# ESTAT3 Inhibitor AG-490 Inhibits the Growth of Prostate Cancer by miR-503-5p Both In Vivo and In Vitro

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## Abstract

**Objective:** To explore the effect and the related mechanism of STAT3 inhibitor AG-490 on inhibiting the proliferation of prostate cancer cells. **Methods:** PC3 cells and DU145 cells were cultured stably and treated with AG-490 to detect the changes in the activity of PC3 cells and DU145 cells. Thirty 6-8 weeks male BALB/c nude mouse were randomly divided into a control group, a DMSO group, and an AG-490 group to detect differences in various indexes . **Results:** The overexpression of miR-503-5p depends on the activation of STAT3. After treatment with AG-490, The proliferation and invasion of PC3 cells and DU145 cells and the expression of miR-503-5p were all reduced. Luciferase reporter assay demonstrated that the target proteins of miR-503-5p include PDCD4, TIMP-3, and PTEN. After treatment with AG-490, the expression of PDCD4, TIMP-3, and PTEN in cells was significantly up-regulated. IL-6-induced overexpression of miR-503-5p and restored the expression of STAT3, demonstrating the correlation between STAT3 and miR-503-5p. AG-490 can inhibit tumor growth and induce tumor cell apoptosis in the PC3 BALB/c nude mouse xenograft model. Western blotting and immunohistochemical staining showed that the expression levels of STAT3, Ki67, Bcl-2 and MMP-2 in the AG-490 group were significantly reduced, and the expression of PDCD4, TIMP-3 and PTEN increased. **Conclusion:** AG-490 can inhibit the growth of prostate cancer cells in a miR-503-5p-dependent manner by targeting STAT3. AG-490 is expected to become a new candidate drug for the treatment of prostate cancer.

## Introduction

Prostate cancer is the malignant tumor with the fifth highest incidence worldwide, with approximately 1 million new cases each year.[1] However, the 5-year survival rate after diagnosis of prostate cancer is still low, which is significantly lower than other cancers, including thyroid cancer and colorectal cancer. Therefore, finding a more targeted and more effective prostate cancer drug target, and in-depth understanding of the molecular mechanism of tumorigenesis are essential for early intervention and treatment of prostate cancer.[2] microRNAs (MiRNAs) are small, highly conserved non-coding RNAs that control gene expression by binding to the seed sequence of the target

3'-untranslated region (3'UTR), resulting in translational inhibition or mRNA degradation. miRNAs are regulatory factors of human carcinogenesis, embryonic development, and cell aging.[3] Previous studies[4] have shown that the abundance of

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miRNAs is abnormally expressed in human tumor tissues, especially malignant tumors of epithelial origin. Several genes have been shown to be direct targets of miR-503-5p in human cancer,[5] including programmed cell death 4 (PDCD4), phosphatase tensin homology deleted on chromosome ten (PTEN), etc. Studies[6] have shown that down-regulation of miR-503-5p can inhibit the proliferation of cancer cells by enhancing the expression of PTEN in hepatocellular carcinoma. In addition, miR-503-5p down-regulates tissue inhibitor of metalloproteinase-3 (TIMP-3) to affect the migration and invasion ability of cancer cells. Signal transducers and activators of transcription (STAT) proteins are the major signaling proteins of mammalian cytokines and growth factors. STAT3 responds to signals such as IL-6, IL-10, and VEGF. STAT3 is essential in tumorigenesis and cancer-induced immunosuppression. There is evidence[7] showing that STAT3 can promote cancer cell growth by activating miR-503-5p. However, the exact biological function and molecular mechanism of the STAT3/miR-503-5p network in prostate cancer are unclear. In this study, we analyzed the correlation between miR-503-5p and STAT3 expression in prostate cancer tissues in order to provide more potential targets for the prevention and treatment of prostate cancer.

## Materials and Methods

### Cell Culture and Tissue Samples

The human prostate cancer cell lines PC3 and DU145 were purchased from China Center for Type Culture Collection (Wuhan) and stored in complete culture containing 10% fetal bovine serum, 2 mM glutamine, 100U/ml penicillin, and 100 mg/ml streptomycin. Incubate in an incubator at 37°C and 5%$CO_2$. STAT3 small molecule inhibitor AG-490 was purchased from Calbio Chemem Company, Germany, and IL-6 was purchased from Sigma Company, USA. AG-490 was added to the cells at a final concentration of 5 μM and treated for 48 hours. This study was approved by the institutional review board of Tianjin Medical University. Prostate cancer tissue specimens were collected in the urology department of our hospital.According to the classification of prostate cancer TNM stage (2010), a total of 45 highly differentiated, 39 moderately differentiated, and 72 poorly differentiated prostate cancer specimens were collected. All tumors and tumor marginal tissues were embedded in paraffin for immunohistochemical staining and in situ hybridization.

### Detection of Mir-503-5p by In Situ Hybridization

Oligonucleotide probes modified by locked nucleic acid (LNA) were used for in situ hybridization with in situ hybridization kit (Boster, China). In situ hybridization was used to detect the expression of miR-503-5p in prostate cancer specimens.The LNA/DNA oligonucleotide contains locked nucleic acids at 8 consecutive centrally located bases and has the following sequence: LNA-miR-503-5p, 5'-TCAA CATCAGTCTGATAAGCTA-3'. DAPI karyotype kit (GenMed, USA) was used for reverse staining, and Fluo-View confocal laser scanning microscope FV1000 was used for observation.

