

# Overexpression of PER3 reverses paclitaxel resistance of prostate cancer cells by inhibiting the Notch pathway

D.-W. CAI<sup>1</sup>, D. CHEN<sup>1</sup>, S.-P. SUN<sup>1</sup>, Z.-J. LIU<sup>2</sup>, F. LIU<sup>1</sup>, S.-Z. XIAN<sup>1</sup>, P.-S. WU<sup>1</sup>, G.-Q. KONG<sup>1</sup>

<sup>1</sup>Department of Urology, Beijing Luhe Hospital, Capital Medical University, Beijing, China

<sup>2</sup>Central Laboratory, Beijing Luhe Hospital, Capital Medical University, Beijing, China

**Abstract.** – **OBJECTIVE:** To investigate the levels of period circadian protein homolog 3 (PER3) in paclitaxel-resistant prostate cancer patients and the effect of PER3 on paclitaxel-resistant prostate cancer cell lines.

**PATIENTS AND METHODS:** A total of 38 patients diagnosed with prostate cancer in our hospital from June 2013 to June 2016 were divided into paclitaxel-resistant group (n=19) and non-resistant group (n=19) according to the follow-up treatment effects. Fluorescent quantitative polymerase chain reaction (PCR) was performed to evaluate the levels of PER3 in drug-resistant and non-resistant groups as well as the relative levels of PER3 before and after treatment. PER3 was overexpressed or knocked down in a paclitaxel-resistant prostate cancer cell line, followed by measuring its IC50 as well as changes in cell cycle and apoptosis. Using Western blot, we detected down-regulation of Notch pathway and related receptor proteins when PER3 was overexpressed.

**RESULTS:** The results of fluorescence quantitative PCR showed that the expression of PER3 in the paclitaxel-resistant prostate cancer group was lower than that in the non-resistant group, and the relative expression of PER3 was decreased after treatment. Fluorescent quantitative PCR and Western blot showed that the expression of PER3 in paclitaxel-resistant prostate cancer cells was higher than that of the untreated counterparts. After overexpression of PER3 by transfecting prostate cancer-resistant cell lines with plasmids, the IC50 was significantly reduced, the cell cycle was arrested, and the apoptosis was significantly increased. Subsequently, we detected decreased expression of Notch1 in PER3 over-expressed paclitaxel-resistant cell lines by Western blot; this attenuated resistance in paclitaxel-resistant cell lines.

**CONCLUSIONS:** PER3 can induce sensitivity of paclitaxel-resistant cell lines to paclitaxel by inhibiting the expression of Notch1.

*Key Words:*

Prostate cancer, Paclitaxel, PER3, Notch pathway.

## Introduction

Prostate cancer is a common form of cancer in male. Its incidence in China ranks sixth in the whole male malignant tumors, which is approximately 9.92 per 100,000 people<sup>1,2</sup>. At present, docetaxel-based chemotherapy is the first-line treatment for hormone-refractory prostate cancer HRPC and can appropriately improve the survival rate of patients. However, chemotherapy drug resistance is still a main obstacle in the treatment of prostate cancer<sup>3</sup>. Drug resistance in tumor cells not only delays the treatment cycle, but also increases the toxicity and side effects of chemotherapeutic drugs, which has become one of the major causes of chemotherapy failure<sup>4</sup>. Endocrine therapy is the first choice treatment for PCa, but most patients relapse after one year of treatment and evolve into hormone refractory prostate cancer (HRPC). This is often accompanied by highly malignant bone metastases. At present, docetaxel-based chemotherapy is the first-line treatment for HRPC<sup>5</sup>, which can appropriately improve the survival rate of patients. However, the toxicity, side effects, and multidrug resistance after chemotherapy also severely constrain their therapeutic effects<sup>6</sup>. As a broad-spectrum antitumor drug, docetaxel has been studied for its drug resistance mechanisms. For example, mutation of the target site will lead to a decrease of the concentration of docetaxel in the tumor cells. The high expression of anti-apoptotic proteins, such as Bcl2, XIAP, survivin, and clusterin, inhibit drug-induced apoptosis<sup>7</sup>. In addition, inflammatory factors such as IL6 and tumor necrosis factor (TNF- $\alpha$ ), are also involved in cell multidrug resistance, invasion and metastasis. Notwithstanding, prostate cancer multidrug resistant cells have their own special features, such as elevated P-gP and interleukin 6 (IL6) expression<sup>8,9</sup>. At the same time, single-targeted drugs such as blocking Bcl2 with antisense

