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Atorvastatin regulates the migration and invasion of prostate cancer through the epithelial-mesenchymal transformation and matrix metalloproteinase pathways

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Purpose: Our purpose was to verify the effects of atorvastatin (ATO) on prostate cancer (PCa) proliferation, apoptosis, invasion, and metastasis and to further explore the drug's mechanism of action.

Materials and Methods: We used cell counting kit-8 (CCK8) and clone formation experiments to study the effect of ATO on the proliferation of PC3 cells. Flow cytometry and Hoechst 33342 staining were used to detect cell apoptosis. Cell migration and invasion were detected through wound healing experiments and transwell experiments. Western blotting was applied to detect apoptosis-related proteins (BAX, Bcl-2, PARP, and Caspase-3), epithelial-mesenchymal transformation (EMT) proteins, and matrix metalloproteinase (MMP) expression. A mouse xenograft tumor model was established, and tumor volume and weight were determined. The expression levels of the above-mentioned proteins were determined through western blot.

Results: ATO inhibited PC-3 cell proliferation and promoted cell apoptosis in a dose-dependent manner. ATO significantly up-regulated the expression of BAX, PARP, and Caspase-3 and inhibited the expression of Bcl-2. Wound healing and transwell experiments showed that ATO inhibited invasion and metastasis in PC-3 cells, possibly because ATO could inhibit the EMT and the expression of MMPs in PC-3 cells. Studies in nude mice showed that ATO significantly reduced tumor volume and weight; the expression levels of related proteins were consistent with the *in vitro* results.

Conclusions: ATO inhibits the occurrence and development of PCa and regulates the migration and invasion of PCa cells by inhibiting the EMT and MMPs.

Keywords: Atorvastatin; Epithelial mesenchymal transition; Matrix metalloproteinases; Prostate cancer

INTRODUCTION

Prostate cancer (PCa) is a malignancy of the prostate tissue in the male genitourinary system. Data from the 2020 World Cancer Report showed that PCa ranks 6th in incidence among male malignant tumors and 9th in terms of mortality rate [1]. According to statistics from the International Agency for Research on Cancer of the World Health

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Organization, the incidence of PCa in China in 2020 was about 15.6 per 100,000, with more than 110,000 new cases and more than 50,000 deaths [2]. The current conventional treatment methods are mainly radical surgery and endocrine therapy, supplemented by radiotherapy and chemotherapy, which can achieve better clinical treatment effects [3]. However, some patients are not candidates for surgery or are not sensitive to endocrine therapy, radiotherapy, and chemotherapy, which are treatment issues that need to be resolved [4]. Therefore, the discovery of an effective drug with less toxicity is essential to improve the overall survival rate of PCa.

Atorvastatin (ATO) is a selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. It is mainly used clinically to reduce cholesterol in the blood and the risk for cardiovascular and cerebrovascular diseases. Current studies have found that ATO has anti-tumor effects in cervical cancer, liver cancer, cholangiocarcinoma, and PCa [5-10]. Statins, especially ATO, can improve the survival of patients with PCa and are expected to become complementary treatment options for PCa, but the specific mechanism of their drug effect is unclear [11]. Research has linked the increased risk for aggressive PCa with elevated cholesterol levels. The findings of Hamilton et al. [12] showed that there is a good link between the use of statins and the survival outcomes of patients who start androgen-deprivation treatment.

The epithelial-mesenchymal transformation (EMT) refers to the transdifferentiation of epithelial cells into mesenchymal cells under specific physiologic and pathologic conditions. The EMT is regarded as a pathologic process leading to tumor progression, and is related to invasion and metastasis. The degradation of extracellular matrix and basement membrane is a key link in tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are the only enzymes known to degrade the extracellular matrix and are also involved in the formation of primary tumors, tumor cell growth, and apoptosis. Some studies have shown that long-term use of statins can reduce the risk for several human malignancies [13,14]. Therefore, in this study, we selected ATO and further validated its role as a potential treatment for PCa.

MATERIALS AND METHODS

1. Reagents

Both ATO and dimethyl sulfoxide (DMSO) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). We dissolved the ATO in DMSO to a final DMSO concentration of 0.1%.

2. Cell culture

PC-3 cells were obtained from the Shanghai Cell Bank of China Academia Sinica (Shanghai, China). We cultured the PC-3 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, and then placed the samples in an incubator containing 5% CO_2 at 37℃.

