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Mechanism of Yishen Tonglong Decoction inhibiting TLR4/p38 MAPK/NF-κB signaling pathway against prostate cancer via upregulating miR-145-5p

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ABSTRACT

Objective To investigate the mechanism of Yishen Tonglong Decoction (益肾通癃汤, YSTLD) inhibiting the toll-like receptor 4/p38 mitogen activated protein kinases/nuclear factor kappa-B (TLR4/p38 MAPK/NF-κB) signaling pathway against prostate cancer by up-regulating miR-145-5p.

Methods miRNA microarray technology was used to detect the changes of miRNA expression profile in prostate cancer PC-3 cells treated with YSTLD, and miRNAs with marked differences in miRNA microarray results were screened and validated by real-time polymerase chain reaction (qRT-PCR). Lentiviral transfection of miR-145-5p into prostate cancer PC-3 cells, Cell Counting Kit-8 (CCK8) assay, and scratch assay were adopted to detect the effects of miR-145-5p on prostate cancer PC-3 cell proliferation and migration. qRT-PCR and Western blot were employed to detect the effects of miR-145-5p on TLR4/p38 MAPK/NF-κB signaling pathway and the expression levels of apoptosis-related genes caspase3, tumor necrosis factor-α (TNF-α), Bax, and Bcl-2. qRT-PCR and Western blot were used to detect the effects of serum containing YSTLD on miR-145-5p, TLR4/p38 MAPK/NF-κB signaling pathway, and the expression levels of apoptosis-related genes caspase3, TNF-α, Bax, and Bcl-2.

Results The expression levels of 35 miRNAs in prostate cancer PC-3 cells treated with YSTLD were significantly different from those in the control group, with miR-145-5p being the most significantly different; qRT-PCR validation revealed that the miR-145-5p levels in prostate cancer PC-3 cells treated with YSTLD were significantly higher than those in the DMSO control group ($P < 0.05$). After lentiviral transfection of miR-145-5p into prostate cancer PC-3 cells, miR-145-5p was found to inhibit the proliferation and migration of prostate cancer PC-3 cells. Overexpression of miR-145-5p up-regulated expression levels of caspase3, TNF-α, and Bax mRNA, and down-regulated expression levels of p38 MAPK, p65 NF-κB, and Bcl-2 mRNA in prostate cancer PC-3 cells ($P < 0.05$), while up-regulated caspase3 protein expression levels in prostate cancer PC-3 cells and down-regulated expression levels of TLR4, p38 MAPK, and p65 NF-κB protein ($P < 0.05$). Serum containing YSTLD could up-regulate the expression levels of caspase3, TNF-α, and Bax mRNA, and down-regulate the mRNA expression levels of p38 MAPK, p65 NF-κB, Bcl-2, and TNF receptor-associated factor 1 (TRAF1) in prostate cancer PC-3 cells after intervening prostate cancer PC-3 cells ($P < 0.05$). Simultaneously, it up-regulated the expression levels of caspase3 protein and down-regulated the protein

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expression levels of TLR4, p38 MARK, p65 NF-κB, and TRAF1 in prostate cancer PC-3 cells ($P<0.05$).

Conclusion YSTLD can promote apoptosis of prostate cancer PC-3 cells by up-regulating the expression level of miR-145-5p and inhibiting TLR4/p38 MAPK/NF-κB signaling pathway, which may be an important mechanism of YSTLD against prostate cancer.

