Metformin inhibits the proliferation of human prostate cancer PC-3 cells via the downregulation of insulin-like growth factor 1 receptor

Haruo Kato*, Yoshitaka Sekine, Yosuke Furuya, Yoshiyuki Miyazawa, Hidekazu Koike, Kazuhiro Suzuki

Department of Urology, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

ABSTRACT

Metformin is a biguanide drug that is widely used for the treatment of type 2 diabetes. Recent studies have shown that metformin inhibits cancer cell proliferation and tumor growth both *in vitro* and *in vivo*. The anti-tumor mechanisms of metformin include activation of the AMP-activated protein kinase/mTOR pathway and direct inhibition of insulin/insulin-like growth factor (IGF)-mediated cellular proliferation. However, the anti-tumor mechanism in prostate cancer remains unclear. Because activation of the IGF-1 receptor (IGF-1R) is required for prostate cell proliferation, IGF-1R inhibitors may be of therapeutic value. Accordingly, we examined the effects of metformin on IGF-1R signaling in prostate cancer cells. Metformin significantly inhibited PC-3 cell proliferation, migration, and invasion. IGF-1R mRNA expression decreased significantly after 48 h of treatment, and IGF-1R protein expression decreased in a similar manner. IGF-1R knockdown by siRNA transfection led to inhibited proliferation, migration and invasion of PC-3 cells. IGF-1 activated both ERK1/2 and Akt, but these effects were attenuated by metformin treatment. In addition, intraperitoneal treatment with metformin significantly reduced tumor growth and IGF-1R mRNA expression in PC-3 xenografts. Our results suggest that metformin is a potent inhibitor of the IGF-1/IGF-1R system and may be beneficial in prostate cancer treatment.

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1. Introduction

Metformin is a biguanide drug that is widely used for the treatment of type 2 diabetes. In addition to its efficacy in lowering glucose levels, recent reports indicate that this drug may have anti-tumor effects in various cancers, including prostate cancer (PC). This is based on work showing that patients receiving metformin for diabetes exhibit reduced cancer incidence [1]. Furthermore, increased cumulative duration of metformin exposure after PC diagnosis is associated with decreases in both all-cause and PC-specific mortality in diabetic men [2]. The anti-tumor mechanisms of metformin include activation of the AMPK/mTOR pathway, direct inhibition of insulin-like growth factor (IGF)-mediated cellular proliferation, and cell cycle arrest in the G0/G1 phase mediated via a reduction in cyclin D1 levels [3]. However, the anti-tumor mechanism of metformin in PC cell lines has not yet been elucidated.

The IGF system plays a key role in regulating the growth of PC cells, and increased IGF-1 serum levels are associated with an increased risk of PC [4]. IGF-1 may also increase the *in vitro* proliferation of PC cells, whereas the antisense-mediated inhibition of IGF-1R expression suppresses *in vivo* tumor growth and limits PC cell invasiveness [5]. Furthermore, IGF-1R antagonism is associated with decreased androgen-dependent and androgen-independent growth [6]. The activated IGF-1R phosphorylates signaling molecules via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways [7]. These signals play important roles in PC cell proliferation and inhibition of apoptosis; therefore, the inhibition of IGF-1R actions is a potentially novel approach to PC treatment.

In the present study, metformin inhibited IGF-1/IGF-1R signaling by decreasing the expression of IGF-1R in androgen receptor (AR)-negative PC-3 cells. These results suggest that metformin may provide a novel therapeutic approach for PC.

* Corresponding author. Department of Urology, Gunma University Graduate School of Medicine and Gunma University Hospital, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Fax: +81 27 220 8307.

E-mail address: hal.kato@gunma-u.ac.jp (H. Kato).

