

# Metformin mitigates PLC $\epsilon$ gene expression and modulates the Notch1/Hes and androgen receptor signaling pathways in castration-resistant prostate cancer xenograft models

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**Abstract.** The present study aimed to establish a mouse model of patient-derived castration-resistant prostate cancer (CRPC) xenograft tumors, and to evaluate the effects of various doses of metformin on phospholipase C $\epsilon$  (PLC $\epsilon$ ) expression and the neurogenic locus notch homolog protein 1 (Notch1)/hair cell enhancer of split 1 and androgen receptor (AR) signaling pathways via western blotting and reverse transcription-quantitative PCR. Additionally, phorbol 12-myristate 13-acetate was used to activate PLC, and Jagged1 was used as a Notch activator to verify whether metformin could suppress CRPC development via the PLC $\epsilon$ /Notch1/AR pathways. The results confirmed that metformin may serve critical roles in CRPC by significantly inhibiting the occurrence, growth and proliferation of CRPC tumors by decreasing PLC $\epsilon$ /Notch1 expression and AR nucleation.

## Introduction

Castration-resistant prostate cancer (CRPC), i.e., prostate cancer that responds poorly to anti-androgens, is characterized by strong local invasion, recurrence and distant metastasis, a low survival rate and a poor prognosis (1). It has been observed that metformin may decrease the risk of prostate

cancer in patients with type 2 diabetes. The drug appears to be beneficial in delaying the development of castration resistance and improving overall survival time in patients with prostate cancer (2). However, there is still no definitive evidence. A meta-analysis conducted by Chen *et al* (3) demonstrated that there is no clear association between metformin and prostate cancer incidence in type 2 diabetes populations. Metformin may have potential protective effects on prostate cancer incidence in an Asian population with type 2 diabetes; however, no statistically significant difference and similar protective effects were found in a Western population with type 2 diabetes (3). Meanwhile, a clinical study confirmed that metformin improved the overall survival outcomes of patients with prostate cancer who were also diagnosed with type 2 diabetes (4). It should be noted that the retrospective studies introduced several interference factors. *In vitro* cell experiments revealed that metformin could inhibit the proliferation of prostate cancer cells, but this has not been demonstrated in any animal models. Ge *et al* (5) indicated that metformin inhibited the malignant biological behaviors of prostate cancer cells through alternative pathways between N-cadherin-expressing cells and N-cadherin-deficient cells. Yang and Wu (6) argued that the effect of metformin on the biological behavior of CRPC PC-3 cells may be activated by inhibiting the phospholipase C $\epsilon$  (PLC $\epsilon$ ) gene-mediated neurogenic locus notch homolog protein 1 (Notch1)/hair cell enhancer of split (Hes) and androgen receptor (AR) signaling pathways.

In the current study, a mouse model of patient-derived xenograft (PDX)-CRPC tumors was established to investigate the effects of metformin on CRPC. Furthermore, the mechanism related to PLC $\epsilon$  gene expression and the Notch1/Hes and AR signaling pathways associated with the exogenous intervention of metformin or corresponding activators was elucidated upon.

## Materials and methods

### PDX-CRPC mouse model

**Mice.** Male mice with severe combined immunodeficiency NOD-NPG (age, 8 weeks; weight, 20 $\pm$ 2 g; n=29) were purchased from Beijing Viton Lihua Experimental Animal Co., Ltd. (production license number: SCXK

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**Abbreviations:** PDX, patient-derived xenograft; CRPC, castration-resistant prostate cancer; PLC $\epsilon$ , phospholipase C $\epsilon$ ; AR, androgen receptor; PSA, prostate-specific antigen; RT-qPCR, reverse transcription-quantitative PCR; ADT, androgen deprivation therapy

**Key words:** metformin, CRPC, patient-derived xenotransplantation, PLC $\epsilon$ , Notch1/Hes signaling pathway, androgen receptor signaling pathway

(Zhejiang) 2018-0001). The mice were housed in an aseptic environment with an ambient temperature of 22-26°C and a relative humidity of 40-60%. A 12/12 h light/dark cycle was maintained, and adequate food and drinking water were provided.

**Human CRPC case.** A single case of CRPC in a male patient (58 years old), treated in Changshu Hospital (Suzhou, China) between January and April 2020 was selected for establishing a PDX-CRPC mouse model. Selection criteria: The serum testosterone of the patient was at castration level (42.5 ng/dl), and the prostate-specific antigen (PSA) level was 62% higher than the highest value recommended (4.0 ng/ml). According to the Eighth Edition of Tumor-Node-Metastasis staging issued by American Joint Committee on Cancer (7), the CRPC was a progressive tumor (T4), as confirmed by prostate computed tomography and a failure to respond to anti-androgen withdrawal treatment (flutamide and bicalutamide dose was decreased to 0 for 4 weeks, and treatment with goserelin was 3.6 mg/28 days). Written informed consent was obtained from the patient.