1 Introduction

Prostate cancer is one of the most common malignancies in men in China [1]. Castration resistance and distant metastasis of prostate cancer are the main causes of death. Although the incidence of prostate cancer in Chinese men remains lower than that in developed countries in Europe and the United States, it still demonstrates a continuous rapid growth trend. Meanwhile, the early symptoms of prostate cancer are insidious, and most patients are already in the advanced stage or develop distant metastasis at the time of presentation, but there is no established and effective treatment currently [2-5]. At present, the pathogenesis of prostate cancer has not been fully clarified. The commonly recognized pathogenesis of prostate cancer is mainly related to prostatic inflammatory stress, tumor cell immune tolerance, oxidative/antioxidant imbalance, protease/antiprotease imbalance, abnormal cell proliferation/apoptosis, cell cycle disorders, tumor internal environment imbalance or tissue remodeling [6-8]. In the treatment of prostate cancer, surgical treatment poses the risk of incomplete resection and residual cancer foci. Western medicine treatments mainly include Luteinizing Hormone Releasing Hormone (LHRH) analogues and non-steroidal antiandrogens. They are not completely effective and will be accompanied by many side effects and adverse reactions including allergy, osteoporosis, cardiovascular system damage, male breast feminization, and sexual dysfunction during medication, which can easily transform this disease into hormone-independent prostate cancer, resulting in poor therapeutic efficacy and unsatisfactory prognosis [9]. However, the therapeutic strategy of multi-target of traditional Chinese medicine (TCM) compound has certain advantages and plays an important role in treating prostate cancer [10]. TCM can be a multi-level, phased treatment, conducive to coping with the long course of prostate cancer and complex and variable syndrome features, with prominent advantages [11]. Yishen Tonglong Decoction (益肾通癃汤, YSTLD) is a proven and effective prescription used by Professor CHEN Qihua, an instructor of the Teaching and Inheritance of Experience of Famous and Veteran Doctors of Traditional Chinese Medicine, who adopted it in the clinical treatment of male diseases for many years. It has the effects of tonifying kidney Qi, transforming stasis and dissipating nodules, and removing toxins and fighting against tumors [12]. This prescription combines reinforcing healthy Qi with eliminating pathogenic factors, which reinforces healthy Qi and transforms Qi through tonifying kidney Qi, attaching importance to transforming stasis, dissipating nodules, and removing toxins to eliminate pathogenic factors and fight against cancer. A large number of previous animal experiments and clinical studies have shown that YSTLD can not only improve the specific symptoms of nude mouse models of human prostate cancer cells, but also delay endocrine tolerance, enhance oxidative stress and inflammatory response [13-16].

It has been reported that miR-145-5p was closely associated with prostate cancer [17-19]. The key role of miRNAs in tumor development has long been demonstrated, and they are involved in almost all tumor-related processes including proliferation, apoptosis, angiogenesis, and immune response. miRNAs can act as both tumor suppressors and oncogenes by the targeted negative regulation of mRNAs to degrade or translationally repress them. MiR-145-5p is a tumor-associated miRNA that functions at multiple stages of cancer progression by regulating downstream target genes [20]. It has been reported that miR-145-5p expression was down-regulated in prostate cancer and overexpression of miR-145-5p inhibited prostate cancer growth and metastasis processes, which acted as a potential target for prostate cancer therapy [17]. LI [18] pointed out that miR-145-5p was closely related to clinicopathological parameters of prostate cancer in low-risk diseases. ZHAO et al. [19] showed that the miR-145-5p cluster promoter region and rs 4705342 gene mutation were markedly associated with prostate cancer pathogenesis in the Chinese Han population. These studies suggest that miR-145-5p may have some inhibitory effects on the development of prostate cancer. MONTALTO et al. [21] demonstrated that tumor-suppressive miR-145-5p could induce growth arrest of cancer cells by targeting toll-like receptor 4 (TLR4) and its downstream target gene tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1) in prostate cancer cells; the transient introduction of miR-145-5p could cause PC-3 cell cycle arrest in prostate cancer and transfection with miR-145-5p inhibitor may inhibit tumor formation in nude mice. JIANG et al. [22] indicated that miR-145-5p could reduce the degree of inflammatory response between body tissues by regulating the toll-like receptor 4/p38 mitogen activated protein kinases/nuclear factor kappa-B (TLR4/p38 MAPK/NF-κB) signaling pathway. miR-145-5p may therefore serve as an effective treatment for prostate cancer, and the TLR4/p38 MAPK/NF-κB signaling pathway may be a crucial targeted signaling pathway. Inflammatory responses have been shown to be directly or