2. Materials and methods

2.1. Cells and chemicals

The human PC cell line PC-3 and LNCaP were purchased from Dainippon Pharmaceutical (Tokyo, Japan) and cultured in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Moregate, Bulimba, Australia). LNCaP-LA was used as an *in vitro* model of castration resistant prostate cancer (CRPC), and were derived from LNCaP cells cultured with 10% charcoal-stripped FBS for 3 months. Antibodies (rabbit anti-ERK1/2 polyclonal antibody, rabbit anti-phospho-ERK1/2 [Thr202/Tyr204] polyclonal antibody, rabbit anti-Akt polyclonal antibody, rabbit anti-phospho-Akt [Ser473] polyclonal antibody, rabbit anti-IGF-1Rβ monoclonal antibody, and rabbit anti-human β-actin monoclonal antibody) were purchased from Cell Signaling (Beverly, MA, USA). Metformin and IGF-1 were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell proliferation assay using human PC cells

Approximately 2×10^3 PC-3 cells, 4×10^3 LNCaP cells or LNCaP-LA cells per well were seeded into a 96-well microtiter plate containing 100 μL culture medium (CM) for MTS assay and a 12-well plate containing 1 mL CM for cell counting with 10% FBS. These plates were incubated for 48 h at 37 °C under an atmosphere of 5% CO_2. Thereafter, the CM was aspirated and the cells were incubated in CM with or without metformin for 48 h at 37 °C under 5% CO_2. After this second round of incubation, the number of living cells was determined by cell counting and MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). The absorbance of the cell lysate was expressed as fold change.

2.3. Migration assay

Cells were plated on a 12-well plate and grown to confluence. Thereafter, the medium was aspirated, and cells were incubated in medium containing 10% FBS for 24 h before each experiment. One thousand-microliter tips were used to make a denuded area. Cells were washed twice with phosphate buffered saline (PBS) and incubated with or without metformin (1–10 mM) for 24 h. Mitomycin C (0.5 μM) was added to block cell proliferation during the entire 48 h period of the study. Photographs were taken at 0 and 24 h, and cell migration distance was determined by subtracting the values. The quantitation was performed using ImageJ (National Institutes of Health, Bethesda, Md). Migration distances were expressed as fold change over the control.

2.4. Invasion assay

Matrigel invasion chamber plates (Becton Dickinson/Biocoat, Bedford, MA) were used for this assay. Cells were incubated in RPMI containing 10% FBS for 24 h before each experiment. Cells were plated in the upper chamber with RPMI containing metformin; the lower chamber contained RPMI plus 10% FBS and the same test ligands as the upper chamber. After 48 h, non-invading cells were removed using a cotton swab. The number of cells that adhered to the bottom surface of the membrane was counted with a microscope in several fields of the membranes. The invasion index was expressed as fold change over the control.

2.5. Quantification of mRNA levels

Transcript levels were quantified using the CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Total RNA extraction and cDNA synthesis were performed as described previously [8]. Amplification was performed in 10 μL Premix Ex Taq (TaKaRa, Tokyo, Japan) using 2 μL cDNA and IGF-1R primer (No. S100011165, Applied Biosystems, Foster City, CA, USA). Next, PCR was performed for one cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The transcription of β-actin (No. 4326315E, Applied Biosystems, Foster City, CA, USA) was also examined as an internal control. Relative quantitation values were calculated, using the comparative C_T method, also known as the $2^{-\Delta\Delta C_T}$ method.

2.6. Western Blotting assays

Cell lysates were prepared in RIPA buffer (Pierce, Rockford, IL), containing 1 mM sodium orthovanadate (Sigma) and protease inhibitors (Complete TM-Without EDTA, Roche Diagnostics, Penzberg, Germany). Equal amounts of proteins (30–40 μg/lane) were electrophoresed on 4–12% SDS–PAGE gels and then transferred to nitrocellulose membranes. Each membrane was incubated with the primary antibodies described above. Blots were developed using a 1:1000 dilution of the horseradish peroxidase -conjugated secondary antibody (Cell Signaling). Proteins were visualized using Immobilon Western HRP Reagent (Millipore, Billerica, MA, USA).

2.7. Small interfering RNA

Cells were seeded into a 6 well microtiter plate in 2000 μL (Western Blotting and invasion assay), a 12 well microtiter plate in 1000 μL (migration assay) and a 96 well microtiter plate in 100 μL (MTS assay) with 10% FBS. Thereafter, cells were transfected with ON-TARGETplus Non-targeting Pool (no. D-001810-10-05, Dharmacon, Lafayette, IN, USA), ON-TARGETplus IGF1R small interfering RNA (siRNA; no. L-003012-00-0005, Dharmacon), using DharmaFect (Dharmacon). The cells were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere after transfection.

