

## ORIGINAL ARTICLE

# The effect of the overexpression of miR-490 on the biological function of prostate cancer cell PC-3

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## Summary

**Purpose:** To investigate the effect and molecular mechanism of miR-490 in prostate cancer.

**Methods:** qRT-PCR was used to detect the expression of miR-490 in human prostate cancer PC-3 cell line (the control group B) and normal human prostate cells (the control group A), the overexpression vector (the positive group) and human prostate cancer PC-3 cells transfected by negative control (the negative group) were built, and the results of the transfection were detected by qRT-PCR.

**Results:** The relative expression levels of miR-490 in the cells in the control group A, the control group B and the negative group were lower than that in the positive group ( $p < 0.05$ ).

The OD values of the cells in the negative and positive groups both increased continuously over time ( $p < 0.05$ ). The OD values of the cells in the positive group at each time point were lower than those in the negative group ( $p < 0.05$ ).

**Conclusion:** The low expression and overexpression of miR-490 in prostate cancer may inhibit the proliferation, migration and invasion of prostate cancer cells and promote apoptosis by inhibiting the phosphorylation of AKT signal pathway.

**Key words:** biological behavior, miR-490, PC-3, AKT, prostate cancer

## Introduction

Prostate cancer is an epithelial malignant tumor that seriously endangers the life of males. It is a disease that has the highest morbidity in the male reproductive system malignant tumors and one of the main causes of male cancer death, accounting for 26% of the total number of new cancer cases and 9% of the death toll that is related to cancer, just second to lung cancer [1,2]. In recent years, with the increase of the aging of the population, the incidence of prostate cancer is also on the rise, especially for male over 70 years old [3]. At present, the pathogenesis of prostate cancer is not completely clear; it is currently believed that the occurrence of prostate cancer is affected by

some complex and comprehensive factors such as genes, environment and sex hormones [4]. The early symptoms of prostate cancer are often not obvious and the diagnosis is late. Current treatments, including surgery, radiotherapy, chemotherapy and hormone therapy, have limited efficacy in the treatment of prostate cancer [5-7]. Therefore, it is very necessary to find new therapeutic targets.

Gene therapy has always been a hot trend in the therapy and research of cancer. MicroRNAs, small, non-coding, endogenous RNAs, influence the pleiotropy of gene expression [8,9], and have been widely used in cancer treatment. In recent years, some studies have reported the effect of miR-490

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in malignant tumors such as breast cancer, ovarian epithelial cancer, and renal cancer; the low expression and overexpression of miR-490 in these tumors effect the inhibition of tumor cell growth and also display other effects [10-12]. We speculated that miR-490 may also play a role as tumor suppressor gene in the occurrence and development of prostate cancer. However, in recent reports, there are only few studies about miR-490 and prostate cancer, and the effects of miR-490 on the biological behavior of prostate cancer cells is unclear.

In this study, the overexpression of miR-490 and its effects on the biological behavior of prostate cancer cell line PC-3 were studied to provide the experimental basis for finding new targets in the treatment of prostate cancer.

## Methods

### *The source of the cells*

Human prostate cancer cells line PC-3 (item number(R)Cells-0367) and normal human prostate cells (item number(R)Cells-0345) were both purchased from Shanghai Qiming Biotechnology Co., Ltd. They were cultured in RPMI1640 medium (Shanghai Haoran Biotechnology Co., Ltd.) +10% fetal calf serum (Shanghai Yiwon Biological Products Co., Ltd.) + 100 µ/mL penicillin-streptomycin solution (Shanghai Rongweida Industrial Co., Ltd.), with pH from 7.2 to 7.4, 37°C, 5% CO<sub>2</sub>, and humidity from 70 to 80%.

