

## **Metformin prevents proliferation of prostate cancer by regulating IGF1R/PI3K/Akt signalling in a mouse model.**

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### **Abstract**

**Objective:** To elucidate the inhibition mechanism of metformin on prostate cancer growth, we studied the effect of metformin in a mouse model.

**Methods:** Six-week old male BALB/c nude mice in SPF level were divided into three groups after tumour initiation and were administered once daily intraperitoneally for 3 weeks: control group, metformin-treated group and picropodophyllin-treated (PPP, IGF1R inhibitor) group. We assessed the effect of metformin on tumour volume, tumour weight and IGF1R/PI3K/Akt signalling pathway in mice.

**Results:** Metformin treatment significantly reduced tumour weight compared with those of untreated control group and picropodophyllin-treated group. IGF-1R mRNA levels and PI3K, p-Akt and Akt protein levels of metformin-treated group were significantly decreased than control group and picropodophyllin-treated group.

**Conclusion:** Metformin inhibits the growth of prostate cancer by inhibiting IGF1R/PI3K/Akt signalling axis and it may be beneficial for prostate cancer treatment.

**Keywords:** Metformin, IGF1R, Prostate cancer, Picropodophyllin

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### **Introduction**

Prostate cancer is the second common male malignant tumours worldwide. It is reported there are 6-7 million new cases every year in China, becoming a threat to the health of elderly men [1]. About 30% patients have been developed into advanced stage of prostate cancer when diagnosed, and there is less chance of surgical resection [2]. After the endocrine therapy for 18-24 months, large of patients would step into castration-resistant prostate cancer (CRPC) with a poor clinical prognosis [3]. Earlier studies confirmed hyperinsulinemia and insulin resistance were independent risk factors of prostate cancer [4]. Metformin, as an insulin sensitizer, reduces hyperinsulinemia and possesses anti-cancer effects to colon cancer, ovarian cancer, prostate cancer [5-7]. In addition, piles of studies showed insulin-like growth factor 1 receptor (IGF1R) played a crucial role in tumour cell growth, apoptosis, development, other than glucose and lipid metabolism [8]. It was demonstrated that targeting IGF-1R/Akt signalling pathway with glucosamine was effective for treating human non-small cell lung cancer [9].

In this study, we implanted prostate tumour cell line into prostate of immunocompetent rat and investigated the response of prostate tumours to metformin. Further, we evaluated the inhibition mechanism of metformin on prostate cancer via mediating IGF1R/PI3K/Akt signal axis in prostate tumour tissues, to provide new targets and theoretical basis for tumorigenesis and intervention.

### **Materials and Methods**

#### **Cells and animals**

DU145 human prostate tumour cell lines were purchased from Cell Bank of Shanghai Institutes for biological Sciences (Chinese Academy of Sciences). Cells were cultured in RPMI 1640 (Gibco) medium containing 10% foetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub>. Cells were harvest by 0.5% trypsin for 3 min and washed by 4°C ice-cold sterile PBS.

A total of 21 BALB/c male nude mice aged 4-6 weeks in SPF level with an average weight of 18-20 g were purchased from Shanghai Sangon Cell Experimental Center, and were housed in a well-ventilated room under 25°C with free access of food and water.  $2 \times 10^4$  cells were suspended in 10  $\mu$ L PBS and were carefully injected into one of the ventral prostate lobes continuously for 7d. Tumour-bearing mice were divided into three groups, including control group (n=7), metformin-treated group (n=7) and picropodophyllin-treated group (n=7). Control group received 150  $\mu$ L saline injection i.p. Metformin-treated group received metformin (120 mg/kg in 150  $\mu$ L saline). Picropodophyllin-treated group was dosing with picropodophyllin (0.1  $\mu$ M in 150  $\mu$ L saline) 4h before metformin (120 mg/kg in 150  $\mu$ L saline) treatment.

Mice were sacrificed 10 days after 3-week agent treatment. The tumour-containing prostates were removed, weighed, frozen in liquid nitrogen, and stored in -80°C. Tissues from

untreated mice were served as controls. Metformin and picropodophyllin was bought from Sigma-Aldrich.