3. Cell counting kit-8 (CCK-8) determination

We detected the viability of PC-3 cells by use of CCK-8 (Beyotime, Nanjing, China), and the detection method was carried out in accordance with the instructions (Dojindo, Kumamoto, Kyushu, Japan). PC-3 cells were seeded on a 96-well plate (2,000 cells/well). We added different concentrations of ATO (0, 2.5, 5, 10, 20, and 40 µM) to the culture for 24 hours. After incubating for the specified time, 10 µL of CCK-8 solution was added to 90 µL of DMEM and the cells were incubated at room temperature for 3 hours. Then, the absorbance at a wavelength of 450 nm was measured in a microplate instrument (Bio-Rad, Hercules, CA, USA). Three replicates were tested for each sample.

4. Clone formation assay

We seeded the PC-3 cells in a 6-well plate (800 µL/well), and added different concentrations of ATO (0, 2.5, 5, 10, 20, and 40 µM) to the cells and cultured them for 24 hours. We then replaced the original medium with a fresh medium containing 10% serum. After 2 weeks, the medium was removed and the cells were fixed, stained, photographed, and counted.

5. Hoechst 33342 staining

PC-3 cells were seeded into a 6-well plate. After the cells were allowed to stick to the wall, we added different concentrations of ATO (0, 10, 20, and 40 µM) to the culture. We discarded the supernatant, washed the cells twice with phosphate-buffered saline (PBS), added 1 mL Hoechst 33242 fluorescent dye, and incubated the cells at 37℃ for 15 minutes. We then discarded the fluorescent dye, washed the cells with PBS, observed the cells, and took pictures under an inverted fluorescent microscope. The experiment was repeated three times.

6. Flow cytometry determination

PC-3 cell apoptosis was detected by flow cytometry (BD Biosciences, Franklin Lake, NJ, USA). Groups of cells were treated with four different concentrations of ATO (0, 10, 20, and 40 µM), and the cells were then collected with 0.25% trypsin, washed twice with precooled PBS, resuspended with

100 µL 1× binding buffer, and stained with the kit solution at 37°C in the dark for 15 minutes. After incubation, 400 µL of 1× binding buffer was added to the mixture, and the percentage of apoptosis was analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA).

7. Wound healing assay

We detected migration of the PC-3 cells by use of the wound healing assay. First, we seeded the cells in a 6-well plate and allowed them to culture overnight. When the cell healing degree reached 85% to 90%, after one scratch, we treated the cells with the indicated concentration of ATO (0, 10, or 20 µM), which was recorded as 0 hour. After culturing for 24 and 48 hours, we collected images and analyzed the scratched area covered by cells in each group.

8. Transwell assay

We added a 200-µL cell suspension ($1×10^5$ cells) into the upper chamber of a transwell insert and added 600 µL of medium containing 10% fetal bovine serum or ATO at the indicated concentration into the chamber bottom. After being allowed to incubate for 24 hours, the cells were fixated and stained, observed under an inverted microscope, and the number of transmembrane cells counted. The cell migration experiment was cultured for 12 hours; the cell invasion experiment was cultured for 48 hours.

9. Western blotting

We used lysis buffer containing PMSF (Roche, Basel, Switzerland) and protease inhibitor cocktail (Roche) and the protein extraction reagent RIPA (Invitrogen, Life Technologies, Carlsbad, CA, USA) to extract total protein from PC-3 cells and tumors. Subsequently, the concentration of the extracted total protein was detected by use of the Bio-Rad protein detection kit (KeyGen Biotech, Nanjing, China). A 30-µg protein sample was subjected to 12% SDS-PAGE for electrophoresis, and then transferred to a 0.22-µM PVDF (Millipore, Burlington, MA, USA) membrane. The membranes were incubated with the corresponding primary antibody overnight at 4°C and were then washed three times and incubated with enzyme-labeled secondary antibody at 37°C for 1 hour. Finally, the enhanced chemiluminescence detection system (Tanon, Shanghai, China) was used to visualize the target protein. Anti-b-cell lymphoma-2 related protein X (BAX), anti-Bcl-2, anti-Caspase-3, anti-PARP, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-MMP-2, anti-MMP-9, and anti-actin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The gray value analysis uses ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative expression of the target protein is expressed as target protein/actin.