indirectly involved in the pathophysiological process of prostate cancer development, promoting its invasion, metastasis, injury, and the development of the biological behavior of other malignancies [23, 24]. In the above process, TLR4/p38 MAPK/NF-κB signaling pathway has a protective effect on the body, but when it is excessively activated, it produces pro-inflammatory factors and aggravates the inflammatory response process, so inhibiting the expression of proteins related to this signaling pathway could significantly inhibit the malignant biological behavior of prostate cancer [25]. TLR4 is a receptor family member of pathogen recognition and innate immune activation, which plays an essential role in signal transduction. The relationship between TLR4 and inflammation-related diseases has been drawing attention from many scholars. The relation between the occurrence of most cancers and inflammation is inseparable. Previous studies have found that TLR4, p38 MAPK, and NF-κB can promote the development of the disease by activating a large number of immune-related cells and molecules of pro-inflammatory, chemotactic, and adhesion factors, and interacting and affecting TLR4 and its ligands, causing chronic inflammation [26]. SHIN et al. [27] showed that the proliferation and apoptosis biological processes of prostate cancer DU145 cells may be associated with the activation of inflammatory regulatory signaling pathways. NF-κB is present within eukaryotic cells and acts as a transcription factor with normal physiological functions that mediate immune responses. When the body is infected, some cytokines are transcribed to mediate the immune response to clear the invading pathogens, and inflammatory cells can also affect tumor proliferation, apoptosis, and other biological processes via NF-κB.

We further investigated the role of miR-145-5p in the anti-prostate cancer process of YSTLD based on previous studies, and whether it can affect the progression of prostate cancer by targeting and regulating the TLR4/p38 MAPK/NF-κB signaling pathway. Based on this, miRNA microarray technology was employed to detect the changes in miRNA expression profile after treating prostate cancer PC-3 cells using YSTLD. miRNAs with marked differences in miRNA microarray results were screened and validated to investigate whether YSTLD could delay the malignant biological behavior of prostate cancer and improve the local inflammatory response through miR-145-5p regulation of TLR4/p38 MAPK/NF-κB signaling pathway to achieve the purpose of preventing and treating prostate cancer.

2 Materials and methods

2.1 Experimental cells and animals

Human prostate cancer cell lines PC-3 was purchased from the Cell Bank of the National Collection of Authenticated Cell Cultures, Chinese Academy of Sciences (SCSP-532), and stored in the Science and Technology Innovation Center of Hunan University of Chinese Medicine.

Thirty specific pathogen free (SPF) male Sprague Dawley (SD) rats weighing 180 - 220 g were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. with animal certificate No. SCXK (Xiang) 2019-0004, and facility use certificate No. SYXK (Xiang) 2019-0009. This animal experiment was approved by the Animal Ethics Committee of the Hunan University of Chinese Medicine (LLBH-202202210003). The handling of all experimental animals in this study followed animal ethics.

2.2 Drugs and reagents

Phosphate buffered solution (PBS) buffer (GIBCO, USA); Roswell Park Memorial Institute (RPMI) 1640 medium (Procell Life Science & Technology Co., Ltd., China); fetal bovine serum (GIBCO, USA); trypsin (GIBCO, USA); 0.25% prote-sin-EDTA (GIBCO, USA); miR-145-5p antibody (Proteintech, USA); p38 MARK antibody (Proteintech, USA); p65 NF-κB antibody (Proteintech, USA); caspase3 antibody (Proteintech, USA); TNF-α antibody (Proteintech, USA); Bax antibody (Proteintech, USA); Bcl-2 antibody (Proteintech, USA); TRAF1 antibody (Proteintech, USA); Cell Counting Kit-8 (CCK8) assay kit (Proteintech, USA); fluorescent secondary antibody IgG (Proteintech, USA); RNA extraction kit (Bioflux, USA); reverse transcription kit (Promega, USA); 2 × Taq PCR Master mix (NovoTech, Slovakia); PureLink RNA Mini Kit (TFS, USA).

2.3 Main instruments

Superclean bench (AIRTECH, China, SW-CJ-2F); precision analytical balance (Mettler-Toledo, Switzerland, ME204E); real-time PCR System (Bio-Rad, USA, CFX96); CO_2 incubator (TFS, USA, TS330); vortex mixer (SCILOGEX, USA, S40-02); microplate reader (Bio-tek, USA, ELX800); high-speed refrigerated centrifuge (Thermo Fisher Scientific, China, Fresco 17); Axio Observer inverted fluorescence microscope (ZEISS, Germany, T-6700).