### Quantification of mRNA levels by real-time qPCR

Total RNA in tumour tissues were extracted by Allprep RNA mini kit (Qiagen). The purity of RNA samples was evaluated by NanoDrop™ One (Thermo fisher). Primers were synthesized by Shanghai Sangon Company. IGF1R: F: 5'-ATGCTGTTTGAAGTATGCGCA-3', R: 5'-GCCCCGGCGTTCTTGCTCGCC-3', 354bp; GAPDH, F: 5'-CGCGAGAAGATGACCCAGAT-3', R: 5'-GCACTGTGTTGGCGTACAGG-3', 169 bp. The cDNA was synthesized from 0.5 µg of RNA with a Prime Script Kit (TAKARA, Osaka, Japan). Relative mRNA levels of IGF-1R were quantified by real-time PCR (RT-qPCR) using a Bio-Rad C1000 qPCR Detection System and Power SYBR Green PCR Master Mix as recommended by the manufacturer (Life Technologies). The relative mRNA levels were presented via 2<sup>-Ct</sup> method.

### Protein extraction and western-blot

After tissue homogenate, total cell protein was extracted in a buffer consisted of 25 mM HEPES buffer, pH 7.9, 1 U/µLof Benzonase 1 mM MgCl, 5 mM EDTA and PhosSTOP (Roche) per 10 ml of buffer. 30µg protein was mixed with 4 × Laemmli buffer Laemmli buffer (Bio-rad, Hercules, CA). After boiled, proteins from each sample were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocked by non-fat milk for 1h, the membranes were incubated with primary antibodies (PI3K p110α, 1:1000; phospho-AKT (Ser473), 1:1000; AKT,1:1000; β-actin; 1:5000) and Horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000). All antibodies were obtained from abcam. Reactive bands were by using a chemiluminescence (ECL) detection kit (Millipore).

### Statistical analyses

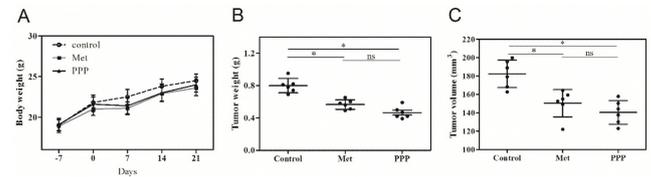
SPSS 13.0 software was used for statistical analysis. Measurement data was presented as mean ± standard deviation. The volumes (V) of the excised tumours were measured with an external calliper and calculated as V=0.52 (length × width × depth). One-way ANOVA was used for comparison among groups. LSD-t test was used for post-hoc comparison. P<0.05 was considered as statistically significant.

## Results

### Metformin inhibits prostate tumour cell growth

During the period of the experiment, no significant difference in body weight was found in 3 groups (Table 1, Figure 1A). There was no death of mice before sacrificed. The tumour weight was 0.79 ± 0.19 g, 0.6 ± 0.12 g and 0.47 ± 0.15 g for control group, metformin-treated group and picropodophyllin-treated group. The average tumour volume was (195.6 ± 32.4) mm<sup>3</sup> in control group, (157.8 ± 21.3) mm<sup>3</sup> in metformin-

treated group, and (164.3 ± 24.2) mm<sup>3</sup> in picropodophyllin-treated group. Tumour weight in metformin-treated group and picropodophyllin-treated group decreased when compared with control group, with the differences statistically significant (Figure 1B, p<0.05), whereas no significant difference was found between metformin-treated group and picropodophyllin-treated group. Similar results were found in tumour volume (Figure 1C, p<0.05).



**Figure 1.** Effect of metformin on tumour weight and tumour volume of mice. (A) Measurements of the mice of three groups. Body weight was measured weekly. Tumour weight (B), tumour volume (C) was measured after mice were sacrificed. Tumour weight and tumour volume of metformin-treated group and picropodophyllin-treated group was significantly lower than control group; \*p<0.05; ns: no significance.

### Metformin inhibits the expression of IGF-1R mRNA

The effects of metformin were investigated by real-time qPCR. We determined whether metformin blocked the IGF1R in prostate tumour of mice. As shown in Figure 2B, metformin treatment as well as picropodophyllin treatment significantly reduced IGF1R mRNA expressions compared to control group. No significance was observed between metformin-treated group and picropodophyllin-treated group.