10. Animal experiments

Male BALB/c nude mice (6 weeks old, 20–22 g) were selected from Experimental Animal Center of Jilin University (Jilin, China) for the experiment. In short, we injected 100 µL of PBS ($5×10^6$ PC-3 cells) subcutaneously into the right side of the mouse to establish a xenograft model [15]. After 2 weeks of inoculation, the mice were divided into two groups with 10 mice in each group. PBS (control group) or ATO (20 µM) was given by gavage daily for 15 days. Calipers were used to monitor the length (a) and width (b) of the tumor tissue every 3 days. The formula for calculating tumor volume is as follows: V (mm^3)=$0.53×ab^2$. All mice were sacrificed after tumor inoculation for a total of 1 month. After that, the xenograft tumor was excised, weighed, and photographed. At the same time, the total protein of tumor tissue was extracted for western blotting detection. All procedures followed the guidance of the Guide for the Care and Use of Laboratory Animals. The animal experiment obtained approval from the Ethics Committee of the First Hospital of Jilin University (approval number: 20110551).

11. Statistical analysis

We use GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) to perform statistical analysis on the data, which were displayed as mean±standard deviation. All experiments were carried out in triplicate. Two groups were compared using Student's t-test. The comparison of three groups was analyzed by using one-way ANOVA with Fisher's LSD *post-hoc* test. We regarded $p<0.05$ as statistically significant.
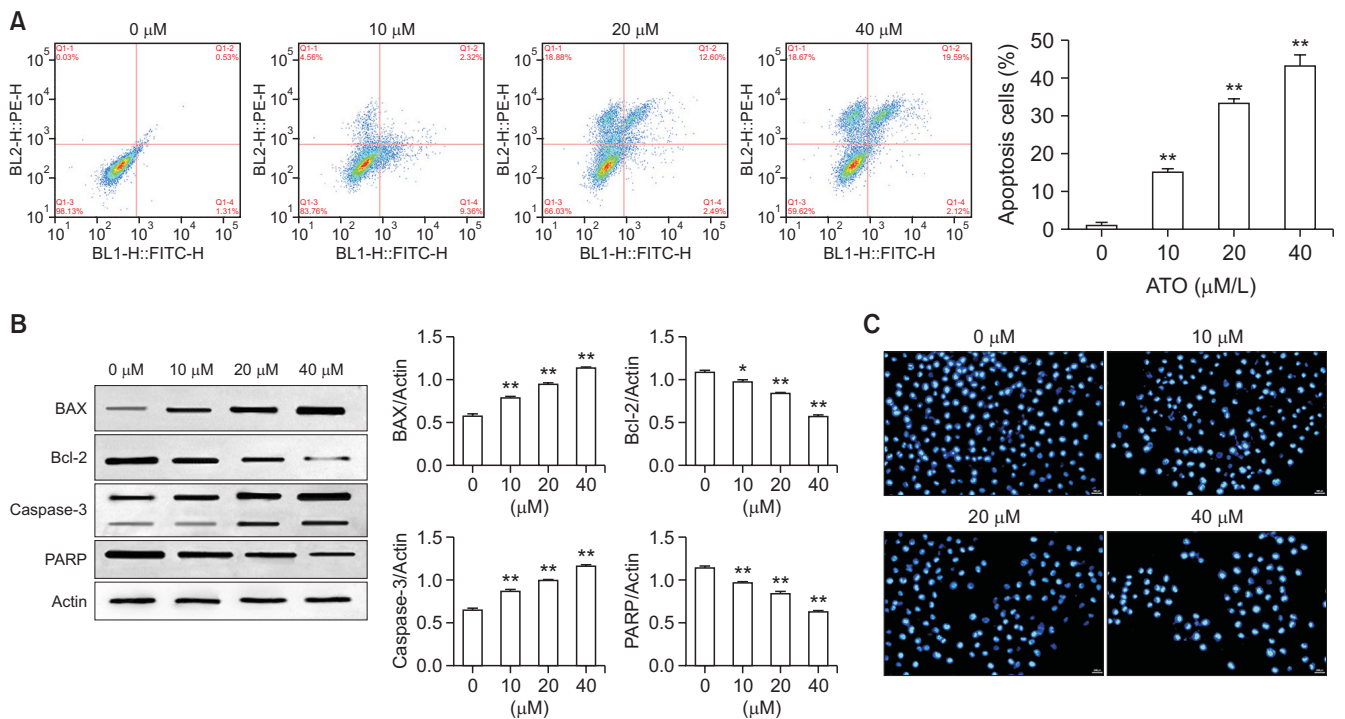
RESULTS