## RT-PCR Detection

Refer to the operating instructions to extract total RNA from cells, patient serum, and tumor tissue specimens, reverse transcribe cellular RNA into cDNA, and then amplify into DNA, all steps are strictly in accordance with the instructions of reverse transcription kit and amplification kit.PDCD4 primer (forward primer, 5'-AAATGAATGGATGTAGAGCAG-3' and. TIMP3(forward primer 5'-GCTGTGGCCTTAAGA GAGTCTCTGG-3' and reverse primer 5'-TTAGTGTCCAA GCTGGGAAGAGGG-3');PTEN (forward primer 5'-ATTT CAGGATCCTCCAACCCAC-3' and reverse primer 5'-CGA AGACGTACTGGTATTTTACCC-3'). All primer sequences were synthesized by Shanghai Bioengineering Technology Co., Ltd. The RT-PCR reaction conditions are as follows: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and a final extension at 70°C for 10 min.

## CCK-8 Detection and Flow Cytometry

Transfected PC3 cells and DU145 cells were seeded in 96-well plates with 5000 cells per well. After transfection at 0 h, 24 h, 48 h and 72 h, add 10 μL of cholecystokinin solution to each well. After incubation for 2 h, the optical density value (OD value) was measured at 490 nm with a microplate instrument and recorded on the cell growth curve. Each group of reactions was repeated 6 times. The transfected PC3 cells and DU145 cells were collected and suspended in 500 μL of binding buffer. Apoptotic cells were double-labeled with 5 μL FITC-Annexin V reagent and 5 μL propidium iodide for 30 min. The cells were then analyzed on a BD FACSCalibur flow cytometer (BD Biosciences) within 1 hour. The percentage of apoptotic cells in the quadrants of Annexin V+/PI− and Annexin V+/PI+ represents the rate of apoptosis. Each sample was repeated 3 times.

## Transwell Detection

The cell invasion ability was measured with a Matrigel-coated Transwell cell. Transfected PC3 cells and DU145 cells (400 cells) were inoculated in 200 μL medium without bovine serum, 500 μL medium containing 10% bovine serum was implanted into the upper cavity, and 500 mmol/L medium containing 10% bovine serum was inoculated into the lower cavity. For cell migration analysis, the cell is not coated with matrix, and the other steps are the same as the invasion analysis. All Transwell experiments were incubated at 37°C for 24 hours. The cells migrating and invading on the lower surface were stained with crystal violet, and 5 predetermined visual fields (× 200) were photographed under a microscope. Repeat 3 times.

## HE Staining and Immunohistochemical Staining

HE staining: The mice were sacrificed after the experiment, the tumor tissue was completely removed, embedded in conventional paraffin, and sectioned, and then sections were baked, and finally they were cryopreserved after cooling. Tissue sections were deparaffinized in xylene and hydrated through graded ethanol. Hematoxylin staining for 30-60 s, rinse for 5 min, eosin staining for 30 s, and rinse for 5 min; dehydrate using graded ethanol, dry and vitrification by dimethylbenzene. Mount with neutral balsam, dry in the air and finally observe under optical microscope. Immunohistochemical staining: take tumor slices, dewax them routinely, dehydration, vitrification and mount after you follow the steps of immunohistochemistry kit step by step, observe under light microscope, take 400 times field of view, and take brown particles as positive indicators. 15 non-overlapping fields outside the medulla were randomly selected, and the average number of positive cells was calculated.

## Western Blotting Detection

The cells and lung tissue of each group were collected to extract the total protein, and the protein concentration was determined by BCA method. Mix the loading buffer, denature by boiling, electrophoresis, membrane transfer, sealing, primary antibody and secondary antibody incubation, all antibodies are diluted in a ratio 1:1000 and ECL method was used to develop. The band intensity was analyzed by Quantity One software, and GAPDH was used as an internal reference to detect the expression of PDCD4 protein, PTEN protein and TIMP-3 protein.

## PC3 Xenograft Tumor Experiment

Thirty SPF male BALB/c nude mouse of 6-8 weeks with body weight ($22 \pm 3$) g, were purchased from the Institute Field Surgery, Third Affiliated Hospital, Third Military Medical University. The number of Animal Qualification Certificate is No.0001517, and the number of Laboratory Animal Use License is: SYXK (Chongqing) 2017-0005. Thirty mouse were randomly divided into a control group, a DMSO group, and an AG-490 group, with different treatment methods. $1 \times 106$ PC3 cells were injected into the axilla of the mice to construct PC3 subcutaneous transplantation model. The treatment in each group is as follows: control group: normal feeding, no treatment. DMSO group: injection of DMSO once every 3 days for 16 days. AG-490 group: local injection of AG-490, 40 mg/kg, once every 3 days for 16 days. The tumor volume is measured with a caliper every 3 days, the formula is: volume = length x width 2/2.Tumor-bearing mice were sacrificed at the end of the observation period, and tumor tissue was removed for detection.