2.8. Apoptosis analysis

Cells were seeded in a 75 mm flask at 40–50% confluence. The cells were incubated with or without metformin (10 mM) for 72 h. For cell apoptosis analysis, cells were trypsinized and washed twice with PBS. Then, cells were fixed with 70% icecold ethanol, followed by analysis with an APO-DORECT kit (Becton–Dickinson, San Diego, CA, USA) according to the manufacturer's instructions. Cell apoptosis histograms were generated after analysis of stained cells by fluorescence-activated cell sorting (FACS), with a Becton–Dickinson FACScan.

2.9. Animal studies

Four-week-old male BALB/c/nu mice (Charles River Laboratories Japan, Yokohama, Japan) were injected subcutaneously (s.c.) into the right flank with approximately 3×10^6 PC-3 cells in BD Matrigel basement membrane matrix (BD Biosciences, Bedford, MA). A high-fat diet and metformin treatment were initiated 2 days beforehand. Five mice were used per group. Mice also received daily intraperitoneal (i.p.) injections with PBS only (control) or 50, 100, or 250 mg/kg metformin. Tumor size was measured weekly during the following 4 weeks and tumor volume was calculated using the formula $A \times B \times C \times \pi/6$, where *A*, *B*, and *C* are the length, width, and height of the tumor, respectively. Mice were euthanized 29 days after the xenograft, and the tumors were collected. All animal experiments were approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus, Japan, and were conducted according to the guidelines of this committee.

2.10. Statistical analysis

Data are expressed as the mean ± SD. Differences between the values were evaluated via one-way ANOVA followed by a *post hoc* Tukey test. ANOVA was also used to compare tumor size in mice after different treatments. In all analyses, *p*-values < 0.05 were considered statistically significant.

3. Results

### 3.1. Metformin inhibits proliferation, migration, and invasion of PC-3 cells

First, we examined the effects of metformin on PC cells proliferation using the MTS assay and cell counting. The numbers of viable PC-3, LNCaP and LNCaP-LA cells in 10% FBS medium decreased significantly after 48 h incubation with metformin (Fig. 1A and B). We also examined whether metformin inhibited the migration of PC-3 cells in a wound healing assay. We found that it significantly inhibited the migration of PC-3 cells in a dose-dependent manner (Fig. 1C and D). It also significantly inhibited the invasion of PC-3 cells in a Matrigel invasion chamber (Fig. 1E). On the other hand, apoptosis was not detected in treatment with metformin for 72 h (data not shown). A representative experiment of three independent experiments is shown in each figure.

### 3.2. Metformin decreases IGF-1R expression

IGF-1 and the IGF-1R system play a key role in regulating the growth of PC cells [5,6], and IGF-1R knockdown suppresses PC-3 cell proliferation [9]. Therefore, we examined whether metformin could inhibit the expression of IGF-1R in PC-3 cells. IGF-1R mRNA expression levels decreased significantly after 48 h incubation in the presence of metformin in PC-3 (Fig. 2A). In addition, IGF-1R protein expression also decreased in all cell lines (Fig. 2B).