### *The transfection of the cells*

The overexpression vector and negative control of miR-490 were designed and synthesized by Thermo Fisher (China) Technology Co., Ltd. The suspension of PC-3 cells that were digested by trypsin was 5\*10<sup>6</sup>, and were inoculated in 6-well plates. The cells were transfected when the cell confluence was up to 40%, and the concentration of the transfection was 20 nM. After 24 hrs of culture, the cells and the two vectors were cultured in RPMI1640 medium (it contained 10% fetal calf serum), then they were incubated for 48 hrs in an incubator at 37°C and 5% CO<sub>2</sub>. They were divided into the positive group and the negative group. The results of the transfection were detected by qRT-PCR. The transfection kit was Lipofectamine™ 2000, purchased from Nanjing Kebai Biotechnology Co., Ltd, Nanjing, China). Normal human prostate cells were used as the control group A and untreated PC-3 cells were used as the control group B.

### *qRT-PCR*

qRT-PCR was used to detect the relative expression level of miR-490. The cells that were transfected for more than 48 hrs in the three groups were collected. TRIzol reagent (American Invitrogen Co.) was used to split the cells to extract their total RNA. After the cells were split, chloroform was used to extract and remove the supernatant. After the precipitate was dried, it was dissolved in water that lacked RNase. The concentration and purity of the extracted total RNA were analyzed by using an ultraviolet/visible light spectrophotometer (American NanoDrop Co.). The A260/A280 value between 1.8 and 2.1 was considered to meet the experimental requirement, and 1% agarose gel electrophoresis (Shanghai Lianshuo Baowei Biotechnology Co., Ltd.) was used to analyze the integrity of RNA. Reverse transcription reaction: 5\*Reverse Transcription Buffer 2 µL, RNase Inhibitor 0.5 µL, AMV reverse transcriptase 0.5 µL, dNTPmixture 1 µL, primer 1 µL, total RNA 2 µg, non-ribonuclease distilled water was added to 20 µL. The reaction condition was 16°C 30 min, 40°C 30 min and 85°C 5 min. After the synthesis of the first chain of cDNA was finished, the amplification reaction was carried out, and the amplification condition of PCR was thermal, including pre-denaturation at 52°C for 1 min, denaturation at 92°C for 5 min, 92°C for 10 s, and 56°C for added 50 s, a total of 40 cycles, and the final melting curve was at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Reaction system: cDNA template: 1.0 µL, 2\*SYBRGreen Realtime PCRmix 10 µL, the upstream primer and the downstream primer 1 µL each, and sterilized deionized water was added to 20µL. GAPDH was used as the reaction internal reference. Each sample was tested in triplicate and the results were analyzed by using 2<sup>-ΔΔCt</sup> method. The reverse transcription kit was purchased from Japanese Takara Co. and the RT qPCR kit/SYBR Green Realtime PCR Master Mix was purchased from Shanghai Xinhaino Biology Co., Ltd, Shanghai, China). The primer sequences are shown in Table 1.

### *Western blot*

Western blot was used to detect the expression level of AKT and phosphorylated AKT (p-AKT) protein. The protein in prostate cancer PC-3 cells was extracted by using the repeated freeze-thawing method. Polyacrylamide gel electrophoresis (Beijing Dingguo Changsheng Co.) was used to split the protein, and the original voltage was 90 V. After the electrophoresis was finished, the membrane was transferred to PVDF membrane. The condition was in 100 V constant voltage for 100 min, and it was closed at 37°C for 60 min. Next, blocking and antibody hybridization were carried out. The membrane

**Table 1.** The primer sequences

	<i>Upstream</i>	<i>Downstream</i>
miR-490	5'-TGCGGTTCAAGTATCAGGA-3'	5'-CCAGTGGGGGTCCGAGGT-3'
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

was incubated with the first antibody (mouse anti-human AKT monoclonal antibody or mouse anti-human p-AKT monoclonal antibody) overnight at 4°C, and it was washed three times by using PBS the next day, 10 min each time. Then it was incubated with goat anti-rabbit IgG secondary antibody, which was labeled by HRP, for 2 hrs at 37°C. After this step was finished, the enhanced chemiluminescence (ECL) reagent was used to develop and photographing fixing. Quantity One software was used to statistically analyze the band that had been scanned by using film. The relative expression level of the protein equalled the gray value of the band/the gray value of the internal reference. All the antibodies were purchased from American Abcam Co., and the western blot test kit was purchased from Shanghai Biyuntian Co.