## Statistical Analysis

At least 3 independent replications were performed. Data are reported as the mean $\pm$ standard deviation (SD). Images were processed by use of Graphpad Prism 5 (GraphPad Software, La Jolla, CA, United States) and Adobe Photoshop (Adobe, San Jose, CA, United States). Student's t-test was used for analysis between 2 groups with only one factor involved. A one-way ANOVA was used for analysis when more than 2 treatments were compared. Significant differences were established at $p < 0.05$.

# Results

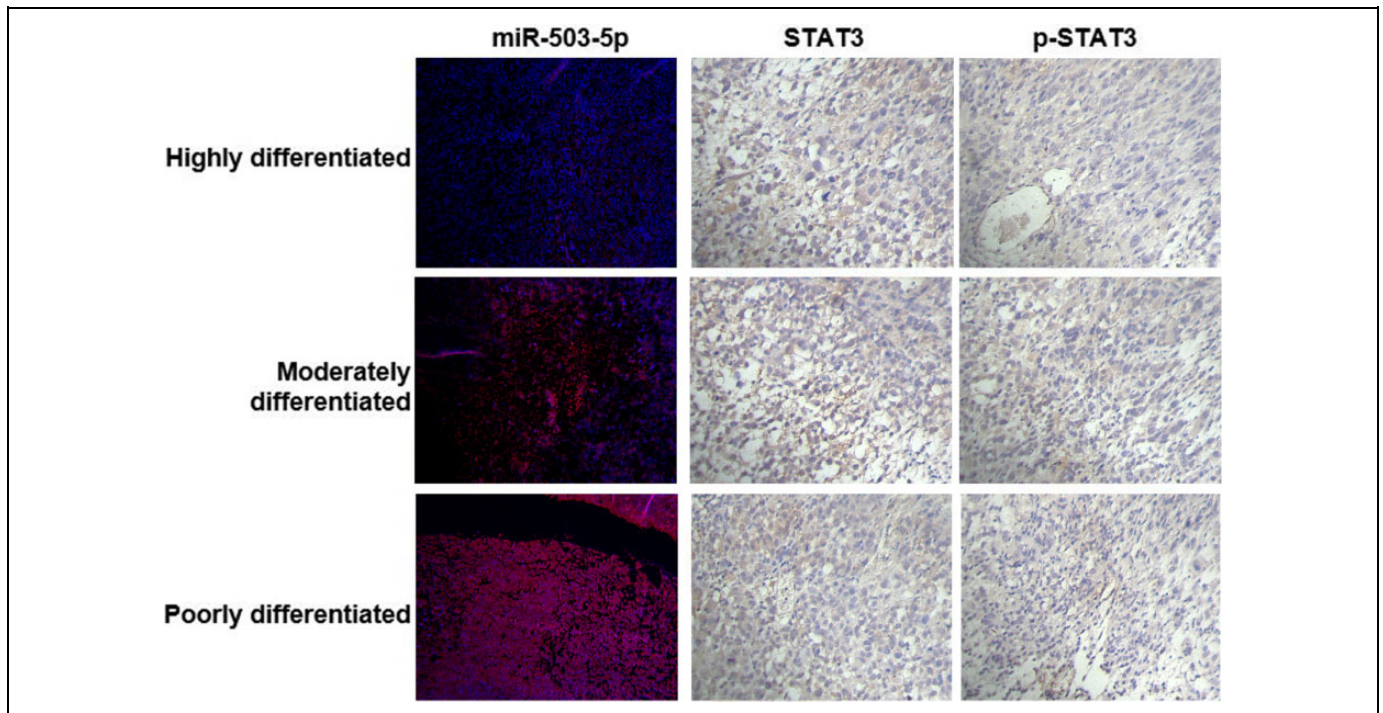## The Expression of STAT3 in Prostate Cancer Tissue Is Related to miR-503-5p

The positive expression rates of miR-503-5p in poorly differentiated, moderately differentiated, and highly differentiated prostate cancers were ($21.4 \pm 9.8$), ($45.0 \pm 15.2$), and ($91.8 \pm 9.20$), respectively (Figure 1). Immunohistochemical staining showed that the STAT3 protein mainly existed in the nucleus and cytoplasm, and the positive expression rates of STAT3 in prostate cancer tissue were ($89.2 \pm 14.5$), ($46.3 \pm 12.5$) and ($25.3 \pm 9.9$) respectively. Spearman rank correlation analysis showed that the expressions of STAT3 and miR-503-5p in prostate cancer tissues with different degrees of differentiation were highly positively correlated ($r = 0.902$, $P < 0.05$).

## AG-490 Reduces the Expression of STAT3 and Inhibit the Growth of Prostate Cancer In Vitro

Cell cloning experiments showed that after treatment with AG-490, STAT3 small molecule inhibitor, the proliferative capacity of PC3 cells and DU145 cells was significantly inhibited, and no significant effect on cell proliferation in the control group (Figure 2A). The survival rate of PC3 cells treated with AG-490 was 56.67%; the survival rate of DU145 cells treated with AG-490 was 62.33% (Figure 2B). Transwell test showed that the number of invasive cells in the AG-490 treated group was reduced compared with the control cells, PC3 cells decreased from ($65.00 \pm 11.14$) to ($17.00 \pm 4.58$), and DU145 cells decreased from ($66.00 \pm 11.53$) to ($16.33 \pm 3.79$) (Figure 2C). Compared with the cells in the control group and DMSO group, the apoptotic cells in AG-490-treated cells increased significantly (10.56% in the PC3 group and 9.77% in the DU145 group) (Figure 2D). Western blotting analysis showed that AG-490 can effectively inhibit the expression of MMP-2 and Ki-67 and Bcl-2 in PC3 and DU145 cells (Figure 2E). Luciferase reporter assay demonstrated that miR-503-5p could bind to PDCD-4, TIMP-3 and PTEN mRNA and inhibit its expression (Figure 2F).