**Model creation.** Fresh CRPC tumor tissue was collected from the patient via surgical resection and divided into two parts: One part was used for the paraffin sectioning and histological analysis, and the other part was cut into 1- to 3-mm<sup>3</sup> tumor fragments and inserted into the renal capsule of the NPG mice using a custom cannula needle. The stable animal model was established by a continuous passage three times. All surgical procedures on the mice were performed under anesthesia, which was induced by 5% isoflurane and maintained by isoflurane (1-1.5%; 21-23% O<sub>2</sub>, with balanced N<sub>2</sub>). All efforts were made to minimize animal suffering. To ensure the health status of the animals, their weight and water consumption were recorded twice a week. After exposing the kidney for 10 sec, a slight natural fold of the renal capsule was observed. Next, the renal capsule was immediately separated from the kidney. A pouch was opened using the pointed glass tube, and 6 pieces of tumor tissue were inserted using the tweezers. Later the kidney was pushed back into the body cavity, and the wound was sewn up with a 6-0 suture line, layer by layer. Mice were afterwards subjected to a 0.5-cm long longitudinal incision on the back and a Testosterone Undecanoate Soft Capsule (40 mg/capsule; NV Organon) was embedded subcutaneously. Near-infrared fluorescent dye (ProSense 680) was injected via the caudal vein to confirm the growth of the transplanted tumor by using the live animal imaging system (USA Caliper Lumina II; Vieworks, Co., Ltd.). Once the recurring tumor exceeded 800 mm<sup>3</sup>, the mice (n=9) were euthanized by CO<sub>2</sub> asphyxiation (CO<sub>2</sub> flow rate, 30-70% of the chamber volume per minute). Death was verified by monitoring for cardiac cessation and respiratory arrest. All the experimental procedures were approved by the Experimental Ethical Committee of Changshu Hospital Affiliated to Nanjing University of Chinese Medicine (Suzhou, China).

**Histological analysis.** Part of the tumor was fixed in 4% formaldehyde for histological analysis to determine the tumor homology, compared with the patient section, and confirm the model. All tissues were fixed with 4% paraformaldehyde at 4°C for 24 h immediately after surgical resection and then embedded in paraffin. The tissue sections (4 μm) were heated at 60°C for 1 h then dewaxed and rehydrated by immersion in

dimethylbenzene and an ethanol series, and hematoxylin and eosin (H&E) staining was performed at room temperature for 1-10 min. Stained sections were observed and images were captured using an optical microscope (Olympus).

**Grouping method.** Tumors under the renal capsule PDXs were surgically removed after tumor growth for 1 month, and the recurrence of tumor after several months was designated as the occurrence of CRPC. Based on the experimental requirements, to analyze the effects of Metformin, 20 CRPC mice (when the tumor exceeded 800 mm<sup>3</sup>) were grouped, with 5 mice in each group, as follows: High concentration group, 270 mg/kg/day; medium concentration group, 90 mg/kg/day; and low concentration group, 30 mg/kg/day (oral administration). The control group was treated with 0.9% physiological saline. The expression of PSA, PLCε, Notch1/Hes and AR proteins was detected in the tumor tissues of each group, and the tumor size was recorded; the maximum tumor sizes observed in the study was 2,318 mm<sup>3</sup>. Metformin, PLC activator phorbol 12-myristate 13-acetate (PMA) and Notch activator Jagged1 were purchased from MilliporeSigma. An intraperitoneal injection of PMA (200 μg/kg) was administered to form the Metformin (90 mg/kg) + PMA group, while an intraperitoneal injection of Jagged1 (500 μg/kg) was administered to form the Metformin + Jagged1 group. The control group was treated with 0.9% physiological saline.

**Immunohistochemistry (IHC).** Paraffin-embedded sections were dewaxed and rehydrated in a graded alcohol series. Antigen retrieval was performed by boiling at 95°C for 10 min in EDTA. Endogenous peroxidase quenching was performed using 3% hydrogen peroxide at room temperature for 30 min, and samples were blocked in 20% goat serum for 40 min at room temperature. Samples were incubated with anti-PSA primary antibody (1:100; catalog no. P07288; Beijing Solarbio Science & Technology Co., Ltd.) at 4°C overnight, and incubation with HRP-conjugated sheep anti-rabbit secondary antibody (1:100; catalog no. SPA134; Beijing Solarbio Science & Technology Co., Ltd.) was performed at room temperature for 60 min. Sections were visualized using 2,2'-diaminobenzidine (MilliporeSigma) and counterstained with hematoxylin. Mouse IgG was used as the primary antibody for the negative control. The results were observed by fluorescent inverted/phase contrast microscope