3.3. Metformin inhibits IGF-1-induced activation of ERK, Akt, and cell proliferation

IGF-1 trans-activates both MAPK/ERK and Akt [7]. Metformin treatment inhibited ERK activation induced by short-term IGF-1 stimulation (40 ng/mL for 15 min) (Fig. 2C). We also examined the effects of metformin on Akt activation. Because PC-3 cells have lost the tumor suppressor gene PTEN, which plays a direct role in the suppression of Akt activation, these cells show Akt phosphorylation even without IGF-1 stimulation [10]. We found that phosphorylated Akt was detected in PC-3 cells even without IGF-1 treatment, and IGF-1-induced Akt phosphorylation was significantly lower in cells that had been incubated with metformin (Fig. 2D). These results show that metformin inhibits signal transduction pathways that are important for cell proliferation.
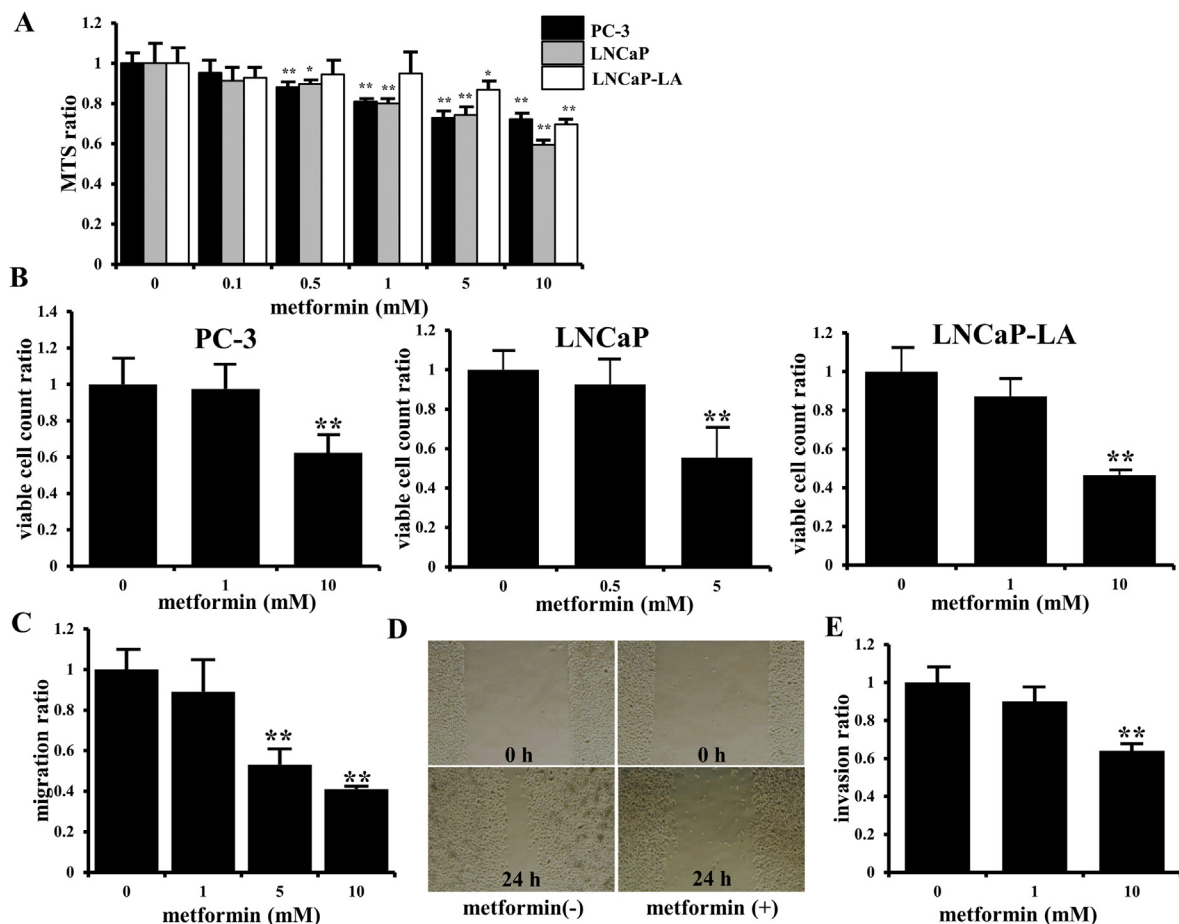
Fig. 1. Effects of metformin on PC-3 cells. (A) Cells were incubated in CM containing 10% FBS with or without metformin (0.1—10 mM). After 48 h, the number of viable cells was determined using the MTS assay. Columns, mean ($n = 6$); bars, SD. **, $p < 0.01$ vs. metformin 0 mM. (B) Cells were incubated in culture medium containing 10% FBS with or without metformin. After 48 h, the number of viable cells was determined by cell counting. Columns, mean ($n = 4$); bars, SD. **, $p < 0.01$ vs. metformin 0 mM. (C, D) Cells were wounded and then cultured in medium containing 10% FBS with or without metformin (1—10 mM) for 24 h. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean ($n = 3$); bars, SD. **, $p < 0.01$ vs. metformin 0 mM. (E) After culturing cells with metformin, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean ($n = 4$); bars, SD. **, $p < 0.01$ vs. metformin 0 mM.
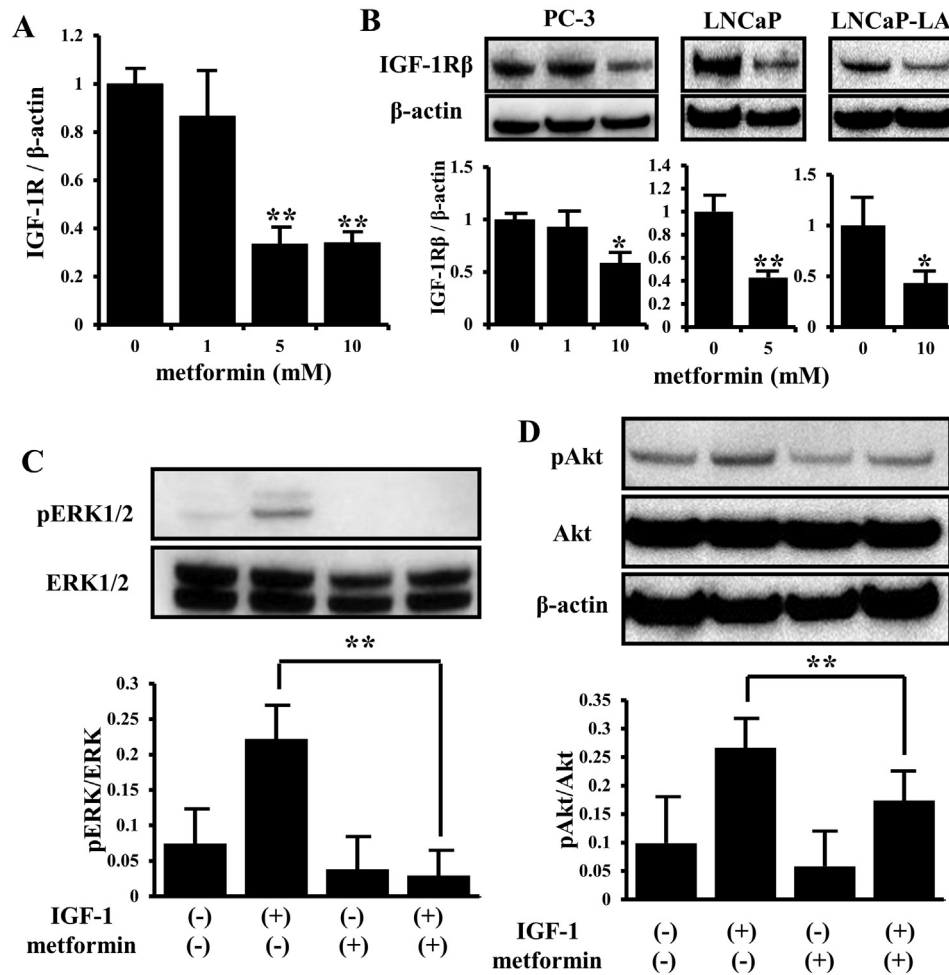
Fig. 2. Effects of metformin on IGF-1R expression and IGF-1/IGF-1R signaling. (A) Cells were incubated in CM containing 10% FBS with or without metformin (1–10 mM). After 48 h, mRNA was isolated and IGF-1R mRNA levels were measured via quantitative real-time PCR. Columns, mean ($n = 4$); bars, SD. **, $p < 0.01$ vs. 0 mM. (B) IGF-1R protein levels were measured via Western blotting. β-actin protein levels were used as the internal control. Cells were incubated in medium containing 10% FBS with or without metformin (1 or 10 mM). After 48 h, total cell proteins were extracted. The results shown are representative of three independently performed experiments. Columns, mean ($n = 3$); bars, SD. *, $p < 0.05$; **, $p < 0.01$ vs. 0 mM. (C, D) PC-3 cells were incubated for 48 h in medium containing 1% FBS with or without metformin (10 mM). Then the cells were stimulated with IGF-1 (40 ng/mL) for 15 min. The cells were harvested and cell lysates were prepared for Western blotting. The results shown are representative of three independently performed experiments. Columns, mean ($n = 3$); bars, SD. *, $p < 0.05$; **, $p < 0.01$.