## AG-490 Reduces the Expression of STAT3 and Inhibits the Growth of Prostate Cancer In Vivo

AG-490 inhibits PC3 growth in a xenograft model. In vitro experiments indicate that STAT3 is a potential target for the treatment of prostate cancer. We conducted a proof-of-

**Figure 1.** Expression of STAT3, p-STAT3 and miR-503-5p in prostate cance. Immunohistochemical staining of STAT3, p-STAT3 and miR-503-5p in prostate cancer tissue.

principle experiment using a PC3 cell xenograft model and AG-490 treatment with DMSO. Ten mice were injected with DMSO in situ as a negative control group, and the other 10 were treated with PBS as a control group. The mean tumor volume of the mice in the control group, DMSO group and AG-490 group had no statistically significant difference at the beginning of treatment ($P > 0.05$). The treatment was monitored every 3 days for 3 consecutive weeks, and the tumor volume of each group of mice was measured. The tumor volume of the AG-490 treatment group was significantly reduced. HE staining showed that the number of atypical tumor cells and necrotic cells in the tumor specimens of the AG-490 group decreased, the volume was reduced (Figure 3A), and the microvessel density and chromatin staining of the tumor tissue decreased. TUNEL staining showed that the number of apoptotic cells in the AG-490 group increased significantly, indicating that inhibition of STAT3 caused tumor cell apoptosis and helped to inhibit the growth of transplanted tumors (Figure 3B). The expression of miR-503-5p in the transplanted tumor of the AG-490 treatment group was significantly reduced ($P < 0.05$) (Figure 3C).

### Immunohistochemistry of Tumor Tissue

Immunohistochemical examination showed that the expression of STAT3 protein and p-STAT3 protein in the tumor tissue of the AG-490 group was significantly inhibited. At the same time, the expression of Ki-67, MMP-2 and Bcl-2 in the tumor tissue also decreased, which is the same as that in vitro

(Figure 4A-B). IHC staining showed that the expression of PTEN, PDCD-4 and TIMP-3 in the tumor tissues of the AG-490 group increased significantly (Figure 4C-D).

### Western-Blot Detection of Protein in Tumor Tissue

AG-490 caused a 50-60% decrease in luciferase activity, indicating that STAT3 promotes miR-503-5p gene transcription (Figure 5A). We tested the expression of miR-503-5p biological targets (PDCD4, PTEN and TIMP-3) at protein levels. Western-blot results showed that compared with the control group and DMSO group, the expression levels of PDCD4 protein, PTEN protein and TIMP-3 protein in the AG-490 group of PC3 cells were significantly reduced. To further illustrate the hypothetical STAT3-miR-503-5p regulatory axis, we recruited IL-6 to up-regulate the activity of STAT3 and tested the expression of miR-503-5p and biological targets. The expression of miR-503-5p was increased in IL-6 treated PC3 cells. PDCD4, PTEN and TIMP-3 protein expression levels increased (Figure 5B).