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was converted to cDNA using a GoScript<sup>™</sup> Reverse Transcription System kit (Promega Corporation) according to the manufacturer's instructions. qPCR was performed using the SYBR Green Step One Plus Real-Time PCR system (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions. Complementary DNA was generated by reverse transcription, also using the Step One Plus Real-Time PCR system kit. PCR amplification conditions were set at 95°C for 10 min, followed by 30 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec. After the final cycle, the reaction was terminated. Results were quantified by the  $\Delta\Delta C_q$  method (8) and measured three times to take the average. Primers used in this study are as follows: PLCε forward,

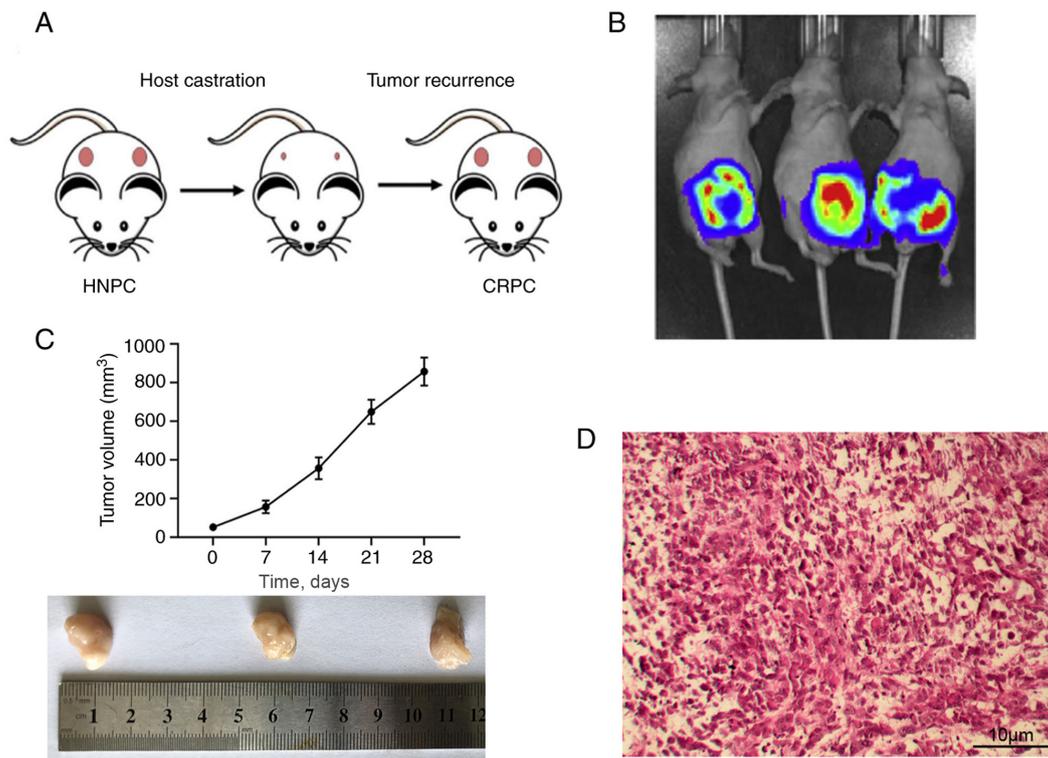


Figure 1. Construction of a model of CRPC xenografts. (A) Overview of the formation of the human to mouse xenograft CRPC model. (B) *In vivo* imaging of the tumor stained with near-infrared fluorescent dye (ProSense 680). (C) Changes of recorded tumor volume. (D) Hematoxylin and eosin staining of histological slide of tumor tissue on the 28th day after tumor recurrence. HNPC, Human prostate cancer; CRPC, castration-resistant prostate cancer.

5'-GGTTTCATCCAGGATCGAGC AGG-3' and reverse, 5'-ACAAAGATGGTCACGGTCTGCC-3'; Hes forward, 5'-ACG ACACCGGAAAACAAA-3' and reverse, 5'-CGGAGGTGCA CTGTCAT-3'; Notch forward, 5'-CATCATCAATGGCTG CAAGGG-3' and reverse, 5'-TCATTCTCACACGTGGCACC-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-CCTGCTTACCACCTTCTTGA-3'.