1. ATO inhibited PC-3 cell proliferation

The results of the CCK8 test showed that ATO had a significant inhibitory effect on the proliferation of PC-3 cells and that this inhibition was dose-dependent. The effect was significant at an ATO concentration greater than 5 µM (Fig. 1A). As the concentration increased, the inhibition of PC3 cell proliferation by ATO increased. The results of the clone formation experiment indicated that ATO inhibited PC-3 cell proliferation (Fig. 1B), which was consistent with the results of the CCK-8 experiment. To further screen for the optimal concentration of ATO, we choose four concentrations of 0, 10, 20, and 40 µM for detection of apoptosis.
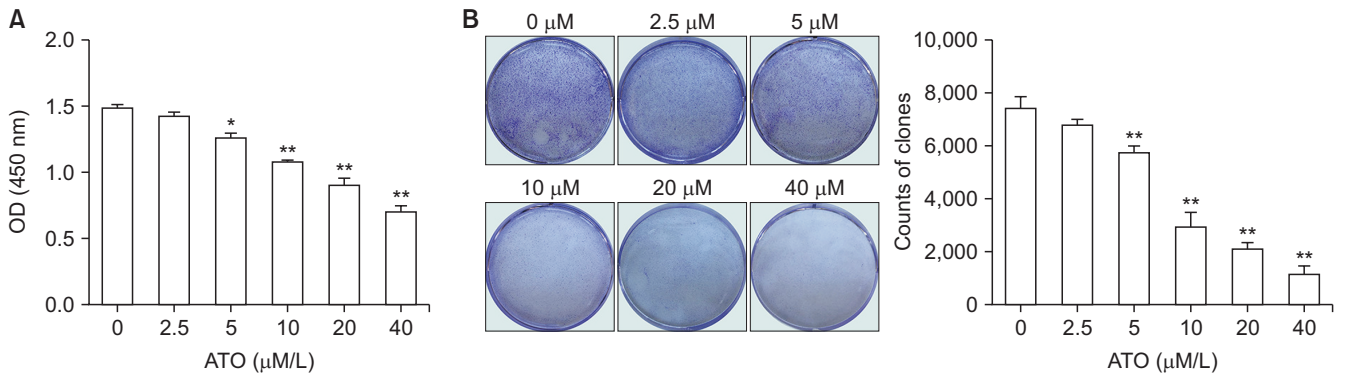
Fig. 1. ATO inhibited the proliferation of PC-3 cells. (A) CCK-8 detects cell viability. (B) The results of the clone formation experiment. OD, optical density; ATO, atorvastatin. *p<0.05, **p<0.01 versus 0 μM.

Fig. 2. ATO promoted PC3 cell apoptosis. (A) Flow cytometry and data analysis. (B) The test results of apoptosis proteins (BAX, Bcl-2, Caspase-3, PARP) and data analysis. (C) Hoechst 33342 staining (magnification, ×200; scale bars, 25 μm). ATO, atorvastatin. *p<0.05, **p<0.01 versus 0 μM.

2. ATO promoted apoptosis in PC-3 cells

We evaluated the effect of ATO on PC-3 cell apoptosis by use of flow cytometry. The results showed that three different concentrations of ATO could significantly promote PC-3 cell apoptosis (Fig. 2A). As the concentration of ATO increased, the rate of cell apoptosis also increased. At the same time, the results of Hoechst 33342 staining showed that ATO promoted PC-3 cell apoptosis, which was consistent with the results of flow cytometry (Fig. 2C). Next, we further verified the role of ATO on the expression levels of related apoptotic proteins BAX, Bcl-2, Caspase-3, and PARP through western blot. BAX and Bcl-2 regulated each other. As the concentration of ATO increased, the expression of BAX significantly increased, and the expression of Bcl-2 showed the opposite trend (Fig. 2B). Caspase-3 and PARP molecules were further detected downstream of the mitochondrial apoptosis pathway. As the concentration of ATO increased, the results consistently showed that the expression of cleaved Caspase-3 and cleaved PARP significantly increased (Fig. 2B). In summary, the effect of ATO in promoting PC-3 cell apoptosis increased with an increase in concentration.