2.4 Preparation of serum containing YSTLD

In this study, the original formula of YSTLD consists of Buguzhi (Psoraleae Fructus) 15 g, Shudihuang (Rehmanniae Radix Preparata) 15 g, Huangqi (Astragali Radix) 30 g, Sanleng (Sparganii Rhizoma) 10 g, and Ezhu (Curcumae Rhizoma) 10 g, all of which were purchased from the Pharmacy of The First Affiliated Hospital of the Hunan University of Chinese Medicine. The above Chinese herbal decoction pieces were soaked in drinking water for 1 h and decocted three times, and after conversion according to the clinical doses of humans and animals in the *Experimental Methodology of TCM Pharmacology* [28], the

Chinese herbal decoction was concentrated to 0.72 g/mL using a rotary evaporator, placed in sterile brown bottles, and stored in a refrigerator at 4 °C until use. Thirty male SD rats were randomly assigned to a blank group (n = 6) and a treatment group (n = 24). Rats in the treatment group were intragastrically administered with YSTLD extract (dose volume 15 mL/kg, once a day), and rats in the blank group were intragastrically administered with the same volume of distilled water for 7 d. The last dose was administered by fasting with water 12 h before gavage (gavage per day). One hour after the last gavage, 10% chloral hydrate was intraperitoneally injected at a dose of 0.5 mL/100 g. After anesthesia, blood was collected from the abdominal aorta of rats and placed in a common blood collection tube; the serum was separated after standing in a refrigerator at 4 °C for 2 h and centrifugation at 3 000 rmp/min for 10 min. The serum of animals in the same group was combined and placed in a constant temperature water bath at 56 °C for 30 min to inactivate the complement. After inactivation, a 0.22 μm microporous filter membrane was used to filter, dispense bacteria, and stored at – 20 °C for future use. In this research, 15% of medicated serum concentrations were selected for intervention following the results of a previous study [13].

2.5 DNA microarray

Prostate cancer PC-3 cells were treated with serum containing YSTLD and dimethyl sulfoxide (DMSO) for 48 h, respectively. Total RNA was extracted, and its concentration and purity were measured. Expression profiles were detected using the Affymetrix GeneChip miRNA 4.0 Array (for miRNA detection) and Affymetrix Human Genome U133 Plus 2.0 Array (for transcriptome detection). The data were scanned and analyzed using the GenePix 4000B microarray scanner and PANAGENE software. Testing was repeated three times, and data obtained from microarray testing were analyzed.

2.6 Effects of YSTLD on miR-145-5p expression level in prostate cancer PC-3 cells

Prostate cancer PC-3 cells were treated with serum containing YSTLD and DMSO for 48 h, respectively. Total RNA was extracted by Trizol, and the relative expression levels of endogenous miR-145-5p in prostate cancer PC-3 cells were detected by real-time polymerase chain reaction (qRT-PCR), with GAPDH as an internal reference gene. Among them, the primer sequences for miR-145-5p were: F: 5'-GACTGTTAATGCTAATCGTGATAG-3', R: 5'-GTGCAGGGTCCGAGGTATTC-3'; and the primer sequences for GAPDH: F: 5'-GGAGCGAGATCCCTCCAAAAT-3', R: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The mRNA reverse transcription reaction system is shown in Table 1.

Table 1 mRNA reverse transcription reaction system

Reagent	Reaction system (20 μL)	Final concentration
dNTP mix (2.5 mmol/L)	4 μL	500 μmol/L
Primer mix	2 μL	–
RNA template	7 μL	50 pg - 5 μg
5 × RT buffer	4 μL	1 ×
DTT (0.1 mol)	2 μL	10 mmol/L
HiFiScript (200 U/μL)	1 μL	–

– represents no final concentration.

2.7 Construction of prostate cancer PC-3 cells with overexpressing of miR-145-5p

Lentiviral vectors with green fluorescent protein (GFP) screening markers [negative control virus CON137, overexpression miR-145-5p lentiviral vector LV-hsa-miR-145 (4174-1)] were constructed and synthesized from Shanghai Genechem Co., Ltd.. Lentiviral transfection was performed as follows: prostate cancer PC-3 cells in the logarithmic phase were adjusted to a concentration of 3 × 105 cells/mL, and the cell suspension was inoculated into a six-well plate containing 2 mL of complete medium, gently shaken, and placed in a 5% CO_2 incubator at 37 °C. When the cells were cultured to a density of about 20%, lentivirus from the negative control group and lentivirus of the target gene were taken. The virus was diluted with complete medium to the required concentration (MOI = 40), while polybrene (5 μg/mL) was added to increase the infection efficiency, gently mixed, and placed in the incubator for 16 h, and then replaced with conventional culture medium to continue the culture. The cell culture status was observed, and 72 h after infection, the expression of the GFP gene was observed by fluorescence microscopy. miR-145-5p expression (/GAPDH) was detected by qRT-PCR. The cells in good condition and the qualified infection efficiency group were used for subsequent experiments.