### Discussion

Prostate cancer is a malignant tumor with high incidence worldwide, with high malignancy, poor prognosis, and high mortality. Epidemiological survey shows that the incidence of prostate cancer is increasing year by year. Although surgical resection is an effective method for the treatment of prostate cancer, due to the lack of effective early diagnosis, its 5-year survival rate is very low, from a rapid decline of
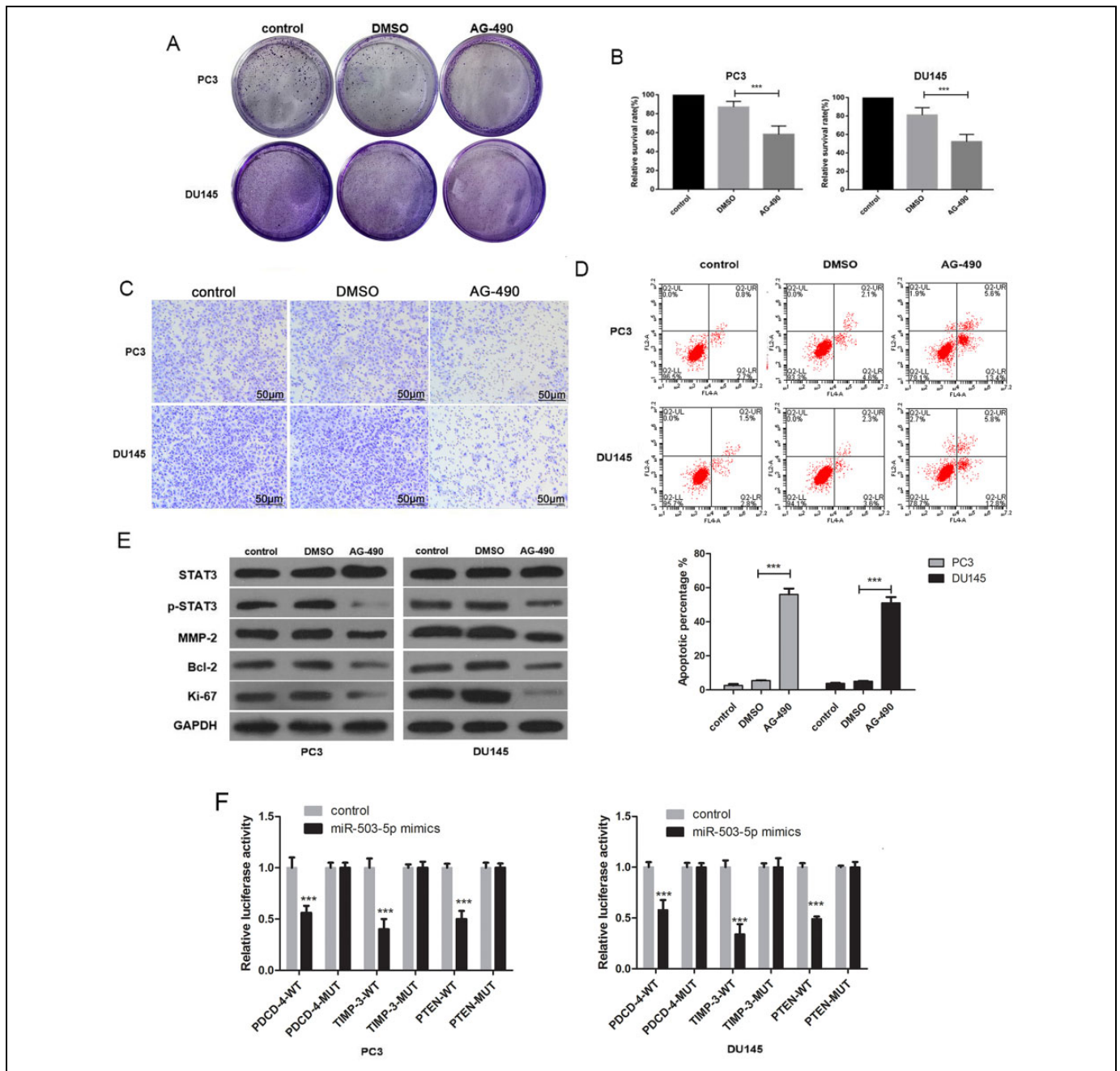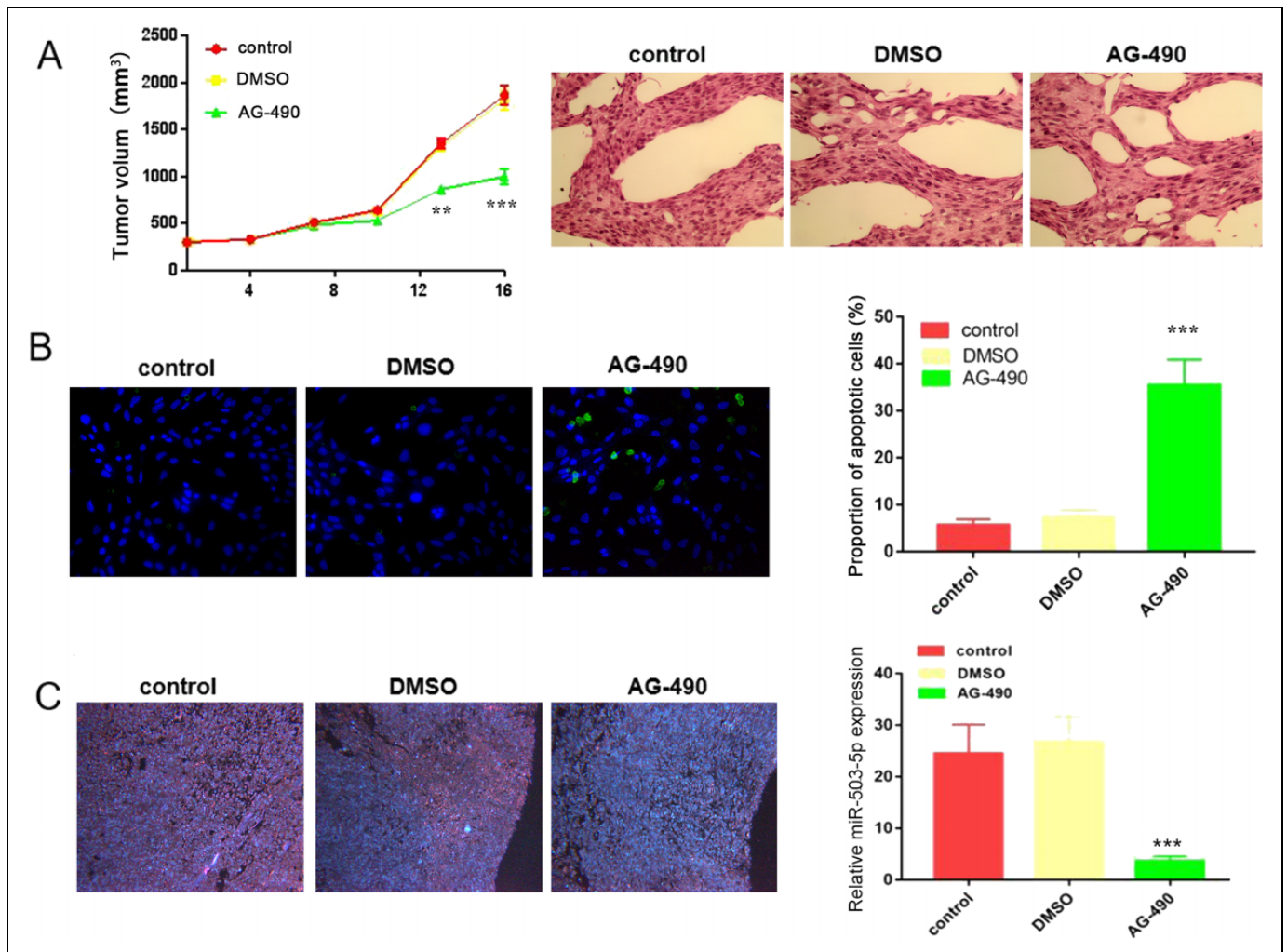
**Figure 2.** AG-490 reduces STAT3 expression and inhibits prostate cancer growth *in vitro.* A: Representative images of cell colony formation. B: CCK-8 assay was used to detect cell viability. C: Transwell assay was conducted to evaluate cell migration and invasion (magnification 200×). D: Apoptotic rates were analyzed by using flow cytometry. E: The protein expression was examined by Western blot. F: Luciferase activity in cells cotransfected with miR-503-5p mimics and luciferase reporters containing PDCD-4, TIMP-3, PTEN or mutant transcripts.