**Western blotting.** RIPA buffer (MilliporeSigma) was used for tumor tissue cell lysis, and the extraction and purification of intracellular protein.  $\beta$ -actin was used as the internal reference gene. BCA reagent (Thermo Fisher Scientific, Inc.) was used to quantify the protein concentration. A total of 30  $\mu$ g protein were separated on an 8% gel using SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked in 5% skimmed milk in 1X TBST at room temperature for 2 h and then incubated with rat anti-mouse Notch1, Hes, AR and  $\beta$ -actin primary antibodies (catalog nos. ab27526, ab71559; ab244058 and ab8226, respectively; dilution, 1:2,000; Abcam) overnight at 4°C. After washing with 1X TBST three times, corresponding secondary antibody, HRP-conjugated anti-mouse IgG or Alexa Fluor 488-conjugated goat anti-rabbit IgG (catalog nos. ab131368 and ab150081, respectively; dilution, 1:500; Abcam) was added to the membrane and incubated at room temperature for 4 h. After washing again with TBST, SuperSignal<sup>®</sup> ECL kit (Pierce; Thermo Fisher Scientific, Inc.) was applied to develop the chemical signal. The Lab Works 4.5 gel imaging software (Invitrogen; Thermo Fisher Scientific, Inc.) was used to perform semi-quantitative analysis.

**Nuclear separation.** The tumor tissues were sonicated (4°C; 20-25 kHz; 1 min), and then lysed on ice with a cytoplasmic lysis buffer (Enzo) for 15 min. Supernatant was collected after centrifugation (12,000 x g, 10 min, 4°C) to obtain the cytoplasmic protein. Precipitate was re-suspended using a cytoplasmic lysis buffer without a protease inhibitor and washed 3 times with cytoplasmic lysis buffer, and then cleaved with a nuclear lysis buffer (Pierce Biotechnology, Inc.). The cleavage process was performed on a vortex oscillator at 4°C for 30 min. Finally, the supernatant was collected as the nuclear protein fraction after centrifugation (12,000 x g, 10 min, 4°C) for western blot analysis as aforementioned. Histone was used as a nuclear protein internal reference.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SD. Statistical analysis was performed using SPSS 20.0 statistical software (IBM Corp.). One-way ANOVA followed by Tukey's post hoc test, and unpaired Student's t-tests, were used to compare multiple groups or two groups, respectively.  $P < 0.05$  was used to indicate a statistically significant difference.

## Results

**PDX-CRPC mouse model.** As depicted in Fig. 1A, surgical resection of the PDX tumor (pink) under the renal capsule of the mice delayed the tumor recurrence for a few months, which was characterized as CRPC. The growth of the tumor was monitored via real time imaging system *in vivo* (Fig. 1B). The tumor size measurements are presented in Fig. 1C. Mice were euthanized after the tumor size exceeded 800 mm<sup>3</sup>.