nucleic acids or blocking IL6 with antibodies have not achieved ideal therapeutic efficacy<sup>10</sup>. Therefore, the search for key molecules related to drug resistance phenotype will be of great significance for elucidating the drug-resistant mechanism and therapeutic targets of prostate cancer.

The mechanism of drug resistance in tumor cells is closely related to escape from apoptosis and overexpression of multidrug resistance transporters. The period (Per1, Per2, Per3) genes are mostly associated with the circadian rhythm. Circadian rhythms affect many physiological and pathological processes including tumors<sup>11</sup>. Biological clock per gene expression changes can affect the cell's differential damage response to radiation<sup>12</sup>. Studies have shown that the overexpression of the circadian clock genes, Per1 and Per2, can increase the sensitivity of apoptosis induced by DNA damage in human tumor cells<sup>13</sup>. However, there are few studies on the relationship between PER3 and tumor drug resistance. The aim of this study was to explore the effect of PER3 on paclitaxel-resistant prostate cancer and its role in prostate cancer sensitivity to paclitaxel.

## Patients and Methods

### Tumor Samples

The prostatic carcinoma specimens were collected from 38 patients in our hospital from June 2013 to June 2016. The prostate tissues were divided into paclitaxel resistant group (n=19) and non-resistant group (n=19). The collection was approved by the Medical Ethics Committee of our hospital and the patients signed the informed consent. The median age was 56.8 (42-69).

### Cell Culture

The prostate cancer cell line, PCa was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C in a humidified incubator at 5% CO<sub>2</sub>. PCa was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell was passed on when 80% of cell fusion was reached with a seeding density of 1:2.

### siRNA Transfection and pcDNA Transfection

The cells were seeded in 6-well plates and transfected with lipofectamine 2000 (pcDNA with Entranster-R4000) at a confluence of

approximately 60%. They were treated with 500 μL of serum-free suspension containing 10 μL of lipofectamine 2000 and 10 μL of 20 nM siRNA-Notch1 (pcDNA with 4 μL of Entranster-R4000 and 2.68 μg of pcDNA-PER3). 1.5 mL of 1640 medium (Gibco, Rockville, MD, USA) were then added to the cells. The control group was treated with lipofectamine 2000 and siRNA-control (pcDNA with Entranster-R4000 and pcDNA-control) in equal amounts to the experimental group. The medium was changed 6 h after transfection. Si-Notch1 sequence 5'-CUGUCA AUGGCAAGCCA UdTdT-3', 5'-dTdTGACAGUUACCGU UCCGGUA-3'

### qRT-PCR (Quantitative Reverse Transcriptase-Polymerase Chain Reaction)

Total RNA was extracted according to the instructions of TRIzol (Invitrogen, Carlsbad, CA, USA), and 50 μL reaction system were prepared according to qRT-PCR instruction. Reverse transcription reaction was performed using the following conditions: 50°C reverse transcription reaction for 30 min and 92°C reaction for 3 min to denature reverse transcriptase. The resulting complementary deoxyribose nucleic acid (cDNA) was subjected to amplification under the following conditions: denaturation at 92°C for 10 s, annealing at 55°C for 20 s, extension at 68°C for 20 s, and amplification for 40 cycles. Using β-actin gene as an internal reference, the 2<sup>-ΔΔCt</sup> method was used to calculate the relative expression of CRNDE. The primer sequences are as follows.