#### MTT cell proliferation experiment

MTT cell proliferation experiment was used to detect the condition of the proliferation of prostate cancer PC-3 cells in the two groups. These cells were made into cell suspension in single row, and were routinely inoculated at a concentration of  $4 \times 10^6$  cells/mL. The 96-well culture plate was used to culture the cells, and 5 collection time points were set, namely the 24, 48, 72th, 96, and the 120th hour. Three parallel well plates were set at each time point. When the culture time of the cells was up to each time point, 20  $\mu$ L MTT (5 mg/mL) solution was added into the holes, then the solution was further cultured at 37°C for 4 hrs. The supernatant containing impurities was removed, the dimethyl sulfoxide preparation was added into the solution which was placed on a horizontal shaking table for 10 min. Finally, in the light absorption microplate reader VersaMax (Meigu molecular instrument' Shanghai Co., Ltd.), the absorbance at 570 nm was measured. The MTT test kit was purchased from American Sigma-Aldrich Co.

#### Tunel cell apoptosis experiment

Four percent of neutral formaldehyde was used to fix the prostate cancer PC-3 cells ( $5 \times 10^7$ /mL) that were at the 48th hour at room temperature for 10 min. PBS was used to wash them twice to remove superfluous liquid, with a duration of each time 5 min. PBS that contained 2% peroxide was disposed at room temperature for 5 min to remove superfluous liquid, and it was washed twice with PBS for 5 min. Cell staining was performed according to the instructions of the Tunel kit (Xiamen Huijia Biotechnology Co., Ltd., Xiamen, China). Image analysis software (Image-proPlus5.0) was used to count the number of Tunel-positive cells under five  $40 \times 10$ x field; the cumulative optical density value represented the total number of Tunel-positive cells, and this step was repeated 3 times.

#### Scratch wound healing assay

After prostate cancer PC-3 cells had been transfected for 48 hrs, they were cultured until the cell confluence became 90%. A straight line of scratching in one direction for 3 times in each hole was performed. Inverted microscope (Guangzhou Mingmei Photoelectric Technology Co., Ltd., Guangzhou, China) was used after

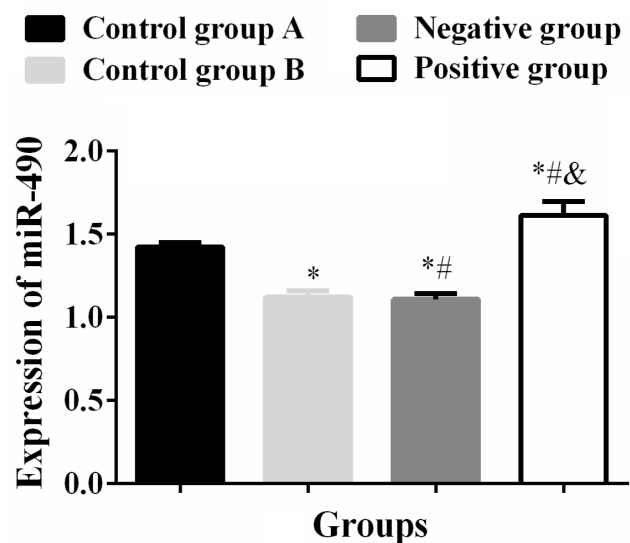
the 0th hr, the 12th hr, and the 24th hr to observe the scratch width.