3. ATO inhibited the migration and invasion in PC-3 cells

To verify the effect of ATO on the migration ability of PC-3 cells, we conducted a wound healing and transwell
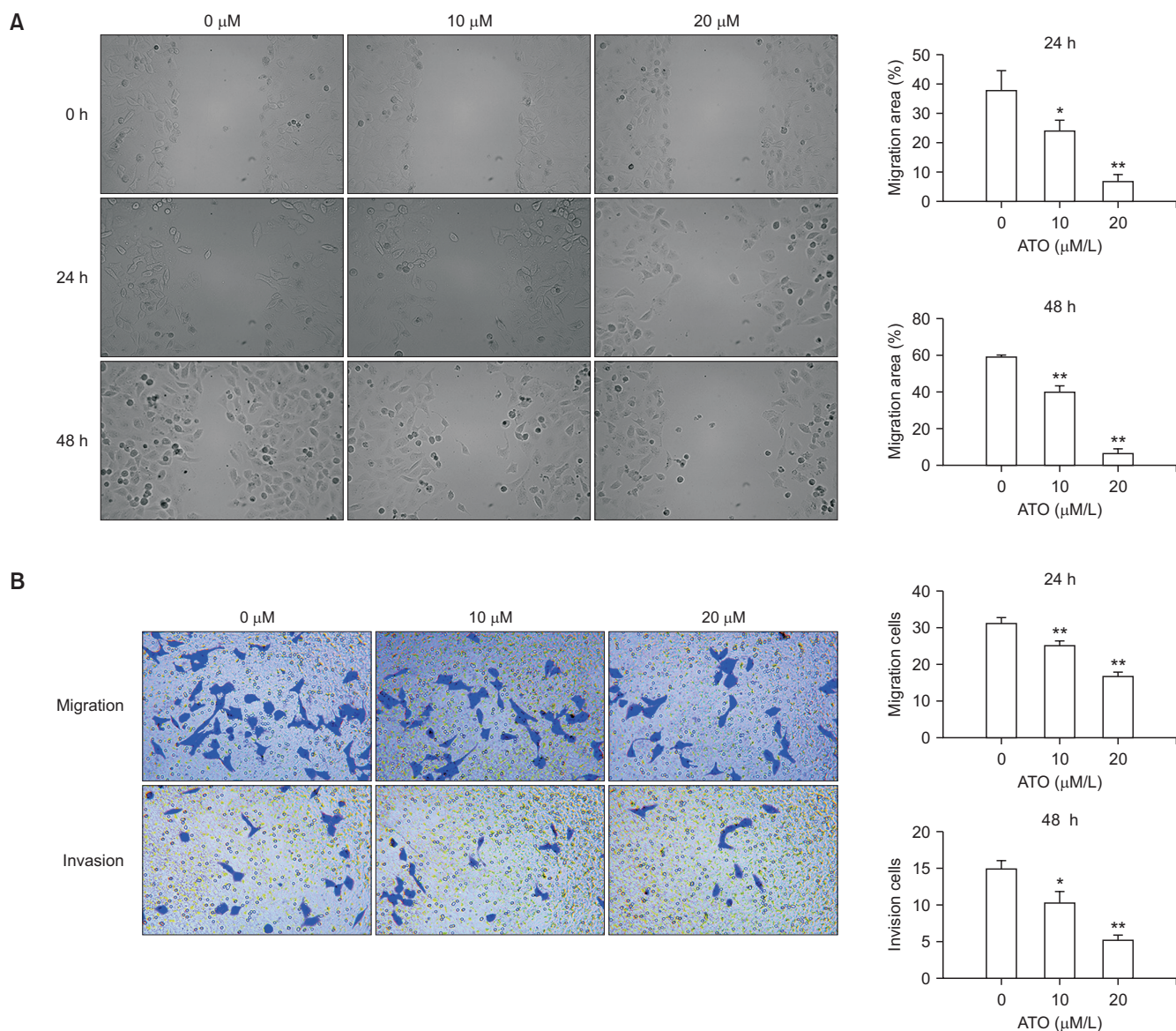
Fig. 3. Effects of ATO treatment on the invasion and migration ability of PC3 cells. (A) The results of wound healing experiments. PC3 cells were cultured until reaching approximately 80–90% cell density, the culture was scratched as described in methods, and further cultured with various concentrations of ATO (0, 10, and 20 μM) for 24 h and 48 h, after which the cells were fixed and photographed. (B) The results of the transwell experiment to detect invasion and migration. Cells were treated using ATO with a concentration gradient for 24 h, and then the cells were fixed and photographed using a microscope after crystal violet staining. Magnification, ×100; scale bars, 100 μm. ATO, atorvastatin. *p<0.05, **p<0.01 versus 0 μM.

experiment. To avoid the influence of the inhibition of proliferation on the experimental results, we chose 10 and 20 μM ATO for the experiment. We found that ATO could significantly inhibit the migration in PC-3 cells (Fig. 3A). After 48 hours, the migration rate of the 0-μM group was 59.85%, whereas the migration rates of the 10-μM group and the 20-μM group were 40.44% and 7.06%, respectively; the inhibitory effect of 20 μM was more obvious than that of 10 μM. transwell migration experiments showed that compared with the 0-μM group, ATO significantly inhibited the migration and invasion of PC-3 cells, and the inhibition effect of 20 μM was

stronger (Fig. 3B). The above results indicate that ATO can inhibit the migration and invasion of PC-3 cells.