### Metformin alleviates PI3K, Akt and phosphorylated Akt (p-Akt) protein levels

We examined the effect of metformin on PI3K, p-Akt and Akt activation by looking for changes in the level of total protein (Figure 2A). Metformin and picropodophyllin treatment significantly attenuated the activation of PI3K and AKT and phosphorylation of AKT on Ser473, compared to control group (Figures 2C-2E, p<0.05). Picropodophyllin treatment significantly suppressed the activation of AKT and phosphorylation of AKT on Ser473 (Figures 2C-2E, p<0.05).

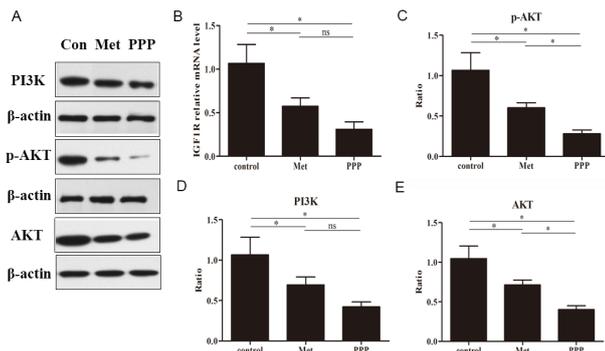
**Table 1.** Characteristics of three groups of mice.

| Variable                                 | Control (n=7) | Met (n=7)    | PPP (n=7)    |
|--|---------------|--------------|--------------|
| Pancreatic tumor weight, g               | 0.79 ± 0.19   | 0.6 ± 0.12   | 0.47 ± 0.15  |
| Pancreatic tumor volume, mm <sup>3</sup> | 195.6 ± 32.4  | 157.8 ± 21.3 | 164.3 ± 24.2 |

## Discussion

It is known that metformin inhibits tumour through activating AMP-activated protein kinase (AMPK), thereby inhibiting mTOR pathway activity. Moreover, metformin could down-regulate Cyclin D1 and inhibit tumour cell mitosis [10]. Data

has shown that [11] metformin can reduce hepatic gluconeogenesis, stimulate muscle tissues to take in glucose and reduce insulin levels and reduce hyperinsulinemia through mediating AMPK. Under high insulin concentration, metformin was able to activate the downstream ERK and PI3K signalling pathways through binding to insulin receptor (IR) and IGF1R on tumour cell membrane, consequently promoted the malignant progression of tumour cells. However, the mechanism of metformin of inhibiting tumorigenesis and development may not involve the changes in blood glucose and insulin levels, but the influence on IGF1R/STAT3 signal axis [12].



**Figure 2.** Effects of metformin on IGF-1R expression and IGF-1R/PI3K/Akt signalling. (A) The panel above shows representative results of Western blot. (B) Relative mRNA expressions of IGF1R in prostate tumours. (C-E) Protein expressions of PI3K, p-AKT and AKT; \* $p < 0.05$ ; ns: no significance.

As mentioned, metformin can inhibit the development of castration-resistant prostate cancer and reduce the mortality of prostate cancer. It has been showed that metformin treatment for 12226 patients with prostate cancer significantly reduce the risk of prostate cancer [13,14]. Clinical data from more than 350 primary care centres in Britain for retrospective cohort study and found that metformin treatment can improve the survival rate of malignant tumour patients with type 2 diabetes mellitus [15]. In this study, we did observe a significant decrease of tumour weight and tumour volume induced by metformin, suggesting that metformin had potential to be a novel approach for prostate tumour prevention.

Accordingly, when IGF1 or insulin binds to the receptors IGF1R thereby inducing a change in the transmembrane  $\beta$  subunits, phosphorylation of IRS is able to activate phosphatidylinositol 3-kinase (PI3K) and consequently of AKT. Picropodophyllin has been reported to specifically inhibit IGF1R activity via blocking IGF1R phosphorylation and downstream signalling, including Akt and MAPK phosphorylation [16,17]. Also, picropodophyllin induces apoptosis of malignant cells in different tumour models [18,19]. At present, exact mechanism of action of metformin on prostate cancer has not been fully elucidated. It remains unknown whether metformin inhibits prostate cancer progression through IGF1R /PI3K/Akt signal axis. In this study, we showed that metformin effectively inhibits IGF-1R-mediated PI3K/Akt signal transduction in prostate tumour.

Taking metformin helps prevent prostate cancer and improve the prognosis of high-grade prostate cancer, which is a conventional drug in new use.

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