β-actin, Forward 5'-CTCCATCCTGGCCTCGCT-GT-3', Reverse 5'-GCTGTCACCTTCACCGT-TCC-3'. PER3, Forward 5'-GCAGGTCTATGCCAGTGTGA-3', Reverse 5'-TGCCTTGTGGTTCTGTTTGT-3', Notch1, Forward: 5'-CCTTTGTGCTTCTGTTCTTCG-3', Reverse: 5'-CCACTCATTCTGGTTGTCGTC-3'.

### Cell Counting kit-8 (CCK8) Assay for Cell Proliferation

The transfection time point was 0 h. Cells of the control group and the treatment group were inoculated into a 96-well plate. Each well had 6 replicates, 5×10<sup>3</sup> cells were placed in each well, and five 96-well plates were repeatedly inoculated. After 6 hours, the activity of the adherent cells was measured (0 h). At 24 h, 48 h, 72 h, and 96 h, 20 μL of CCK8 (Dojindo, Kumamoto, Japan) solution were added to each well and placed in a 37°C, 5% CO<sub>2</sub> incubator. At 2-3 h,

the absorbance (OD value) was measured using a microplate reader at a wavelength of 450 nm. Wells containing only CCK8 solution and RPMI-1640 medium were served as the blank controls.

### **Western Blotting Assay**

Cells mentioned above were lysed with lysis buffer with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) to harvest total cellular protein. Standard Western blot experiments were performed as previously described. The concentration of extracted protein was quantified by bicinchoninic acid (BCA) protein assay kit. An equal amount of protein sample was loaded onto a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After the membrane was blocked with skimmed milk, it was incubated with primary antibody overnight at 4°C and incubated with secondary antibody for 2-3 hours on the next day. At last, images of the protein bands were captured by Tanon detection system (Shanghai, China).

### **Flow Cytometry**

After the cell supernatant was collected in a pre-labeled flow tube, the cells were digested with trypsin without Ethylene Diamine Tetraacetic Acid (EDTA). The cells were then suspended, pipetted into a corresponding flow tube and centrifuged twice with phosphate-buffered saline (PBS). After centrifugation, 200 µL of calcium-containing binding buffer were added to the pellet, and then the target antibody was incubated in the dark. Dual-channel wavelength detection was performed on the flow cytometer (Partec AG, Arlesheim, Switzerland).

### **Statistical Analysis**

Statistical product and service solutions (SPSS 22.0, Armonk, NY, USA) statistical software were used for data analysis, and Graph-Pad Prism 6.0 (La Jolla, CA, USA) was used for image editing. Measured data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference).  $\chi^2$ -test was used as the classification data.  $p < 0.05$  indicated that the difference was considered statistically significant. \* $p < 0.05$ , \*\* $p < 0.01$ .