4. ATO inhibited the EMT pathway and MMP expression

Tumor infiltration and metastasis are closely related to the EMT. Western blot was used to detect the expression levels of EMT proteins. The EMT is often accompanied by low expression of E-cadherin and high expression of N-cadherin and vimentin. We found that after ATO treatment, the expression levels of E-cadherin were significantly up-
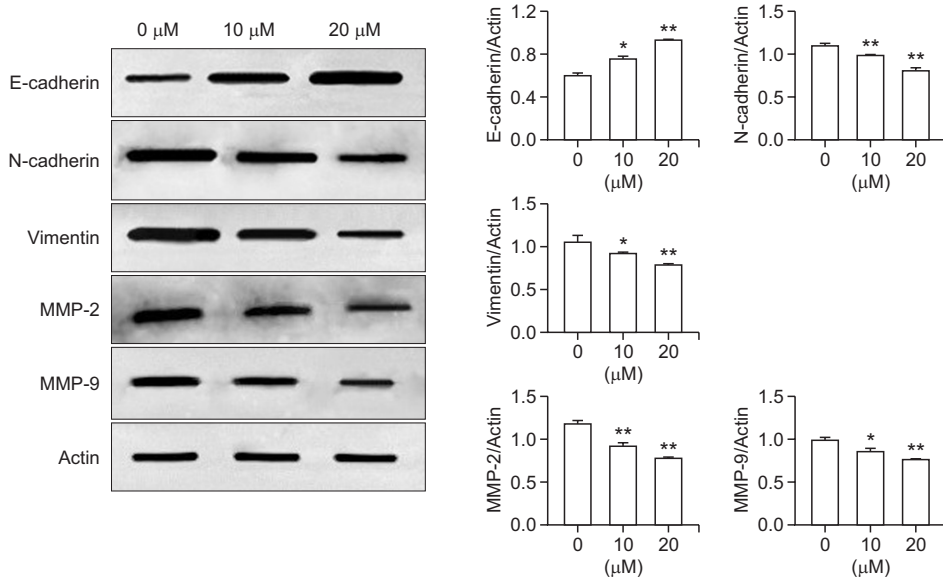
Fig. 4. Protein expression in relation to the migration and invasion of PC3 cells as shown by western blot. *p<0.05, **p<0.01 versus 0 μM.
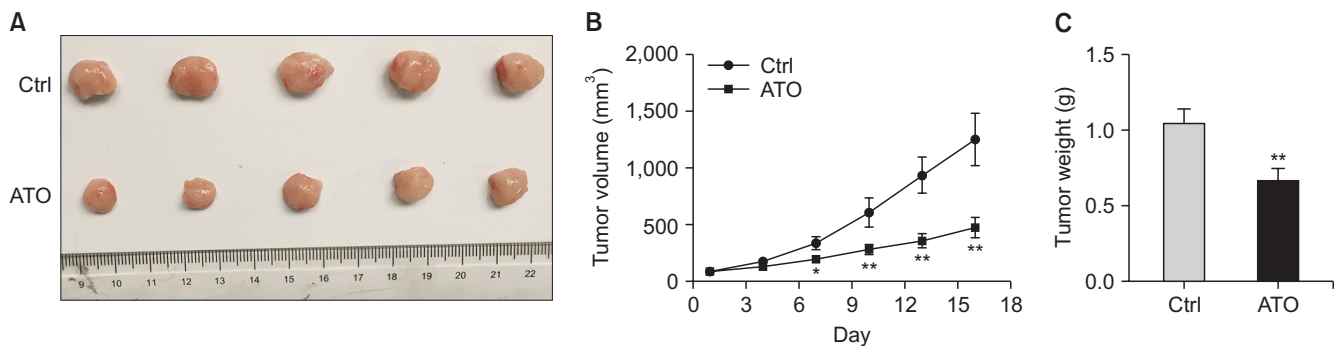
Fig. 5. ATO inhibits the growth of PCa *in vivo*. (A) Representative photographs of the tumors. (B) The growth curve of tumor volume. (C) Tumor weight. Ctrl, control; ATO, atorvastatin. *p<0.05, **p<0.01 versus the Ctrl group.

regulated, whereas the expression levels of N-cadherin and vimentin were significantly down-regulated (Fig. 4). MMPs are key enzymes related to the degradation of the extracellular matrix and are believed to play a key role in metastasis. The present study further tested the expression levels of MMP-2 and MMP-9. The results indicated that ATO down-regulated the expression levels of MMP-2 and MMP-9 in PC-3 cells (Fig. 4). In summary, ATO can inhibit the invasion and metastasis of PC-3 cells through different signaling pathways.