#### Transwell cell invasion experiment

Transwell chambers were preliminarily coated with matrigel, and prostate cancer PC-3 cells were made into a single array of  $1 \times 10^5$ /mL/mL cell suspension. A hundred  $\mu$ L of them were inoculated in Transwell chambers, and a total of 100  $\mu$ L of RPMI1640 medium + 10% of fetal calf serum + cell suspension were added into the upper chamber; a total of 500  $\mu$ L of RPMI1640 medium + 20% fetal calf serum + cell suspension were added into each chamber and were cultured at 37°C and 5% of CO<sub>2</sub> for 48 hrs. They were stained with crystal violet and the number of the cells that passed was counted. Six chambers were evaluated, 6 fields of view were counted in each chamber and 3 parallel experiments were performed simultaneously. Transwell chambers and the related reagents were purchased from Shanghai Yuanzi Biotechnology Co., Ltd, Shanghai, China.

#### Statistics

SPSS19.0 was used. Percents were used to express the quantitative data and  $\chi^2$  test was used in the comparison of qualitative data. Mean  $\pm$  standard deviation was used to express the enumeration data; t-test was used



**Figure 1.** The test results of the relative expression levels of miR-490 in the cells tested by qRT-PCR in the four groups. The relative expression levels of miR-490 in the cells in the four groups were significantly different ( $p < 0.05$ ); the relative expression levels of miR-490 in the cells in the control group A, the control group B and the negative group were lower than that in the positive group ( $p < 0.05$ ); the relative expression levels of miR-490 in the cells in the control group B and the negative group were significantly lower than that in the control group A ( $p < 0.05$ ); there was no significant difference in the relative expression level of miR-490 in the cells between the control group B and the negative group ( $p > 0.05$ ). \*Compared with the control group A,  $p < 0.05$ ; #Compared with the control group B,  $p < 0.05$ ; and compared with the negative group,  $p < 0.05$ .

in the comparison between the two groups. One way ANOVA was used in the comparison between the groups. The repeated ANOVA measurement experiments were used in the comparison within the groups at different time points. Spearman's correlation analysis was used to analyze the correlation between the relative expression level of miR-490 in PC-3 cells and p-AKT protein.  $P < 0.05$  was considered as statistically significant.

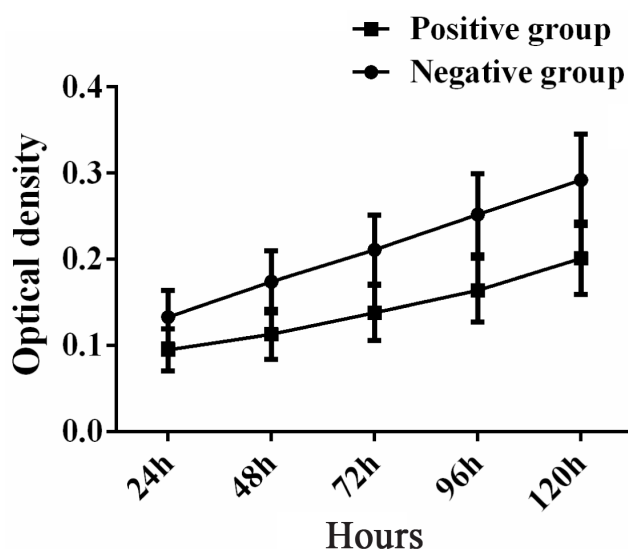
## Results

### The test results of qRT-PCR

The test results of the relative expression levels of miR-490 in the cells tested by qRT-PCR in the four groups showed that the relative expression levels of miR-490 in the cells in the four groups were significantly different ( $p < 0.05$ ). The relative expression levels of miR-490 in the cells in the control group A, the control group B and the negative group were significantly lower than those in the positive group ( $p < 0.05$ ). The relative expression levels of miR-490 in the cells in the control group B and the negative group were significantly lower than those in the control group A ( $p < 0.05$ ). There was no significant difference in the relative expression level of miR-490 in the cells between the control group B and the negative group ( $p > 0.05$ ; Figure 1).