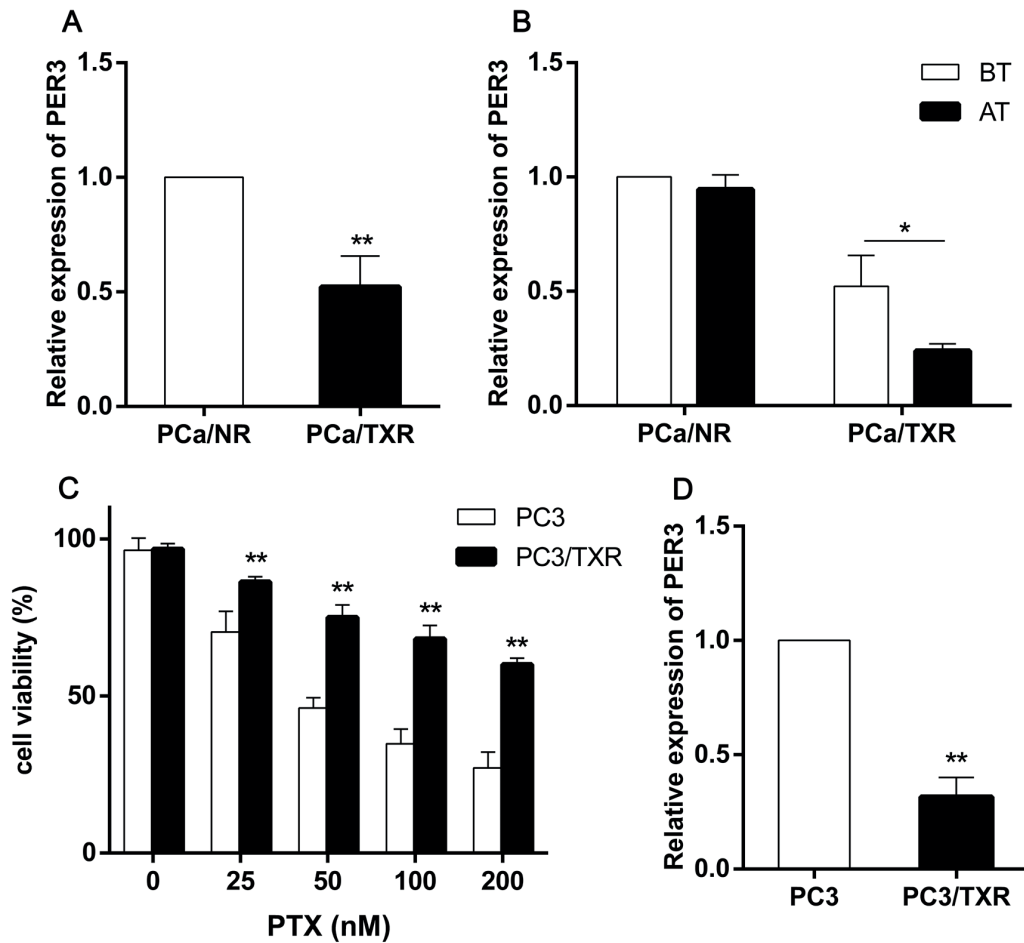
## **Results**

### ***PER3 is Down-Regulated in Paclitaxel-Resistant tissues and Cells of Prostate Cancer***

We selected prostate tissues of patients diagnosed with prostate cancer at our hospital from June 2013 to June 2016 and classified them into paclitaxel-resistance group (n=19) and non-resistance group (n=19) in accordance to the therapeutic effect of one year's follow-up. Due to the small size of the biopsy sample, we only performed the detection of PER3 expression at the mRNA level. Fluorescent quantitative PCR results showed that the expression of PER3 in the prostate cancer-resistant paclitaxel-resistant group was decreased compared with the non-resistant group (Figure 1A). After treatment, the relative expression of PER3 was significantly decreased compared to that before treatment, but not in the non-resistance group (Figure 1B). Subsequently, paclitaxel was added to the cultured prostate cancer cell line PCa to screen for drug-resistant strains. CCK-8 results showed that the selected paclitaxel-resistant cell line (PCa/PTX) was resistant to paclitaxel at different paclitaxel concentrations. Both are significantly higher than the parental cells (Figure 1C). At the same time, the result of Western blot demonstrated that the level of PER3 in paclitaxel-resistant strains was especially lower than that of the parental cells (Figure 1D). Overall, PER3 expression was downregulated in prostate cancer-resistant paclitaxel-resistant tissues and cells.