about 50% in early patients to 20% in advanced patients.[8] Most patients do not have the opportunity to undergo radical surgery and can only rely on adjuvant therapy such as radiotherapy and chemotherapy. This may prolong the life of the patient to a certain extent, but the effect is relatively low. Whether it is imaging technology or serological markers, the sensitivity and specificity of prostate cancer diagnosis are relatively poor.[9] Therefore, in order to improve the

prognosis of prostate cancer patients, there is an urgent need to develop diagnostic markers with higher specificity and less aggressiveness. miRNAs are a class of small noncoding RNAs that have important biological functions, by binding to the complementary sequences in the 30 untranslated regions of their target mRNAs, they cause mRNA degradation or inhibit translation.[10] The regulation of miRNA on gene expression plays an important role in tissue

**Figure 3.** AG-490 reduces the expression of STAT3 and inhibits the growth of prostate cancer *in vivo*. A: cells were subcutaneously injected in nude mice. Tumor volume was evaluated. B: TUNEL analysis of cell apoptosis. C: Immunofluorescence staining analysis of miR-503-5p expression.

differentiation, cell proliferation and apoptosis. miRNAs have different expressions in different types of tissues and play a key role in tumorigenesis. Cancer is a complex genetic disease caused by uncontrolled gene expression. Prostate cancer is one of the most deadly cancers in humans, traditional diagnosis and treatment methods focus on understanding the mechanism of prostate cancer development and searching for new diagnosis and effective treatment methods.miR-503-5p has a significant effect on the tumor epithelial-mesenchymal transition (EMT) process, and has different effects on the occurrence and development of malignant tumors. Studies[11] have reported that the most basic pathogenesis of prostate cancer includes activation of the STAT family and regulation of abnormal expression of miRNAs. In this study, we found that miR-503-5p and STAT3 had higher co-expression in human prostate cancer tissues compared to adjacent tissues. In some medium and large-scale map experiments aimed at detecting

dysregulated miRNAs in human cancer, miR-503-5p is one of the most overexpressed miRNAs. In breast cancer, glioma, hepatocellular carcinoma, oral squamous cell carcinoma, myeloma, and colon cancer, overexpression of miR-503-5p can increase cell proliferation, migration, and invasion.[12]