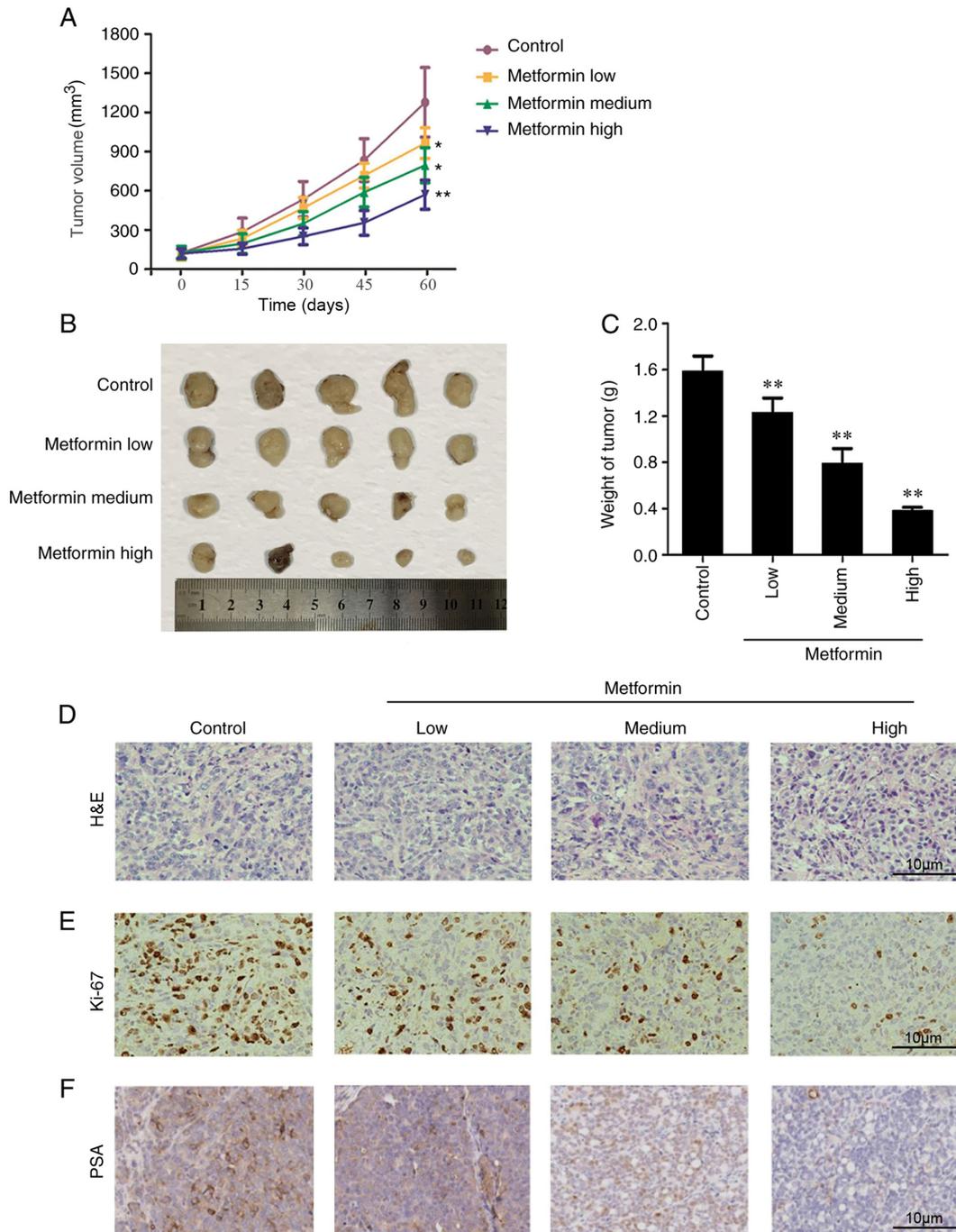


Figure 2. Effects of various doses of metformin (270, 90 and 30 mg/kg/day) on CRPC growth. (A) Recorded tumor volume in the control group and different metformin-treated groups. (B) Images of tumors in the control group and different metformin-treated groups. (C) Recorded tumor weight in the control group and different metformin-treated groups. Data are represented as the mean  $\pm$  SD of three individual experiments. (D) Tumor tissue samples in the control or metformin-treated groups stained with H&E. (E) Expression of Ki-67 in the control or metformin-treated tumor tissues. (F) Expression of PSA in the control or metformin-treated tumor tissues. Magnification,  $\times 400$ . Scale bar, 10  $\mu\text{m}$ . \* $P < 0.05$  and \*\* $P < 0.01$  vs. control. CRPC, castration-resistant prostate cancer; H&E, hematoxylin and eosin; PSA, prostate-specific antigen; vol., volume.

Histological analysis of the tumors indicated that the CRPC model was successfully established, as indicated by deeper HE staining, and stacked or inlaid cell clusters (Fig. 1D).

*Effects of metformin on CRPC growth.* Tumor sizes and weights were further observed in mice after 2 months of daily treatment with different doses of metformin (low, medium and high, and control) (8,9). As shown in Fig. 2, the results showed significant differences among tumor weights

and sizes in a dose-dependent manner, with higher doses associated with lower tumor weight and size, indicating that metformin significantly inhibited tumor growth. Histological analysis (Fig. 2D) showed that metformin improved cell and nuclear morphology (H&E), inhibited tumor cell proliferation (Ki-67) and downregulated the proportion of PSA-positive cells. Overall these results indicated that an increase in metformin dose significantly inhibited prostate cancer proliferation.