### ***Upregulation of PER3 in Prostate Cancer-Resistant Paclitaxel-Resistant Cell Lines Sensitizes the Prostate cancer Cell Lines to Paclitaxel***

To investigate the influence of PER3 on the sensitivity of prostate tumor cells to paclitaxel, a PER3 overexpression plasmid was transfected into paclitaxel-resistant cell lines. Quantitative PCR results showed that after transfection of pc-PER3, prostate cancer cells were resistant to paclitaxel. The expression of PER3 in the drug-resistant cell line was significantly increased (Figure 2A). Next, we measured the IC<sub>50</sub> of PCa/PTX cell lines that overexpressed or did not overexpress PER3. The results showed that the IC<sub>50</sub> of paclitaxel-resistant cell lines after overexpression of PER3 was lower than that of the control group (Figure 2B), indicating that prostate cancer up-regulated PER3 expression in paclitaxel-resistant



**Figure 1.** Expression of PER3 in paclitaxel-resistant tissues and cells of prostate cancer. *A*, The expression of PER3 in the paclitaxel-resistant prostate cancer group was significantly lower than that in the non-resistance group. *B*, The relative expression of PER3 in the prostate cancer-resistant paclitaxel-resistant prostate cancer group decreased after treatment. *C*, Cellular viabilities detected by CCK-8 assay after treatment of paclitaxel-resistant prostate cancer cells and untreated cell lines treated with different concentrations of paclitaxel. *D*, The expression of PER3 in drug-resistant cell line after screening by paclitaxel was significantly lower than that of untreated cell line.

cell lines. This upregulation can significantly increase the sensitivity of drug-resistant cell lines to paclitaxel.

**Cell Cycle Arrest and Apoptotic Rate Increased in Prostate Cancer-Resistant Paclitaxel-Resistant Cells After Overexpression of PER3**

To further explore the effects of PER3 on prostate cancer-resistant paclitaxel-resistant cell lines, we investigated its effect on the PCa/PTX cycle by flow cytometry. The flow cytometry results showed that the cell cycle of the paclitaxel-resistant cells was blocked in the G1 phase when PER3 was overexpressed (Figure 3A). Subsequently, we verified the subcutaneous tumor *in vivo* in animals. The results (Figure 3B) showed that, after treatment with PTX

in mice, the proliferation rate of subcutaneously formed tumors in the PER3 overexpressing resistant cell lines was significantly slower than that in the non-overexpressing group. At 21 days, we sacrificed the mice and removed the tumors. Similarly, the subcutaneously formed tumors of PER3 overexpressing resistant cell lines were significantly larger than the non-overexpressing group (Figure 3C). In addition, flow cytometry results showed a significant increase in apoptosis of paclitaxel-resistant cells after overexpressing PER3 (Figure 3D).

**PER3 Inhibits Paclitaxel Resistance of Prostate Cancer Cell Lines by Down-Regulating Notch1**

Through a literature search, we found that Notch1 signaling pathway is essential in the resistance of



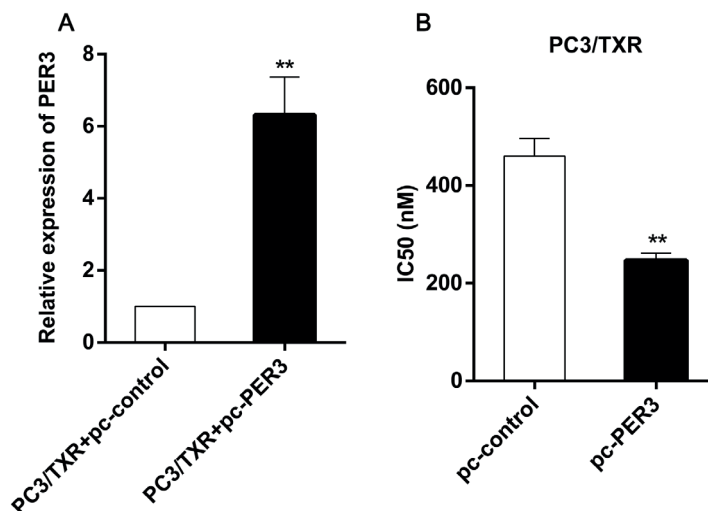
tumor cells<sup>14</sup>. We then detected the expression levels of Notch1 in drug-resistant strains. Western blot results showed that the level of Notch1 was significantly down-regulated in the PER3 overexpressed paclitaxel-resistant cells (Figure 4A). When we overexpressed Notch1 in a PER3 overexpressing resistant cell line, CCK-8 results showed that the proliferation of resistant strains was rescued synchronously treated with PTX (Figure 4B). In addition, we directly interfered with the expression of Notch1 in paclitaxel-resistant strains, and resistant cell lines partially restored the sensitivity to paclitaxel (Figure 4C).