2.8 Inhibitory effect of overexpressing of miR-145-5p on prostate cancer PC-3 cells

Logarithmic phase cells were collected and adjusted for cell suspension concentration. An amount of 100 uL was added to each well of a 96-well culture plate, plated to a cell density of 5 000 cells/well to be tested, and marginal wells were filled with PBS. The black control (Mock), the negative control (miR-145-5p-NC), and the miR-145-5p overexpression (miR-145-5p) groups were established, and five duplicate wells were set for each group. The cells were incubated in an incubator containing 5% CO_2 at 37 °C until the cell monolayer covered the bottom of the

well and tested at 24, 48, 72, 96, and 120 h after plating, respectively. The detection method was as follows: 10 uL of 7Sea-Cell Counting Kit solution in the kit was added to each well, zeroing wells were set synchronously, and the culture was continued for 2 h. The microplate reader was set to a wavelength of 450 nm, and the absorbance (OD) of each well was measured. The results were recorded and plotted, and the cell proliferation inhibition rate was calculated. The cell proliferation inhibition rate = [1 – (OD value of the experimental group/OD value of the normal group)] × 100%.

2.9 Migration ability of prostate cancer PC-3 cells overexpressing miR-145-5p detected by scratch test

About 1×10^5 infected cells of each group were added to each well to ensure the cells reached more than 90% confluence the next day. The low-concentration serum medium was replaced the next day. The scratch tester was used to aim at the central part of the lower end of the 96-well plate, and the scratch was formed by gently pushing upward. The serum-free medium was gently rinsed for three times, and the low-concentration serum medium (0.5% fetal bovine serum) was added. After photographing, the medium was placed in an incubator at 37 °C and 5% CO_2 for culture. According to the preliminary experiment, time points in 48 h were selected for photography to calculate the cell scratch healing rate.

2.10 Effects of serum containing YSTLD on miR-145-5p, TLR4/p38 MAPK/NF-κB signaling pathway, and expression levels of apoptosis-related genes caspase3, TNF-α, Bax, and Bcl-2 in prostate cancer PC-3 cells detected by qRT-PCR

Prostate cancer PC-3 cells in the logarithmic growth phase were inoculated into 6-well plates at a concentration of 1×10^5/mL, and 2 mL of cell suspension was added to each well. The cells were divided into Mock, miR-145-5p-NC, miR-145-5p, YSTLD, and miR-145-5p + YSTLD groups. After 48 h of routine culture, 350 μL Buffer RLT was added to the cells in each well after washing, and the cells were collected into 1.5 mL eppendorf (EP) tubes, mixed by blowing with ethanol and centrifuged. A total of 700 μL Buffer RW1 was added to the adsorption column and centrifuged at 10 000 × g for 15 s; 500 μL Buffer RPE was added, centrifuged at 10 000 × g for 15 s, and the filtrate was discarded. 500 μL Buffer RPE was added, centrifuged at 10 000 × g for 2 min, and the filtrate was discarded. The cartridge was added into a new L collection tube and centrifuged at full speed for 1 min. RNeasy mini adsorption column was transferred into a new 1.5 mL EP tube, and 30 μL RNase-free water was added into the column and centrifuged at 10 000 × g for 1 min. The dissolved RNA was collected and immediately detected by reverse transcription and qRT-PCR. The primer sequences are shown in Table 2.