5. ATO inhibits tumor proliferation and migration in vivo

To determine the effect of ATO on PCa, we established a nude mouse subcutaneous tumor model. The weight difference of each group of mice was not significant (Supplementary Fig.). The tumor changes in the ATO group and the control group are shown in Fig. 5A. We found that ATO significantly inhibited tumor growth. As shown in Figs. 5B and

5C, tumor volume and weight were significantly reduced in the ATO group compared with the control group (p<0.01). This indicated that ATO can significantly inhibit tumor proliferation.

To clarify the effect of ATO on tumor apoptosis, we detected the expression levels of apoptosis- regulated proteins by western blot. We found that the expression levels of BAX and Caspase-3 were significantly increased, and the trend of cleaved PARP and Bcl-2 expression were the opposite (Fig. 6A). At the same time, to explore the mechanism of the effect of ATO on tumor metastasis, we further detected the expression levels of the key molecules of the EMT and of MMP-2 and MMP-9 through western blot. The results are shown in Fig. 6B. After ATO treatment, the expression levels of N-cadherin, vimentin, MMP-2, and MMP-9 was significantly down-regulated (p<0.01), and that of E-cadherin was increased (p<0.01) (Fig. 6B).
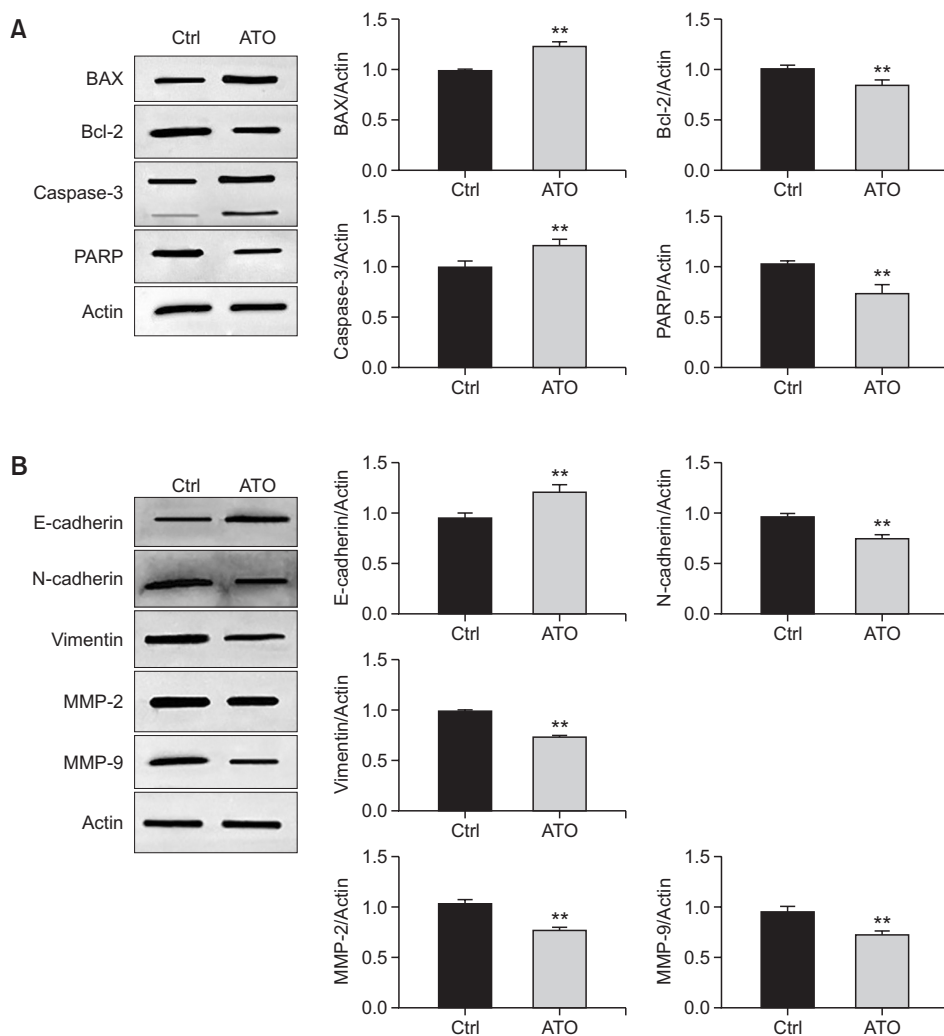
Fig. 6. Expression of proteins related to apoptosis, migration, and invasion of PCa as shown by western blot. (A) The test results of BAX, Bcl-2, Caspase-3, PARP, and statistical analysis. (B) The test results of E-cadherin, N-cadherin, vimentin, MMP-2, MMP-9, and statistical analysis. Ctrl, control; ATO, atorvastatin. **p<0.01 versus the control group.