## Discussion

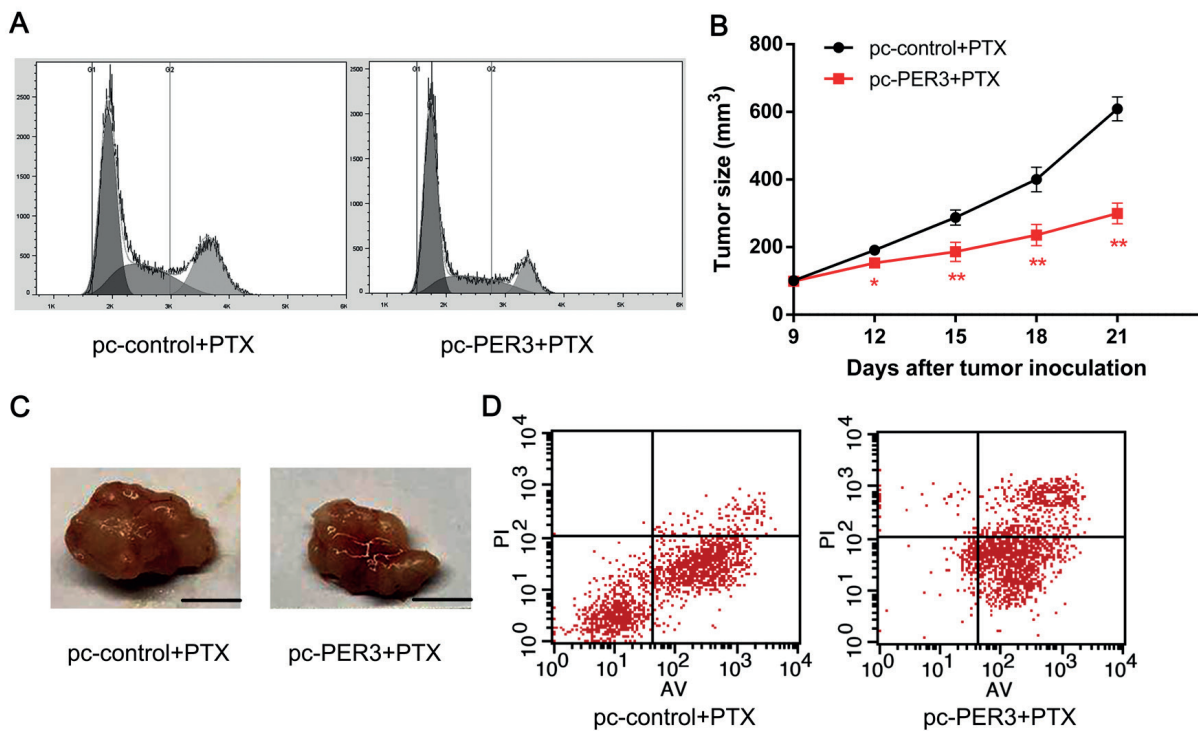
Prostate cancer is one of the most common malignant tumors. It ranks first in the incidence rate in Western countries, and it is only second to lung cancer and bronchial cancer with respect to fatality rate. Due to changes of the environment and lifestyle in China, the incidence of prostate cancer has continued to grow rapidly in recent years, threatening the health of men<sup>15</sup>. There are currently three main treatments for prostate cancer: surgical resection, radiotherapy, and medication<sup>16</sup>. Surgical resection will affect the growth of prostate epithelial cells. Radiotherapy will cause greater damage to normal tissues. Drug thera-

py can achieve efficacy and can be modified to reduce adverse drug reactions. Therefore, drug therapy is still a very promising choice of treatment option<sup>17</sup>.