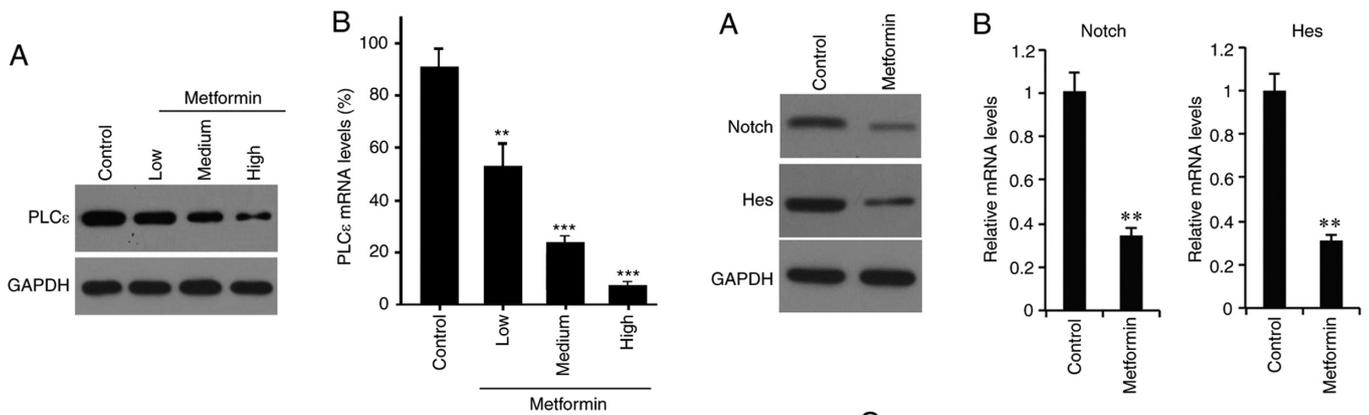


Figure 3. Effects of various doses of metformin on PLCε expression. (A) Expression of PLCε in the tumor tissues treated with different metformin doses, according to western blotting analysis. (B) The mRNA expression of PLCε in tumor tissues of the different metformin-treated groups. \*\*P<0.01 and \*\*\*P<0.001 vs. control. PLCε, phospholipase Cε.

*Effect of metformin on PLCε expression.* Western blotting and RT-qPCR analyses were next used to determine PLCε expression levels. The results indicated that the expression of PLCε was downregulated at the protein and mRNA levels by metformin in a dose-dependent manner (Fig. 3).

*Effects of metformin on expression of Notch, Hes and AR proteins.* It has been well documented that PLCε could affect the development of tumors by regulating the Notch signaling pathway and AR nucleation (6). In the present study, western blotting and RT-qPCR were used to analyze the expression levels of Notch and Hes at the protein and mRNA levels. As shown in Fig. 4A, Notch and Hes expression was downregulated at the protein and mRNA levels in the metformin (medium concentration)-treated group. Moreover, after nuclear separation, the nuclear AR expression was also significantly decreased in the metformin-treated group, indicating that metformin inhibited the nuclear relocation of AR (Fig. 4B).

*Effects of metformin alone and in combination with PMA/Jagged1 on tumor growth.* The effects of PMA/Jagged1 in combination with metformin on tumor growth were further investigated. Each group was monitored continuously for 45 days using a live animal imaging system. The results showed that tumor growth was significantly decreased in the mice treated with a combination of metformin and PLC activator PMA or Notch activator Jagged1, compared with that in the mice treated with metformin only (Fig. 5). This result could be attributed to PMA being able to increase PLC and Notch expression levels, and Jagged1 being able to increase Notch expression levels (Fig. 5A). Moreover, these two agents promoted AR expression (Fig. 5B).

## Discussion

The multiple roles and mode of action of metformin in the treatment of various diseases are yet to be elucidated (9). Several studies suggested that metformin could affect the physiological function of prostate cancer cells by regulating the activity of adenosine 5'-monophosphate-activated protein

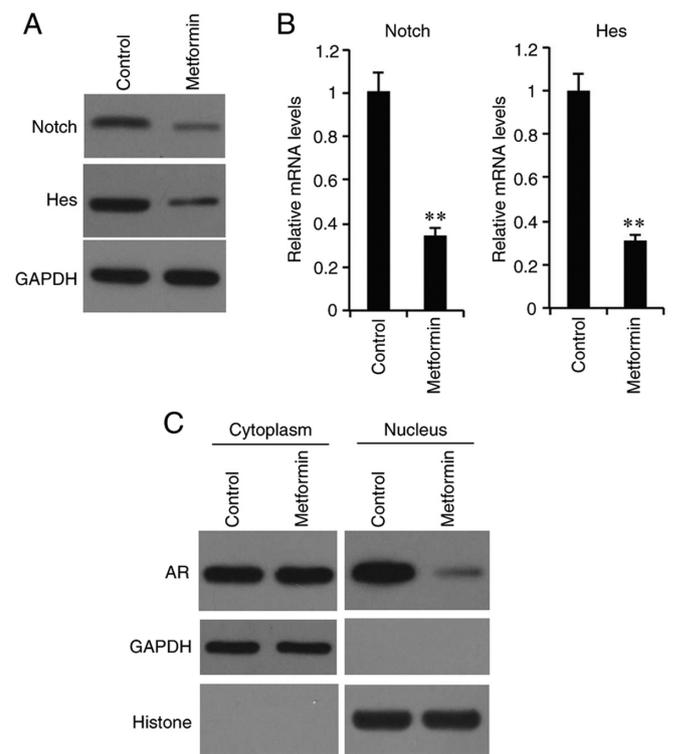


Figure 4. Effects of metformin on the expression of Notch, Hes and AR proteins. (A) Expression of Notch and Hes in the control or metformin-treated tumor tissues according to western blotting analysis. (B) The mRNA expression of Notch and Hes in the control or metformin-treated tumor tissues. \*\*P<0.01 vs. control. (C) Expression of AR in control or metformin-treated tumor tissues according to western blotting analysis. Notch, neurogenic locus notch homolog protein; Hes, hairy and enhancer of split; AR, androgen receptor.