2.11 Effects of serum containing YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and expression levels of apoptosis-related genes caspase3, TNF-α, Bax, and Bcl-2 in prostate cancer PC-3 cells detected by Western blot

Prostate cancer PC-3 cells from each group were lysed in Radio Immunoprecipitation Assay (RIPA) buffer, incubated on ice for 30 min, and centrifuged at 8 000 r/min for 10 min in an ultra-low temperature centrifuge to obtain total protein solution. Following protein quantification, they were mixed with an equal volume of 2 × loading buffer, and incubated in a boiling water bath for 2 min to denature the protein. And 40 μg protein samples were transferred to gel loading wells for SDS-PAGE at 100 V for 90 min. Proteins were then transferred to nitrocellulose (NC) membranes using a wet transfer apparatus at 300 mA for 20 min. After blocking the membranes with 5% nonfat dry milk, NC membranes were incubated with miR-145-5p (1 : 2 000 dilution), TLR4 (1 : 1 000 dilution), p38 MAPK (1 : 500 dilution), NF-κB (1 : 2 000 dilution), caspase3 (1 : 1 000 dilution), and TRAF1 (1 : 1 000 dilution) for 2 h at room temperature, followed by secondary antibodies (1 : 3 000 dilution) for 1 h at room temperature. A chemiluminescent solution was added to develop in the dark, and Image J software was used to analyze the gray values of each band. The target protein expression level was

Table 2 Primer sequences

Target gene symbol	Upstream primer	Downstream primer
miR-145-5p	5’-CAGTCTTGTCCAGTTTTCCCAG-3’	5’-TATGCTTGTTCTCGTCTCTGTGTC-3’
p38MAPK	5’-CTCGCTTCGGCAGCACA-3’	5’-AACGCTTCACGAATTTGCGT-3’
p65 NF-κB	5’-CCTCACCATCATCACACTGGAAGAC-3’	5’-GTCTCTCCCAGGACAGGCACAAAC-3’
Caspase3	5’-AAAGGCCCCAAGGTAGTTATC-3’	5’-TTGTGTTTCAACTGTTCTCGTCG-3’
TNF-α	5’-CGTGTCATAAAATACCAGTGGA-3’	5’-AAATTCTGTTGCCACCTTTCG-3’
Bax	5’-GACGAACTGGACAGTAACATGGAGCT-3’	5’-CGGCCCAGTTGAAGTTGC-3’
Bcl-2	5’-GCCTTCTTTGAGTTCGGTGG-3’	5’-TGCCGGTTCAGGTACTCAGTCATC-3’
TRAF1	5’-ACCTGAAGTTTGAGCAAGTGG-3’	5’-AXXTGACTTCCGAGGACTACTC-3’
GAPDH	5’-CACCCACTCCTCCACCTTTGA-3’	5’-TCTCTCTTCCTCTTGTGCTCTTGC-3’

expressed as the ratio of the target protein to internal reference GAPDH.

2.12 Statistical analysis

Statistical analysis was performed using SPSS 26.0 software. Measurement data were expressed as mean ± standard deviation (SD), and analysis of variance was used for comparison between groups. Data were plotted using GraphPad Prism (version 8.0). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Serum containing YSTLD preparation

Following the above method for the preparation of medicated serum, approximately 8 – 15 mL of blood was collected from each rat, and about 20 – 30 mL of serum could be obtained from each 50 mL of rat blood, which was transparent and yellowish. The prepared blank serum and medicated serum were placed into a 1 mL EP tube for use.

3.2 YSTLD up-regulating the expression level of miR-145-5p in prostate cancer PC-3 cells

DNA microarray was used to investigate the molecular mechanism of YSTLD against prostate cancer, and the assay was repeated three times in YSTLD-treated PC-3 cells. As shown in Figure 1, miRNA microarray assay revealed that 35 miRNAs were significantly expressed in prostate cancer PC-3 cells treated with YSTLD compared with the DMSO group. The most significant differential expression was miR-145-5p and the results of the three assays were consistent, all of which were significantly up-regulated in the YSTLD treatment group.