Prostate cancer (PCa) is a hormone-dependent male malignant tumor. Androgen/androgen receptor (AR)-mediated signaling pathway is critical for the development of prostate cancer<sup>18</sup>. Therefore, withdrawing hormones and inhibiting AR-mediated signaling pathways have become the first choice for PCa therapy and have achieved good therapeutic effects. However, one year after hormone deprivation therapy, almost 80% of patients had tumor recurrence, endocrine resistance, anti-apoptosis, bone and lymph node metastasis and later developed into hormone-resistant prostate cancer (HRPC)<sup>19</sup>. Because the pathological mechanism of HRPC is very complicated and incomplete, there is no effective clinical treatment. At present, chemical drug treatment is still the main method for the treatment of HRPC. Approximately 50% of patients that are intrinsically tolerant to chemotherapy and initially sensitive to chemotherapy will eventually develop chemotherapy resistance. Docetaxel has been used as a first-line chemotherapy drug for 10 years, although it increases the partial survival rate and also has higher toxicity<sup>6</sup>. Long-term use of docetaxel is likely to induce resistance to prostate cancer cells. Once HRPC is resistant to docetaxel, its prognosis is



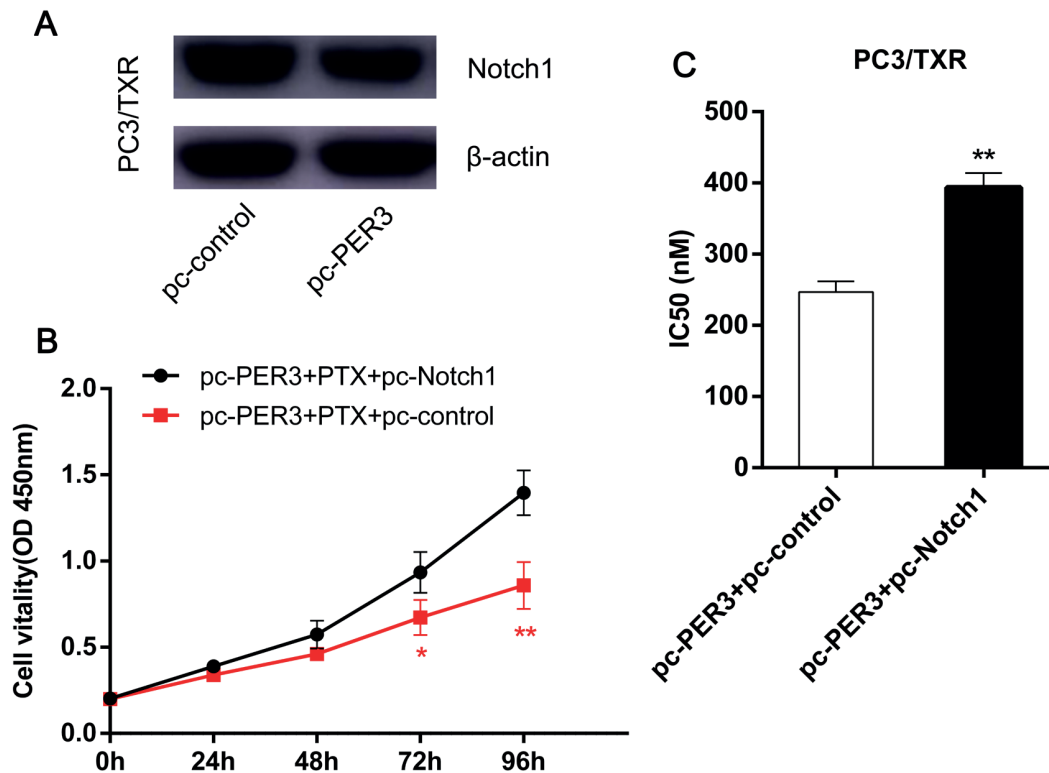
**Figure 2.** Up-regulation of PER3 in prostate cancer-resistant paclitaxel-resistant cell lines reverses the sensitivity of the prostate cancer cell lines to paclitaxel. **A**, The expression of PER3 in prostate cancer-resistant paclitaxel-resistant cell lines was significantly increased after transfection with pc-PER3. **B**, After PER3 overexpression, IC50 of prostate cancer-resistant paclitaxel-resistant cell line decreased compared with the control group.