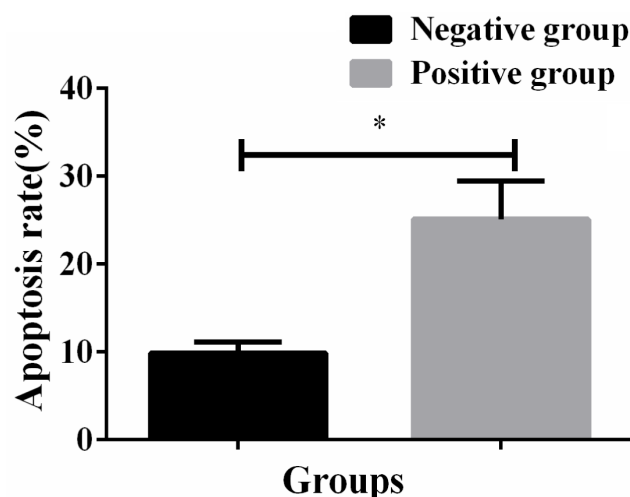
### The test results of MTT cell proliferation experiments

The test results of MTT cell proliferation experiments of the cells in the negative and positive

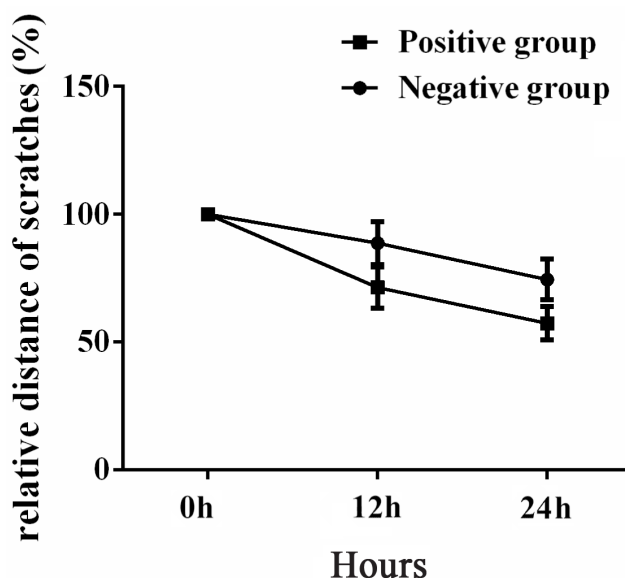


**Figure 2.** The test results of MTT cell proliferation experiments of the cells in the negative and positive groups. The OD values of the cells in the negative and positive groups increased continuously over time ( $p < 0.05$ ); the OD values of the cells in the positive group at each time point were lower than those in the negative group ( $p < 0.05$ ).

groups showed that the OD values of the cells in the negative and positive groups increased continuously over time ( $p < 0.05$ ), and the OD values of the cells in the positive group at each time point were lower than that in the negative group ( $p < 0.05$ ; Figure 2)



**Figure 3.** The test results of TUNEL apoptosis experiments of the cells in the negative and positive groups. There were significant differences in the apoptosis rate of the two groups; the apoptosis rate of the positive group was higher than that of the negative group ( $p < 0.05$ ). \*mean  $p < 0.05$ .

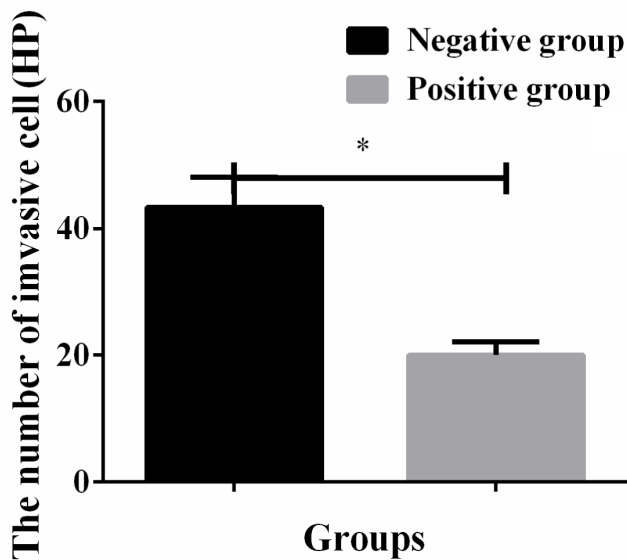


**Figure 4.** The test results of scratch wound healing assay of the cells in the negative and positive groups. There was no statistical difference when the scratch width of the cells between the two groups at the zero hour was compared ( $p > 0.05$ ); the scratch width of the cells in the two groups was lower than that in the previous time point from the 12th hour to the 24th hour ( $p < 0.05$ ), but the scratch width of the cells in the negative group was lower than that in the positive group ( $p < 0.05$ ).