kinase (10-12). It has been documented that metformin could directly regulate the AR, which is closely related to the occurrence and development of prostate cancer, and that it regulates the PI3K/Akt and MAPK signaling pathways, which are the downstream targets in several signaling pathways of insulin receptors. Thus, metformin may serve an important role in regulating insulin resistance and affect the function of prostate cancer cells (13). It was previously suggested that metformin significantly inhibited cell proliferation, invasion and apoptosis in the hormone-resistant prostate cancer PC3 cell line in time- and dose-dependent manners (14). Moreover, metformin targeted and inhibited PLCε gene expression and decrease the molecular expression of the AR and Notch1/Hes signaling pathways, which were closely associated with prostate cancer cell proliferation and invasion (6).

CRPC is recognized as the terminal stage of prostate cancer; it is characterized by distant metastasis and resistance to anti-androgen therapy, radiotherapy and chemotherapy. In a previous study, metformin reversed epithelial-mesenchymal transition in prostate cancer tissue to some extent and enhanced tumor cell sensitivity to neoadjuvant radiotherapy and chemotherapy (15).

There are two main prostate cancer cell lines for models of transplantation: PC3 and LNCaP. However, the probability of losing the three-dimensional structure of the tumor in long-term laboratory culture is high, which means that the heterogeneity of clinical prostate cancer would not

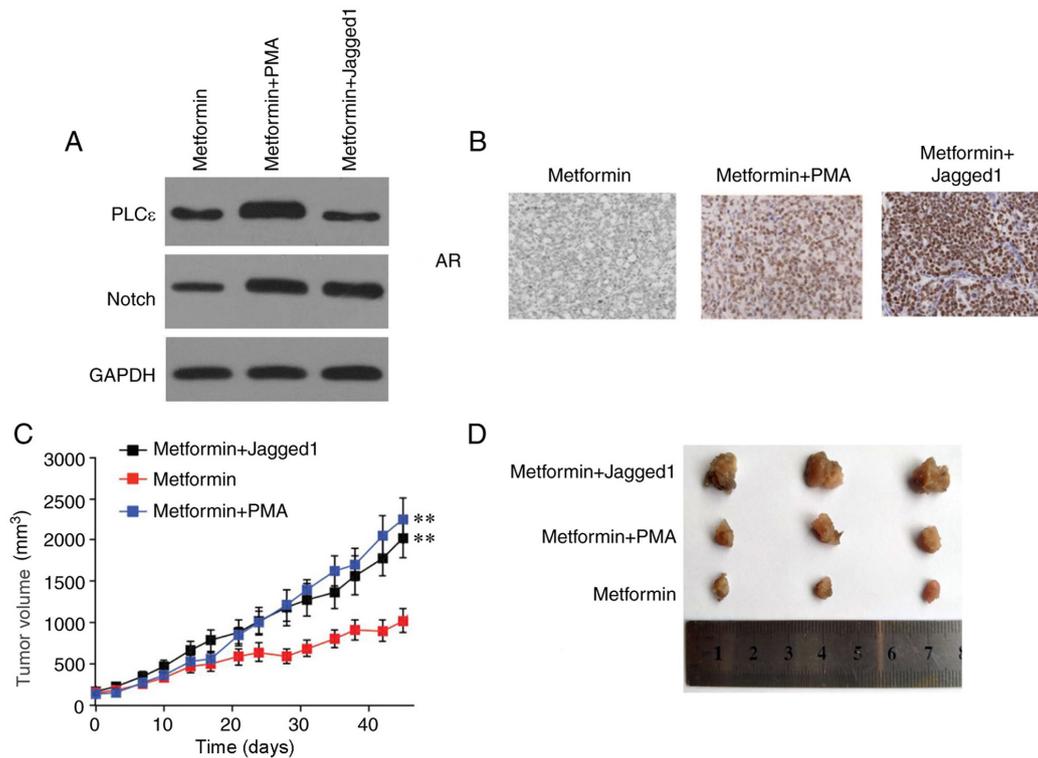


Figure 5. Effects of metformin only or a combination of metformin and PMA/Jagged1 on tumor growth. (A) Expression of PLC $\epsilon$  and Notch in the metformin only group or the combination groups according to western blotting analysis. (B) Expression of AR protein in the metformin only group or the combination groups (x200 magnification). (C) Changes in recorded tumor volume in the metformin only group or the combination groups. \* $P < 0.01$  vs. metformin. (D) Images of tumors in the metformin only group or the combination groups. PLC $\epsilon$ , phospholipase C $\epsilon$ ; Notch, neurogenic locus notch homolog protein; PMA, phorbol 12-myristate 13-acetate; vol., volume.