**Figure 3.** Effects of overexpression of PER3 on cell cycle arrest and apoptotic rate increased in paclitaxel-resistant prostate cancer cell lines. **A**, Prostate cancer paclitaxel-resistant cell line's proliferation is blocked after PER3 overexpression. **B**, After treatment with PTX, the proliferation rate of subcutaneously formed tumors of PER3 overexpressing cell lines decreased significantly. **C**, After the mice were sacrificed at 21 days, the volume and weight of the tumor in the PER3 overexpression group were significantly smaller than those in the control group. **D**, Apoptosis of paclitaxel-resistant cell lines in prostate cancer is significantly increased after overexpression of PER3.

very poor<sup>20</sup>. The induction of HRPC resistance by docetaxel involves the abnormal expression of many proteins and changes in the signaling pathways, such as apoptosis, inflammation, angiogenesis, signal intermediates, drug efflux pumps, and tubulin. Inhibiting HRPC resistance and increasing the efficacy of docetaxel is therefore very challenging. Regardless, one of the biggest obstacles to drug treatment at present is the problem of drug resistance of tumor cells. The period gene family is a central component of the biological rhythm regulation mechanism and plays an important role. At present, it has been confirmed that humans have three kinds of PER proteins (PER1, PER2, PER3)<sup>21</sup>. The PER3 gene is the most important member of the human period gene family and is involved in the important processes of biological rhythm regulation. Some scholars<sup>22</sup> believe that genetic malfunction of the circadian clock is not only related to certain diseases, but also to the occurrence of tumor<sup>23</sup>. Researches have shown that the period gene participates in

the process of tumor development by regulating cell proliferation and apoptosis, participating in DNA damage repair pathways, maintaining genome stability. The study found that the PER3 gene polymorphism is involved in the occurrence of malignant tumors, such as prostate cancer and breast cancer. Overall, our work found that PER3 is downregulated in prostate cancer-resistant paclitaxel-resistant tissues and cells. Upregulation of PER3 expression in prostate cancer-resistant paclitaxel-resistant cell lines can significantly increase the sensitivity of drug-resistant cell lines to paclitaxel. In addition, the overexpression of PER3 in drug-resistant cell lines can decrease the expression of Notch1, and restoration of Notch1 expression can reduce the increased paclitaxel sensitivity resulting from PER3 overexpression. This is the first study to investigate the effectiveness of PER3 on paclitaxel-resistant prostate cancer. The mechanism of how downregulated PER3 causes paclitaxel resistance in prostate cancer still needs further investigation.



**Figure 4.** PER3 inhibits paclitaxel resistance of prostate cancer cell lines by down-regulating Notch1. **A**, Decrease in Notch1 expression in PER3 overexpressing resistant strains. **B**, Overexpression of Notch1 in PER3 over-expressed paclitaxel-resistant cell lines rescued paclitaxel-resistant cell line proliferation. **C**, Down-regulation of Notch1 expression in C-resistant strains, cells restores paclitaxel sensitivity.

## Conclusions

We showed that PER3 can reverse the sensitivity of paclitaxel-resistant cell lines to paclitaxel by inhibiting the expression of Notch1.

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## Conflict of Interest

The Authors declare that they have no conflict of interest.

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