A systematic review and meta-analysis showed that the miR-503-5p test can effectively assess the prognosis of cancer patients, especially in gastrointestinal tumors.[13] The high expression of miR-503-5p is related to the overall survival of gastric cancer. Some researchers believe that miR-503-5p is a potential biomarker for tumor therapy. Therefore, the mechanism of upregulation of miR-503-5p in human tumors needs further study. Several studies have confirmed that miR-503-5p is induced by different mechanisms, such as gene mutations, single nucleotide polymorphisms, methyltransferases, and histone deacetylases. LPS activation of Toll ligand receptor can up-regulate miR-
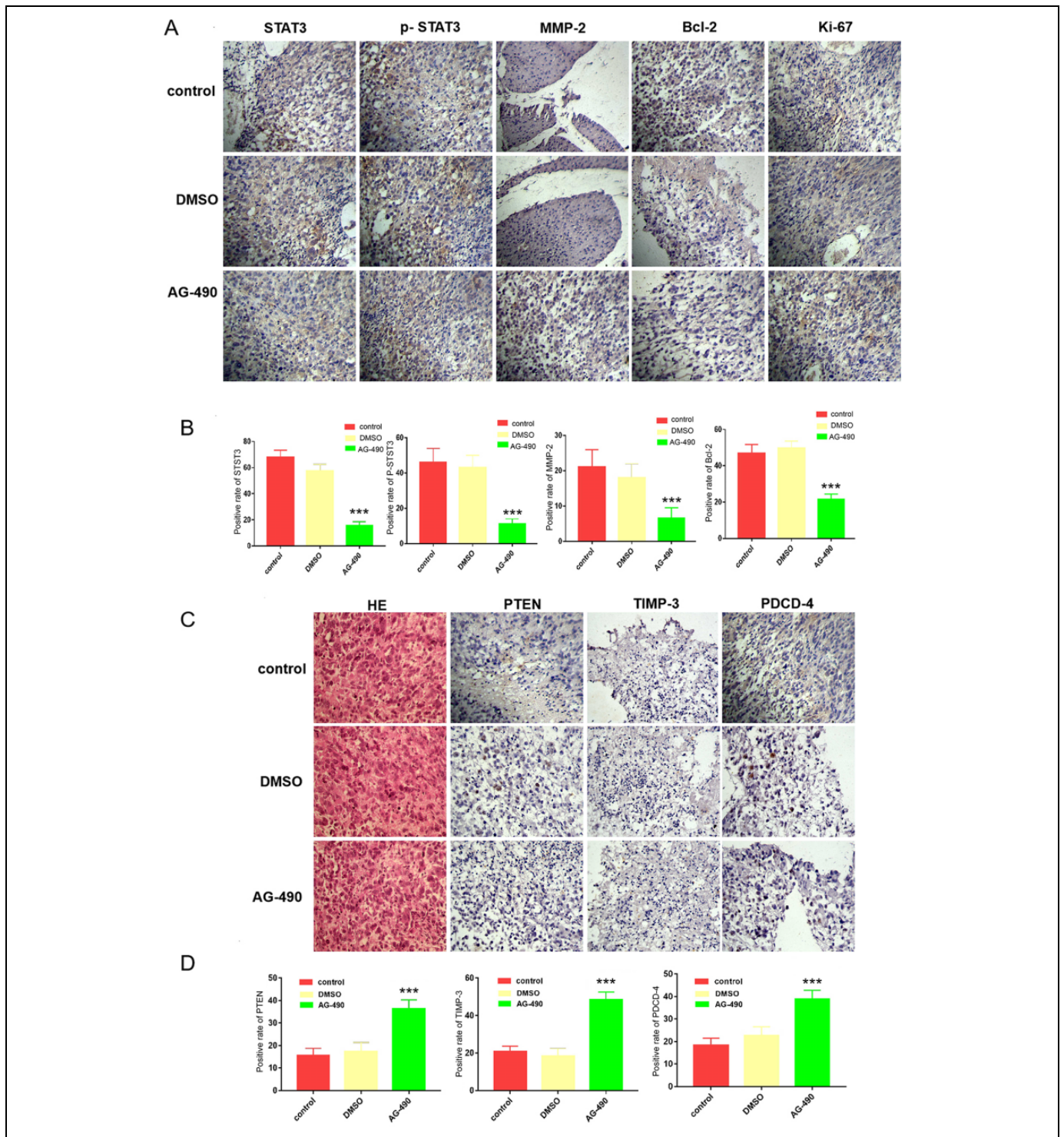
**Figure 4.** Immunohistochemistry of rat tumor tissue. A: Representative photographs of IHC analysis of STAT3, p-STAT3, MMP-2, Bcl-2 and Ki-67 in indicated tumors of mice. B: IHC quantitative analysis of the expression of ROCK, PTEN, p-PI3 K, and p-cofilin-1; C: Representative HE staining of the indicated tumors of mice. Representative photographs of IHC analysis of PTEN, TIMP-3 and PDCD4. D: IHC quantitative analysis of the expression of PTEN, TIMP-3 and PDCD4.
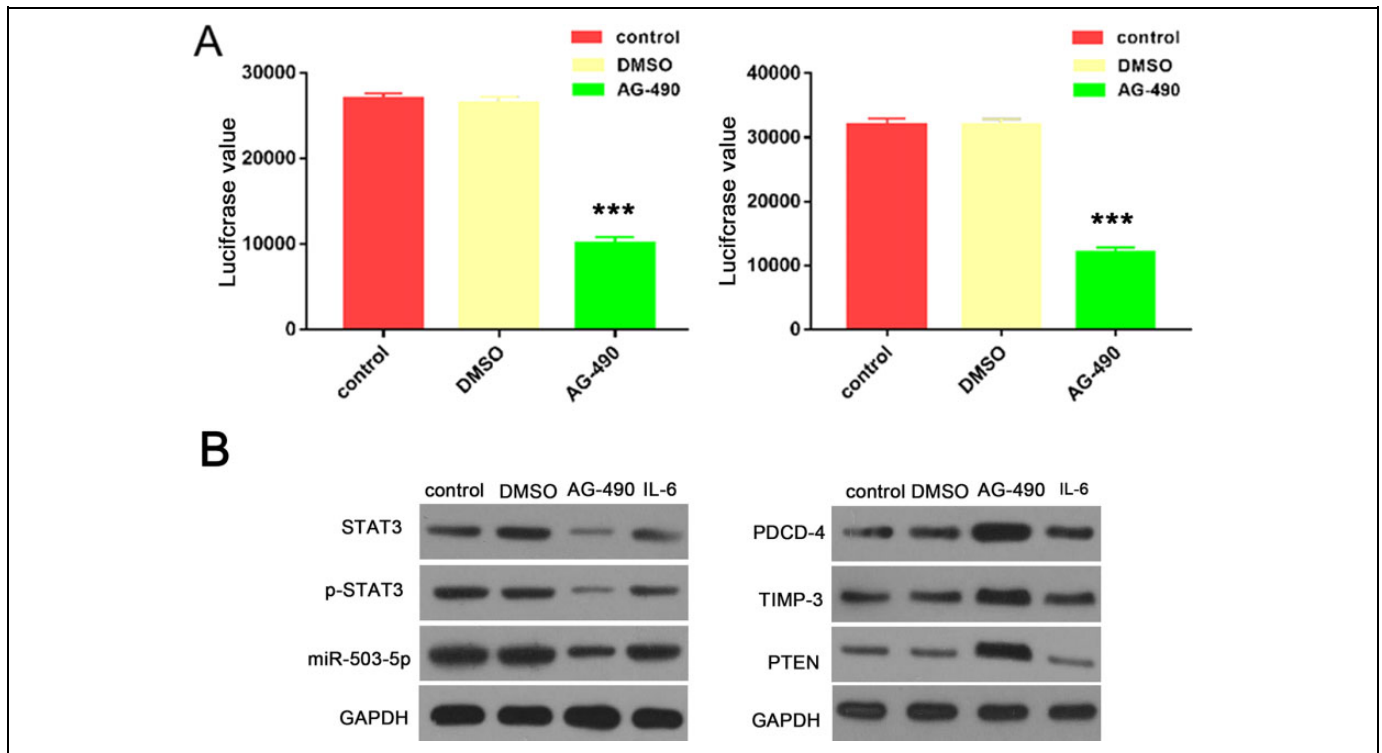
**Figure 5.** Western blotting detection of protein in rat tumor tissue. A: Luciferase activity in cells treated with or without AG-490. Data are presented as the relative ratio of firefly luciferase activity of Renilla luciferase activity. B: Western Blot analysis of STAT3, p-STAT3, miR-503-5p, PTEN, TIMP-3 and PDCD4 expression in PC cells.