### The test results of Tunel apoptosis experiments

The test results of Tunel apoptosis experiments of the cells in the negative and positive groups showed that the apoptosis rate of the negative group was  $9.87 \pm 1.25$ , and the apoptosis rate of the positive group was  $25.12 \pm 4.32$ . There was a significant difference in the apoptosis rate of the



**Figure 5.** The test results of Transwell cell invasion experiments of the cells in the negative and positive groups. There were significant differences in the number of transmembrane cells in the two groups, and the number of transmembrane cells in the positive group was lower than that in the negative group ( $p < 0.05$ ). \*mean  $p < 0.05$ .

two groups, and the apoptosis rate of the positive group was higher than that of the negative group ( $p < 0.05$ ; Figure 3).

### The test results of scratch wound healing assay

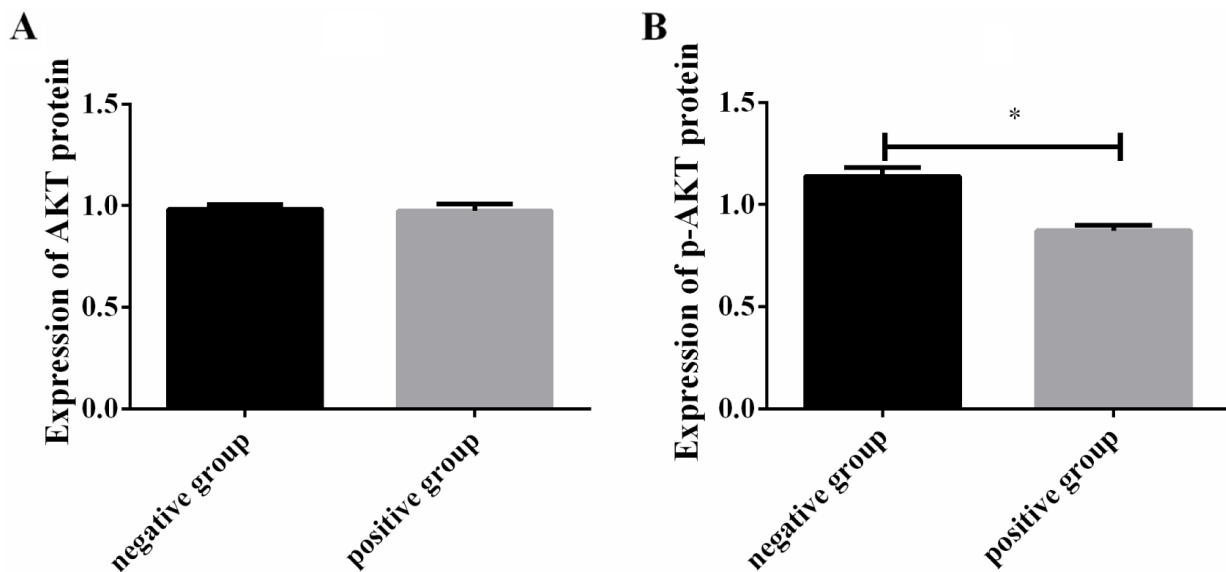
The test results of scratch wound healing assay of the cells in the negative and positive groups showed that the scratch width of the cells between the two groups was not statistically different at zero hr ( $p > 0.05$ ); the scratch width of the cells in the two groups was significantly lower than that in the previous time point from the 12th hr to the 24th hr ( $p < 0.05$ ), but the scratch width of the cells in the negative group was lower than that in the positive group ( $p < 0.05$ ; Figure 4).