DISCUSSION

In present study, we found that as its concentration increased, ATO inhibited cell proliferation, migration, and invasion but promoted apoptosis. Importantly, the results of our *in vivo* and *in vitro* studies showed that ATO regulates the migration and invasion of PCa through the EMT and MMP signaling pathways. Some progress has been made in inhibiting the development of PCa using ATO alone or in combination. For example, Wang et al. [16] showed that metformin combined with ATO inhibits the proliferation, apoptosis, and migration of PCa. In other studies, ATO combined with aspirin [17] or docetaxel [18] was also shown to inhibit the progression of PCa. He et al. [19] found that ATO inhibits the colony formation of PCa and enhances the effect of radiotherapy, which may be related to an interaction between Bcl-2 and MSH2.

Caspase is an executor of apoptosis, and is the first to trigger apoptosis. Caspase-3 is the most important terminal cleavage enzyme in the process of apoptosis and is generated by the activation of pro-caspase-3. During the activation process, pro-caspase-3 is cleaved from Asp28–Ser29 and Asp175–Ser176 to form P17(29–175) and P10(182–277) fragments, and the two subunits are reconstituted into the active form of caspase-3 [20]. In addition to caspases, signal molecules such as PARP have been reported to participate in the apoptosis pathway, which is the main substrate of caspase-3 [21]. Our western blotting results showed that ATO can induce PC3 cell apoptosis in a concentration-dependent manner by increasing the expression of caspase-3 and PARP.

We also explored the anti-cancer effects of ATO on PCa *in vivo*. We found that ATO had a significant inhibitory effect on tumor growth. The xenograft tumors in the ATO treatment group were smaller and lighter in weight compared with the control group.

The EMT is a significant process for the invasion and metastasis of epithelial malignant tumors, among which E-cadherin, N-cadherin, and vimentin are key molecules. Previous studies indicated that E-cadherin is required to maintain stable cell connections. The down-regulation of E-cadherin

expression often occurs in the process of epithelial-to-mesenchymal cell phenotypic transformation and carcinogenesis and can initiate the EMT, leading to tumor invasion and metastasis [22]. It is still unclear whether the signal pathway through which ATO inhibits PCa metastasis is related to the EMT. Our results showed that after adding ATO, the expression of E-cadherin was significantly increased and that of N-cadherin was down-regulated. Vimentin is a major member of the intermediate filament protein family and a key marker of the EMT. Singh et al. [23] showed that down-regulation of vimentin in hormone-resistant PCa CL1 cells can inhibit their invasion and metastasis ability. Our research results for vimentin are consistent with those of Singh et al. [23].

The MMPs belong to the family of zinc-dependent endopeptidases. MMP-2 and MMP-9 participate in the degradation of the tumor basement membrane and the extracellular matrix and play a key role in the occurrence of tumors. MMP-2 and MMP-9 also gradually become the target of tumor treatment. Research by Fromigué et al. [24] showed that ATO reduces the expression or activity of MMP-2, -9, and -14 and TIMP2 in invading cells. This result shows that ATO can inhibit the expression of MMP-2 and MMP-9 in PC-3 cells. Our data support and expand the previous data, showing that ATO affects the proliferation, apoptosis, migration, and invasion of PCa.

Although we carried out relevant studies at the *in vivo* and *in vitro* levels, our study still has some limitations. This study did not explore whether ATO affects normal PCa cells, and only one PCa cell line was selected for research. Multiple cell lines were not selected for repeated verification. In addition, the specific mechanism of ATO needs to be further verified.

CONCLUSIONS

In summary, our research indicated that ATO affects the proliferation, apoptosis, migration, and invasion of PCa in a dose-dependent manner. ATO regulates the migration and invasion in PCa by the EMT and MMP signaling pathways. Further research on the EMT and related signaling pathways will help us to understand the role of ATO in regulating cancer cell proliferation and metastasis. In other words, ATO may become a new drug for the treatment of PCa. Therefore, it is necessary to further explore the clinical application of ATO.

CONFLICTS OF INTEREST

FUNDING

AUTHORS' CONTRIBUTIONS

SUPPLEMENTARY MATERIAL

Supplementary material can be found via https://doi.org/10.4111/icu.20210411.

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