3.4. IGF-1R knockdown suppressed PC-3 cell growth

We used siRNA to inhibit IGF-1R gene expression in PC-3 cells to study the role of IGF-1R in PC-3 cells. IGF1R protein expression decreased significantly following siRNA treatment in PC-3 cells (Fig. 3A). Next, we evaluated the effect of IGF-1R knockdown of expression by siRNA on PC-3 cell proliferation, migration, and invasion. IGF-1R knockdown suppressed cell proliferation, migration, and invasion significantly (Fig. 3B–D). Although metformin inhibited proliferation of control cells to an extent similar to IGF-1R knockdown, no significant difference in proliferation of IGF-1R knockdown cells was observed in the presence or absence of metformin (Fig. 3D). This result indicates that downregulating IGF-1R may be a very important mechanism of the anti-tumoral effects of metformin.

3.5. Metformin inhibits PC-3 cell growth and IGF-1R expression in vivo

To determine whether metformin affects tumor growth *in vivo*, we injected nude mice with PC-3 cells (right flank, s.c.). Then the mice received daily injections (i.p.) with either PBS or one of three doses of metformin (50, 100, or 250 mg/kg). Metformin did not affect body weight (data not shown). However, high-dose treatment with metformin (250 mg/kg) significantly reduced tumor growth by 34% relative to the PBS-injected control (Fig. 4A and B). The tumor growth in mice fed with normal fat diets was not reduced in any of the dose groups (data not shown). To determine whether metformin affects IGF-1R transcription *in vivo*, we examined IGF-1R mRNA expression in xenografts after treatment with metformin. Consistent with the tumor growth data, metformin significantly decreased IGF-1R mRNA expression in tumor cells at a dose of 250 mg/kg/day (Fig. 4C). These results suggest that, similar to our *in vitro* observations, metformin decreases tumor growth by reducing IGF-1R levels.