503-5p in various cell types, including macrophages, fibroblasts and peripheral blood mononuclear cells. IL-6 induces miR-503-5p expression through STAT3 activation and promotes the survival of multiple myeloma cells. The results of in situ hybridization and immunohistochemistry showed that STAT3 and miR-503-5p gene may have a linearly related expression mechanism in the promoter region. We used a specific STAT3 inhibitor AG-490 to suppress STAT3 activation in PC3 and DU145 cells. In prostate cancer cell lines and animal models, the expression of miR-503-5p was significantly down-regulated after treatment with AG-490. The tumor suppressor genes PTEN, TIMP-3 and PDCD4 are the functional targets of miR-503-5p. Inhibition of STAT3 can cause up-regulation of protein expression levels. IL-6-induced STAT3 activation can suppress the expression of these genes. Luciferase analysis showed that the miR-503-5p promoter region contains a STAT3 regulatory sequence. The results suggest that there is a signal transduction pathway STAT3/miR-503 transcriptional regulation axis in prostate cancer. Early studies have shown that HNSCC and its derived cell lines have significantly increased p-STAT3 levels in STAT protein family members,[14] and the structural activation of STAT3 has been confirmed in many types of cancer, including breast cancer, prostate cancer and thyroid cancer. The increase of STAT3 level changed the cell cycle process and promoted the proliferation and survival of tumor cells. AG-490 can significantly reduce the cloning ability of cancer cells in vitro and in vivo on prostate cancer cells, increase the apoptosis rate, and increase cancer cell migration, invasion inhibition and cell cycle arrest in G1 phase. We speculate that the miR-503-5p reduction caused by AG-490 treatment may partially explain the mechanism of this result. In prostate cancer cells, due to the down-regulation of miR-503-5p expression, PTEN expression is increased and the AKT signaling pathway is inhibited. The AKT pathway participates in tumor survival and metastasis by regulating Bcl-2 and MMP2/9. TIMP3, a protease regulatory factor combined with ECM, is a key inhibitor of several MMPs, and its expression has a predictive effect on the prognosis of cancer. Therefore, the restoration of TIMP-3 protein is related to the reduction of prostate cancer cell migration and invasion. According to reports, PDCD4 inhibits AP-1 mediated transactivation and induces the expression of cyclin-dependent kinase inhibitor p21.Therefore, the upregulation of PDCD4 leads to the arrest of the cycle of oral squamous cell carcinoma cells in G1 phase. Our results provide evidence that AG-490 inhibits STAT3, upregulates PTEN, PDCD4, and TIMP-3 by inhibiting miR-503-5p, and the role of the STAT3/miR-503-5p pathway in prostate cancer growth in vitro and in vivo.

In summary, this study found that AG-490 can inhibit the growth of prostate cancer cells in a miR-503-5p-dependent manner by targeting STAT3, and is expected to become a new candidate drug for the treatment of prostate cancer.

## Authors' Note

## Declaration of Conflicting Interests

## Funding

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