### The test results of Transwell cell invasion experiments

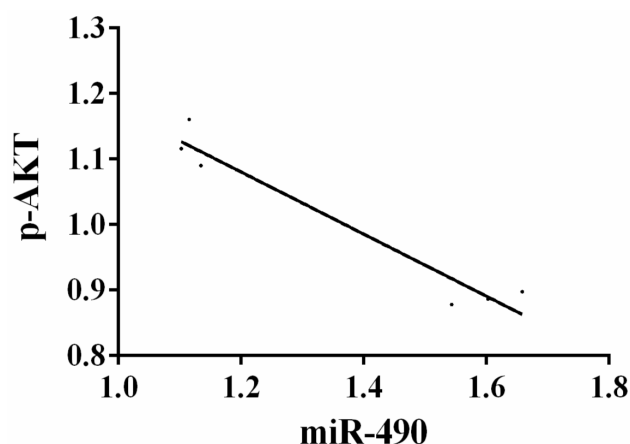
The test results of Transwell cell invasion experiments of the cells in the negative and positive groups showed that the number of transmembrane cells in the negative group was  $43.33 \pm 4.72$ , and the number of transmembrane cells in the positive group was  $20.00 \pm 2.14$  ( $p < 0.05$ ); there were significant differences in the number of transmembrane cells in the two groups, and the number of transmembrane cells in the positive group was lower compared with the negative group ( $p < 0.05$ ; Figure 5).

### The test results of western blot

The test results of the protein related to the AKT signal pathway of the cells tested by western



**Figure 6.** The test results of the protein related to the AKT signal pathway of the cells tested by western blot in the negative and positive groups. **A:** There was no difference in the relative expression levels of AKT protein between the two groups ( $p > 0.05$ ). **B:** The relative expression levels of p-AKT protein in the two groups were significantly different, and the relative expression level of p-AKT protein in the cells in the positive group was significantly lower than that in the negative group ( $p < 0.05$ ). \* mean  $p < 0.05$ .



**Figure 7.** The results of Spearman correlation analysis for the relative expression levels of miR-490 and p-AKT protein in the cells in the negative and positive groups. The relative expression level of miR-490 was negatively correlated with the relative expression level of p-AKT protein. When the relative expression level of miR-490 increased, the phosphorylation level of AKT protein decreased ( $r=-0.9701$ ,  $p=0.001$ ).

blot in the negative and positive groups showed that there was no difference in the relative expression levels of AKT protein between the two groups ( $p>0.05$ ), but the relative expression levels of p-AKT protein in the two groups were significantly different. The relative expression level of p-AKT protein in the cells in the positive group was significantly lower than that in the negative group ( $p<0.05$ ; Figure 6).

#### Spearman's correlation analysis

The results of Spearman's correlation analysis for the relative expression levels of miR-490 and p-AKT protein in the cells in the negative and positive groups showed that the relative expression level of miR-490 was negatively correlated with the relative expression level of p-AKT protein. When the relative expression level of miR-490 increased, the phosphorylation level of AKT protein decreased ( $r=-0.9701$ ;  $p=0.001$ ; Figure 7).

## Discussion

The morbidity of prostate cancer is particularly high in European and American countries, and it is reported that its morbidity and mortality are only second to lung cancer. In recent years, with the aging of the global population, the trend of the morbidity of prostate cancer has also been upward year by year, which raises important concern of the medical community about prostate cancer [13,14]. At present, there are more than 1000 microRNAs that have been discovered. Recently, many stud-

ies have reported that a variety of microRNAs are closely related to the occurrence and development of prostate cancer, such as miR-1290, miR-208, etc [15,16]. There are only few studies about miR-490 in prostate cancer. The present study aimed to test the expression level of miR-490 in prostate cancer and analyze its effect and mechanism to provide the experimental basis for finding new potential therapeutic targets for this disease.

