cells 2013;36:279-87.

15. Perl A. mTOR activation is a biomarker and a central pathway to autoimmune disorders, cancer, obesity, and aging. Ann N Y Acad Sci 2015;1346:33-44.

16. Garofalo C, Capristo M, Manara MC, Mancarella C, Landuzzi L, Belfiore A, et al. Metformin as an adjuvant drug against pediatric sarcomas: hypoxia limits therapeutic effects of the drug. PLoS One 2013;8:e83832.

17. Issaq SH, Teicher BA, Monks A. Bioenergetic properties of human sarcoma cells help define sensitivity to metabolic inhibitors. Cell Cycle 2014;13:1152-61.

18. Duo J, Ma Y, Wang G, Han X, Zhang C. Metformin synergistically enhances antitumor activity of histone deacetylase inhibitor trichostatin a against osteosarcoma cell line. DNA Cell Biol 2013; 32:156-64.

19. Quattrini I, Conti A, Pazzaglia L, Novello C, Ferrari S, Picci P, et al. Metformin inhibits growth and sensitizes osteosarcoma cell lines to cisplatin through cell cycle modulation. Oncol Rep 2014;31:370-5.

20. Chen X, Hu C, Zhang W, Shen Y, Wang J, Hu F, et al. Metformin inhibits the proliferation, metastasis, and cancer stem-like sphere formation in osteosarcoma MG63 cells in vitro. Tumour Biol 2015; 36:9873-83.

21. Perry JA, Kiezun A, Tonzi P, Van Allen EM, Carter SL, Baca SC, et al. Complementary genomic approaches highlight the PI3K/mTOR

pathway as a common vulnerability in osteosarcoma. Proc Natl Acad Sci U S A 2014;111:E5564-73.

22. Han B, Cui H, Kang L, Zhang X, Jin Z, Lu L, et al. Metformin inhibits thyroid cancer cell growth, migration, and EMT through the mTOR pathway. Tumour Biol 2015;36:6295-304.

23. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 2008;27:3576-86.

24. Chou CC, Lee KH, Lai IL, Wang D, Mo X, Kulp SK, et al. AMPK reverses the mesenchymal phenotype of cancer cells by targeting the Akt-MDM2-Foxo3a signaling axis. Cancer Res 2014;74:4783-95.

25. Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell 2005;18:283-93.

26. Muaddi H, Chowdhury S, Vellanki R, Zamiara P, Koritzinsky M. Contributions of AMPK and p53 dependent signaling to radiation response in the presence of metformin. Radiother Oncol 2013; 108:446-50.

27. Tucker GT, Casey C, Phillips PJ, Connor H, Ward JD, Woods HF. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br J Clin Pharmacol 1981;12:235-46.

28. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, Gotlieb WH. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. Biochem Biophys Res Commun 2011;414: 694-9.

29. Dowling RJ, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. J Mol Endocrinol 2012;48:R31-43.

30. Chae YK, Arya A, Malecek MK, Shin DS, Carneiro B, Chandra S, et al. Repurposing metformin for cancer treatment: current clinical studies. Oncotarget 2016 Mar 19 [Epub]. http://dx.doi.org/10.18632/oncotarget.8194.

31. Bonanni B, Puntoni M, Cazzaniga M, Pruneri G, Serrano D, Guerrieri-Gonzaga A, et al. Dual effect of metformin on breast cancer proliferation in a randomized presurgical trial. J Clin Oncol 2012; 30:2593-600.

32. Cazzaniga M, DeCensi A, Pruneri G, Puntoni M, Bottiglieri L, Varricchio C, et al. The effect of metformin on apoptosis in a breast cancer presurgical trial. Br J Cancer 2013;109:2792-7.

33. Schuler KM, Rambally BS, DiFurio MJ, Sampey BP, Gehrig PA, Makowski L, et al. Antiproliferative and metabolic effects of metformin in a preoperative window clinical trial for endometrial cancer. Cancer Med 2015;4:161-73.