4. Discussion

We examined the mechanism by which metformin inhibits the growth of PC cells. Recent studies have suggested that metformin may inhibit PC cell proliferation [11], but the mechanism underlying this intriguing anti-tumor effect remains unclear. To the best of our knowledge, this is the first report showing that metformin
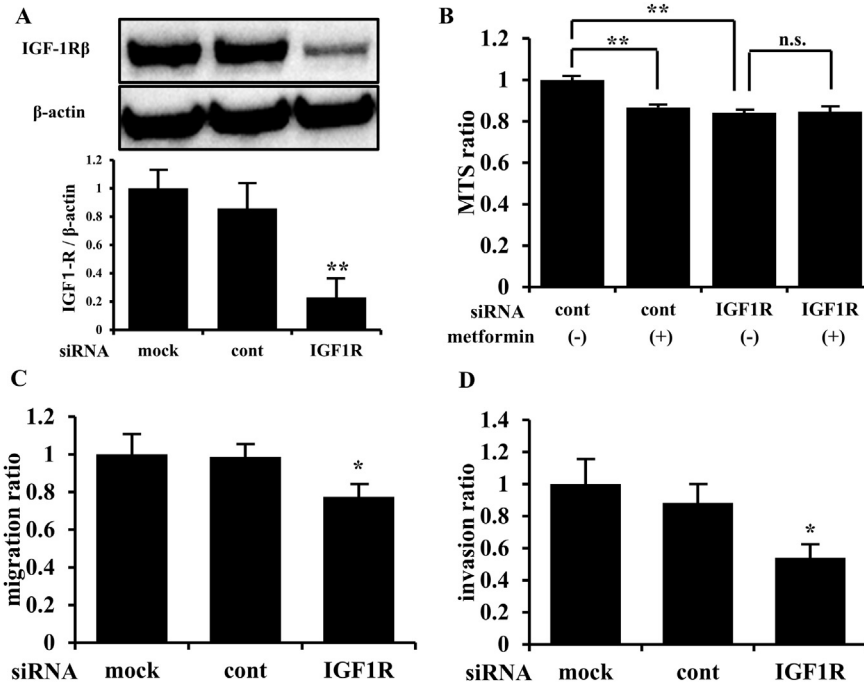
Fig. 3. Effects of IGF-1R knockdown via siRNA in PC-3 cells. PC-3 cells were transfected with IGF-1R siRNA or negative siRNA. Cells that were not transfected (mock) and negative siRNA-transfected cells (cont) were used as controls. (A) After transfection, the cells were incubated for 48 h before harvest for Western blotting. A representative experiment is shown, which was repeated three times with similar results. (B) After transfection, the cells were incubated with CM containing 10% FBS in the absence or presence of metformin (10 mM). The number of viable cells was measured by the MTS assay after 72 h. Columns, mean ($n = 6$); bars, SD. **, $p < 0.01$; n.s., not significant. (C) After transfection, the cells were wounded and cultured in medium containing 1% FBS for 24 h. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean ($n = 4$); bars, SD. *, $p < 0.05$ vs. cont. (D) After transfection, the cells were peeled off and reseeded in a Matrigel invasion chamber plate in CM with 10% FBS. The number of cells that invaded through the Matrigel after 48 h was counted under a microscope. Columns, mean ($n = 3$); bars, SD. *, $p < 0.05$ vs. cont.
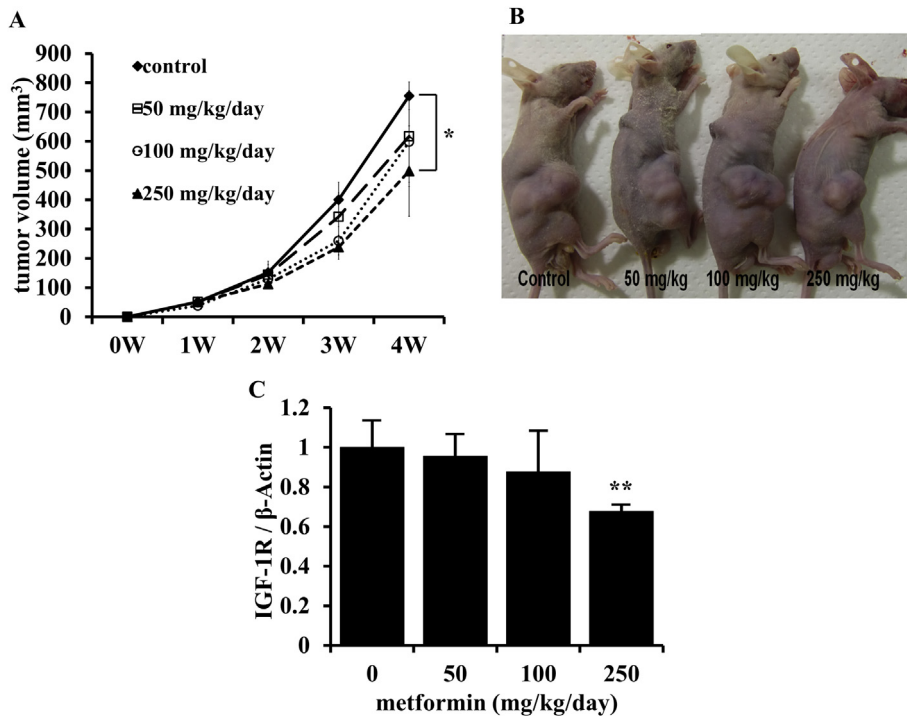
Fig. 4. Effects of metformin on tumor growth and IGF-1R expression *in vivo* using a PC-3 xenograft model. (A, B) Graphic view of mean tumor volumes in PC-3 xenografts. Mice were injected with PC-3 cells (3×10^6 per site) and then subsequently injected with PBS (control) or 50, 100, or 250 mg/kg of metformin (i.p.) daily. Columns, mean ($n = 5$); bars, SD. *, $p < 0.05$ vs. control at 4 weeks. (C) IGF-1R mRNA expression was analyzed via quantitative real-time PCR and compared to that in PBS-treated controls. Columns, mean ($n = 5$); bars, SD. **, $p < 0.01$ vs. control.

reduces the expression of IGF-1R and inhibits PC cell proliferation *in vitro* and *in vivo* in AR-negative PC-3 cells.

Currently, metformin is being studied for its use in cancer prevention and as a potential therapy to treat various cancers, including PC. Recent clinical work has shown that diabetic men with longer cumulative exposure to metformin after PC diagnosis show decreases in both all-cause and PC-specific mortality [2]. In addition, metformin use may improve prostate-specific antigen recurrence-free survival, PC-specific mortality, and overall survival, and reduce the development of CRPC in PC patients [12]. In the present study, we used PC-3 cells, which are known to be androgen-independent PC cells, to show that metformin inhibited cell proliferation and reduced tumor growth *in vivo*. These results suggest that metformin has the potential to slow the development of advanced PC.