We tested the expression levels of miR-490 in human prostate cancer PC-3 cells and human normal prostate cells. The test results of RT-PCR showed that the expression level of miR-490 in human prostate cancer PC-3 cells was significantly lower than that in human normal prostate cells. In previous reports, which are dealing with the expression of miR-490 in tumors, some studies have found that miR-490 is lowly expressed in breast cancer ovarian epithelial cancer and renal cancer, and it acts like a tumor suppressor to inhibit the growth and development of tumor cells [10-12]. Some of their results are similar to our results, such as the expression level of miR-490 in tumor cells is lower compared with normal cells. We also studied the effects of miR-490 on the biological behaviors of prostate cancer cells, such as proliferation and invasion. We constructed the overexpression vector of miR-490, which transfected human prostate cancer PC-3 cells. The test results of the expression level of miR-490 tested by RT-qPCR in the cells in the negative and positive groups showed that the expression level of miR-490 in the cells in the positive group was significantly higher than that in the negative group, suggesting that the transfection was successful. MTT proliferation experiment *in vitro*, Tunel apoptosis experiment, scratch wound healing assay and Transwell cell invasion experiment were used to respectively detect the effect of proliferation, apoptosis, migration and invasion of the cells in the negative and positive group. The test results of this study showed that the ability of proliferation, migration and invasion of human prostate cancer PC-3 cells that had the higher expression of miR-490 were significantly weaker than in those cells that had lower expression of miR-490, but the level of apoptosis was higher compared to the cells which had lower expression of miR-490. The result confirmed our initial speculation, which was that miR-490 may play the role of tumor suppressor gene in prostate cancer. In some related studies the anti-cancer effect of miR-490 was reported. Increasing the expression of miR-490 can inhibit the proliferation and migration of hepatocellular carcinoma and induce apoptosis of liver cancer cells [17]. MiR-490 can also induce apoptosis of colorectal cancer cells

and inhibits the invasiveness of cells by inhibiting the initiation of epithelial mesenchymal transition, and epithelial mesenchymal transition is one of the key mechanisms of the invasion and metastasis of tumor cells [18]. However, our study failed to test the condition of epithelial mesenchymal transition in the negative and positive groups due to the limitations of the conditions. We will further analyze and supplement our results in future studies. From the analysis of the above results, we can believe that the overexpression and low expression of miR-490 in prostate cancer can inhibit the proliferation, migration and invasion of prostate cancer cells, promote apoptosis and play a role as a tumor suppressor gene. Finally, we tested the possible mechanism of miR-490 which regulated prostate cancer cells. Studies have reported that miR-490 inhibits the growth of renal cancer by targeting and inhibiting the AKT signal pathway [12]. AKT signal pathway is a classical way to regulate the proliferation and invasion of cells. It has been reported in many studies that AKT signal pathway is closely related to the occurrence, development, and treatment of prostate cancer [19-21]. In our study we tested the expression level of AKT and p-AKT protein in the cells in the negative and positive groups, and found no difference in the expression level of AKT

protein in the cells between the two groups, while the expression level of p-AKT protein in the cells in the positive group was significantly lower than that in the control group. We then analyzed the correlation between miR-490 and the expression level of p-AKT protein, and found negative correlation. As a result, we speculated that there was a certain regulatory relationship between miR-490 and p-AKT protein, and the biological behavior of prostate cancer cells was regulated in this way.

This study presents some limitations. We only used a cell line of prostate cancer and the *in vitro* experiments could not simulate the intracorporal complex tumor microenvironment, so we hope this study could encourage researchers in the field to carry out more in-depth research and provide more experimental and clinical data so as to find new potential therapeutic targets of prostate cancer.

In conclusion, the overexpression of miR-490 in prostate cancer may inhibit the proliferation, migration and invasion of prostate cancer cells and promote apoptosis by inhibiting the phosphorylation of AKT signal pathway.

### Conflict of interests

The authors declare no conflict of interests.

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