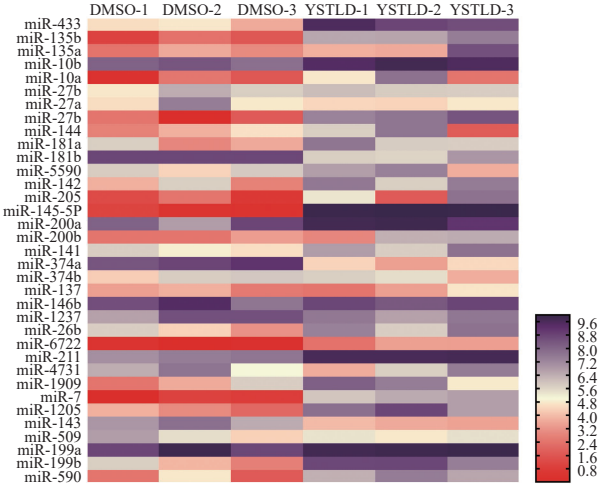

Figure 1 Expression level of miRNA in PC-3 cells treated with YSTLD

3.3 Effects of YSTLD on miR-145-5p expression level in prostate cancer PC-3 cells

Relative expression level of endogenous miR-145-5p in prostate cancer PC-3 cells was measured by qRT-PCR. The results showed that endogenous miR-145-5p expression level in YSTLD-treated prostate cancer PC-3 cells (56.36% ± 4.36%) was significantly increased compared with the DMSO group (12.04% ± 1.92%, $P < 0.05$, Figure 2).

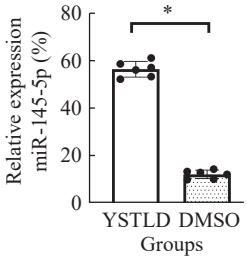

Figure 2 Effects of YSTLD on miR-145-5p expression in prostate cancer PC-3 cells
*$P < 0.05$.

3.4 Construction of prostate cancer PC-3 cells transfected with miR-145-5p overexpressed lentivirus

To verify the effect of miR-145-5p on the function of prostate cancer PC-3 cells, overexpressing miR-145-5p lentivirus were constructed and transfected into prostate cancer PC-3 cells. The transfection efficiency was observed by fluorescence microscopy, and miR-145-5p expression in prostate cancer PC-3 cells was detected by qRT-PCR. As shown in Figure 3A, after prostate cancer

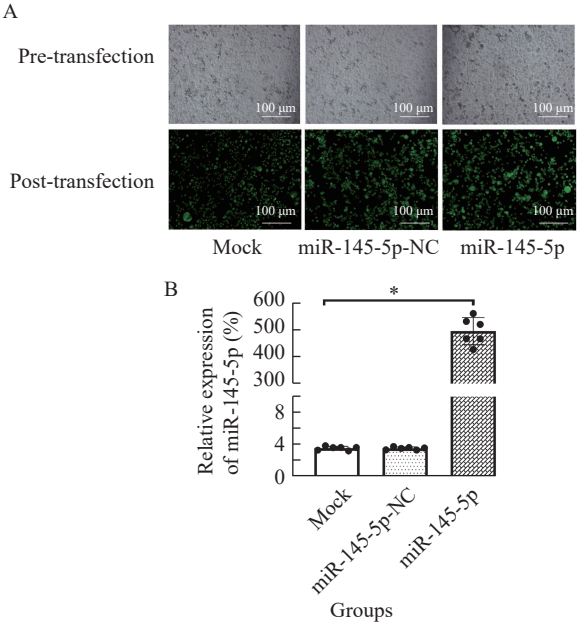

Figure 3 Fluorescence expression after lentiviral transfection of prostate cancer PC-3 cells
A, normal visual field and green 100 fluorescence visual field in the Mock, miR-145-5p-NC, and miR-145-5p groups. B, miR-145-5p expression in each group after transfection. *$P < 0.05$.

PC-3 cells were transfected with negative control and overexpressing miR-145-5p lentivirus, the proportion of fluorescent cells reached over 90%. The transfection efficiency was high, which could be used for subsequent experimental studies. qRT-PCR was adopted to detect the expression level of miR-145-5p in prostate cancer PC-3 cells after lentiviral infection. As shown in Figure 3B, the relative expression level of miR-145-5p in the Mock, miR-145-5p-NC, and miR-145-5p groups were 3.52 ± 0.24, 3.57 ± 0.18, and 473.62 ± 51.45, respectively. The relative expression level of miR-145-5p in the miR-145-5p group was significantly higher than that in the Mock group ($P < 0.05$), suggesting that the overexpression of miR-145-5p prostate cancer cell line was successfully constructed.