Previous studies have shown that IGF-1/IGF-1R signaling is important in the malignant behavior of tumor cells. IGF-1 increases PC cell proliferation *in vitro* [13]. One of the proposed mechanisms through which this may occur is the induction of receptor tyrosine phosphorylation upon IGF-1–IGF-1R binding, followed by activation of the intracellular MAPK and Akt signaling pathways [7]. MAPK signal transduction plays an important role in PC progression, whereas MAPK inhibition reduces growth factor-induced proliferation of androgen-independent PC cells [13]. Moreover, Akt is a well-known regulator of cell survival, with anti-apoptotic effects. In the case of PC-3 cells, Akt activation promotes cell proliferation, whereas Akt inhibition suppresses cell proliferation and the induction of apoptosis *in vivo* [14]. In the present study, metformin inhibited the IGF-1-induced activation of both MAPK and Akt. These results indicate that metformin inhibits cell proliferation via the IGF-1R/ERK or Akt pathway.

IGF-1R is frequently overexpressed in a wide range of tumor types [7] and increased IGF-1R expression is associated with an increased risk of developing various neoplasms [15]. In contrast, silencing IGF-1R strongly inhibits proliferation, colony-forming capability, migration, and the invasive/metastatic potential of pancreatic ductal adenocarcinoma cells [16]. Clinical work on PC has revealed that increased IGF-1R expression is associated with increased levels of prostate-specific antigen and higher Gleason scores, both of which indicate increased aggressiveness and poorer prognosis [17,18]. In this study, we showed that IGF-1R knockdown via siRNA suppressed PC-3 cell proliferation, migration and invasion. It has also been reported that IGF-1R inhibition suppresses PC cell growth *in vivo* and *in vitro* [6,19]. These studies indicate that IGF-1R plays an important role in androgen-independent PC cell growth. On the other hand, previous study has also shown that IGF-1R knock-down induces apoptosis in PC-3 cells [20]. However, the apoptosis was not detected in treatment with metformin. The inhibition of IGF-1R expression by metformin was less than siRNA (Figs. 2B and 3A), which seems to be one of the reasons to the discrepancy.

In PC cells, the expression of IGF-1R is reportedly regulated by androgens [21]. Recent studies have reported that metformin inhibits IGF-1-mediated biological effects by disrupting membrane-initiated AR action responsible for IGF-1R upregulation in AR-positive LNCaP PC cells [22]. In the present study, we selected PC-3 cells as our PC model because they allowed us to evaluate the relationship between metformin and IGF-1R in PC cells without the interference of androgens.

The main limitation of this study is related to the concentration of metformin. We found that metformin inhibited the expression of IGF-1R at a concentration of 10 mM. This concentration is higher than that observed in metformin-treated patients admitted with lactic acidosis (median concentration, 330 μM) [23]. However, specific organs may be exposed to metformin concentrations that are significantly greater than those observed in the serum [24,25]. In addition, in an *in vivo* study that sued an LNCaP xenograft model, intraperitoneal metformin treatment (1 mg/day), a dosage typically used in diabetic patients, led to a 35% reduction in tumor growth [3]. However, in the present study, we found that only the highest metformin dose (5 mg/day) decreased tumor growth in a PC-3 xenograft model. Although this dosage is clearly higher than that used in previous studies, the use of a high dosage did not adversely affect body weight, demonstrating that this concentration did not have marked toxic consequences for mice (data not shown).

In summary, metformin inhibited AR-negative PC-3 cell growth via the downregulation of IGF-1R, both *in vitro* and *in vivo*. These data suggest that metformin may be a potent inhibitor of IGF-1/IGF-1R signaling. Because metformin is a well-tolerated and relatively inexpensive drug with the added co-benefits of mitigating hyperglycemia and metabolic syndrome related to androgen-deprivation therapy, it may prove to be beneficial for the treatment of CRPC.

Conflict of interest

None.

Acknowledgments

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.178.

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