be captured, and ultimately, the important characteristics and diversity of prostate cancer would not be accurately summarized (16). The PDX model not only maintained the interaction between the micro-environmental components inside the tumor, but also accurately simulated the major characteristics of prostate cancer in patients, such as its hormone-dependent or non-dependent nature, and also induce the occurrence of CRPC through androgen ablation in mice (17). The present current study successfully established a mouse model of a PDX-CRPC transplanted tumor. Moreover, the PDX model of prostate cancer was improved by intermittent androgen supplementation and the selection of intrarenal transplantation sites. We consider that the PDX-CRPC transplanted tumor model has a high success rate, good stability and maintains the important characteristics of human prostate cancer, which guarantees the ability to use metformin treatment and information for the subsequent discussions on the molecular mechanisms involved. However, further investigations are required to determine how different metformin concentrations affect the human body. After the intervention with metformin for 2 months, the diameter of the tumors in the low and high concentration groups were significantly smaller than that in the control group, and the tumors in the high concentration group were smaller than those in the low concentration group ( $P < 0.05$ ). Meanwhile, mice in the control group developed multiple tumor metastases. This suggested that metformin intervention could inhibit *in vivo* growth and metastasis of prostate cancer in a dose-dependent manner.

PSA is a specific molecular target of prostate cancer tissue, and its positive expression is often associated with tumor malignancy, castration resistance and a poor clinical prognosis (18). IHC staining showed that expression of PSA was significantly higher in tumor tissues from the control group, compared with that in tumor tissues from the metformin-treated groups ( $P < 0.05$ ). Similarly, the tumors from mice treated with a high concentration of metformin exhibited lower expression levels of PSA compared with the tumors from mice treated with a low concentration of metformin. The results also indicated that the PDX-CRPC mouse model could express PSA, which further confirmed the similarity between the initial tumor of human origin and the PDX-CRPC tumor of the mouse. A previous study found that both the PC3 and LNCaP models lose their expression during passage to passage culture, which means that they do not fully reflect the important characteristics of human prostate cancer (19). In the present study, the expression level of PLC $\epsilon$  mRNA in the control group was significantly higher than that in the metformin-treated groups ( $P < 0.05$ ), and the group with a high metformin concentration exhibited a low level of PLC $\epsilon$  mRNA. This result suggested that metformin may regulate prostate cancer cell proliferation and invasion by inhibiting the expression of the PLC $\epsilon$  gene. Additionally, previous studies reported that the Notch1/Hes and AR cell pathways played an important role in promoting the early onset, castration resistance and distant metastasis of prostate cancer (20,21). The present results showed that the expression levels of Notch1, Hes and AR proteins were lower in tumor tissues from the metformin-treated groups compared with that in tumor tissues

from the control group ( $P < 0.05$ ). Moreover, the group treated with a higher dose of metformin exhibited a lower expression level of these proteins, suggesting that metformin may activate the Notch1/Hes and AR cell pathways to affect prostate cancer cell proliferation and invasion. Based on the aforementioned results and those of previous studies, further studies are required to investigate the potential role of the Notch1/Hes and AR cell pathways in prostate cancer.

Whitburn *et al* (22) indicated that the application of metformin in prostate cancer patients treated with androgen deprivation therapy (ADT) inhibited cancer cell proliferation and improve metabolic syndrome. However, as the number of cases in this study is limited, the randomized trial data provided is still insufficient. Similarly, Hankinson *et al* (15) reported that metformin treatment in patients with type 2 diabetes reversed metabolic conditions by decreasing the androgen levels, thereby leading to high levels of androgen stimulating prostate growth, proliferation and tumorigenesis. Although the antitumor properties of metformin are not obvious in the early stages of prostate cancer development, the drug could be effective in decreasing the mortality of patients with prostate cancer by significantly improving the parameters of metabolic syndrome after ADT, and it could exhibit a therapeutic effect on some patients with asymptomatic or mild metastatic CRPC (23,24). There is potential for metformin to be used as a monotherapy or an adjuvant agent in ADT, or in external therapy or other chemotherapies.

In conclusion, the present study reported that the PDX-CRPC mouse model of transplanted tumors enables investigation of the mechanism of prostate cancer occurrence and drug intervention. Moreover, metformin may have the potential to inhibit CRPC progression by activating the Notch1/Hes and AR signaling pathways, which could inhibit PLC $\epsilon$  gene expression.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

JP and QL conceived and designed the study. KX, JT and ZL analyzed and interpreted the data. JP, QL and KX drafted the manuscript. JT and ZL revised the manuscript. JP, QL and KX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

The present study was ethically approved by the Institutional Review Board of Changshu Hospital Affiliated to Nanjing

University of Chinese Medicine (Suzhou, China). Written informed consent was obtained from the patient prior to the start of the study.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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