3.5 Effects of miR-145-5p on prostate cancer PC-3 cell function

3.5.1 Effects of miR-145-5p on prostate cancer PC-3 cell proliferation CCK-8 assay was used to detect the proliferation ability of prostate cancer PC-3 cells overexpressing miR-145-5p lentivirus in the Mock, miR-145-5p-NC, and miR-145-5p groups. The inhibition rate of drugs on prostate cancer PC-3 cells in each group was calculated. There was no significant difference between the Mock group and miR-145-5p-NC group ($P > 0.05$). The inhibition rate in miR-145-5p group was not significantly different from that in the Mock and miR-145-5p-NC groups within 72 h ($P > 0.05$), but the difference was significant after 72 h ($P < 0.05$, Figure 4).

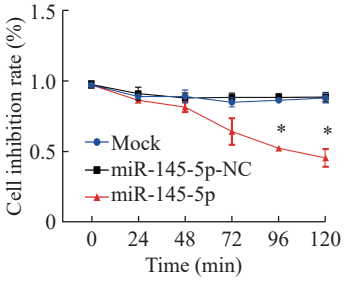

Figure 4 Effects of lentiviral transfection overexpressing miR-145-5p on the proliferation of prostate cancer PC-3 cells
*$P < 0.05$.

3.5.2 Effects of overexpression of miR-145-5p on migration of prostate cancer PC-3 cells The effect of miR-145-5p overexpression on the migration ability of prostate cancer PC-3 cells was examined by cell scratch assay. The results showed that overexpression of miR-145-5p significantly inhibited the migration ability of prostate cancer PC-3 cells after 24 h of culture. The scratch healing rates of the Mock, miR-145-5p, and miR-145-5p-NC groups were 73.7% ± 5.3%, 41.9% ± 4.1%, and 92.8% ± 6.6%, respectively. There was no significant difference between the Mock group and miR-145-5p-NC group ($P > 0.05$). The scratch healing rate of the miR-145-5p group was significantly lower than that in the Mock and miR-145-5p-NC groups ($P < 0.05$, Figure 5).

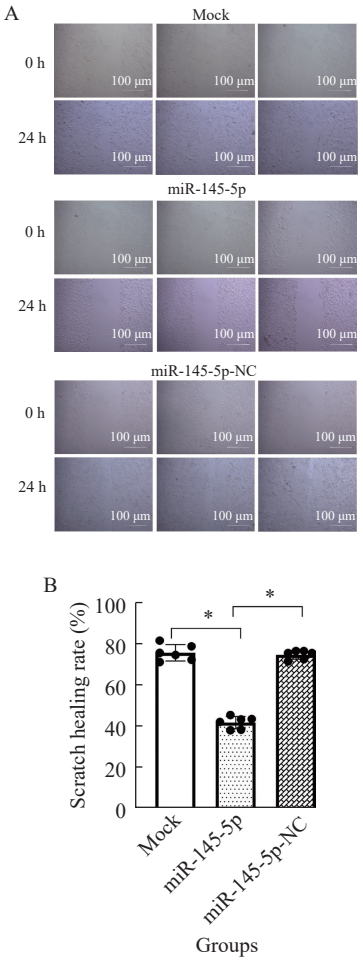

Figure 5 Effects of overexpression of miR-145-5p lentivirus transfection on the proliferation of prostate cancer PC-3 cells
A, migration of PC-3 cells in each group. B, scratch healing rate of PC-3 cells in prostate cancer. *$P < 0.05$.

3.6 Effects of miR-145-5p and YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and mRNA expression of apoptosis-related genes in prostate cancer PC-3 cells

To investigate the effects of miR-145-5p and serum containing YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and apoptosis-related gene caspase3, TNF-α, Bax, and Bcl-2 mRNA expression, prostate cancer PC-3 cells overexpressing miR-145-5p after lentiviral infection and gene expression in prostate cancer PC-3 cells treated with serum containing YSTLD for 24 h were detected by qRT-PCR. The results showed that serum containing YSTLD could up-regulate the expression levels of miR-145-5p, caspase3, TNF-α, and Bax mRNA and down-regulate the mRNA expression levels of TLR4, p38 MAPK, p65 NF-κB, Bcl-2, and TRAF1 in prostate cancer PC-3 cells after intervening prostate cancer PC-3 cells. There were

significant differences between the Mock group and the miR-145-5p and miR-145-5p + YSTLD groups ($P < 0.05$), and between the YSTLD group and the miR-145-5p + YSTLD group ($P < 0.05$, Figure 6).

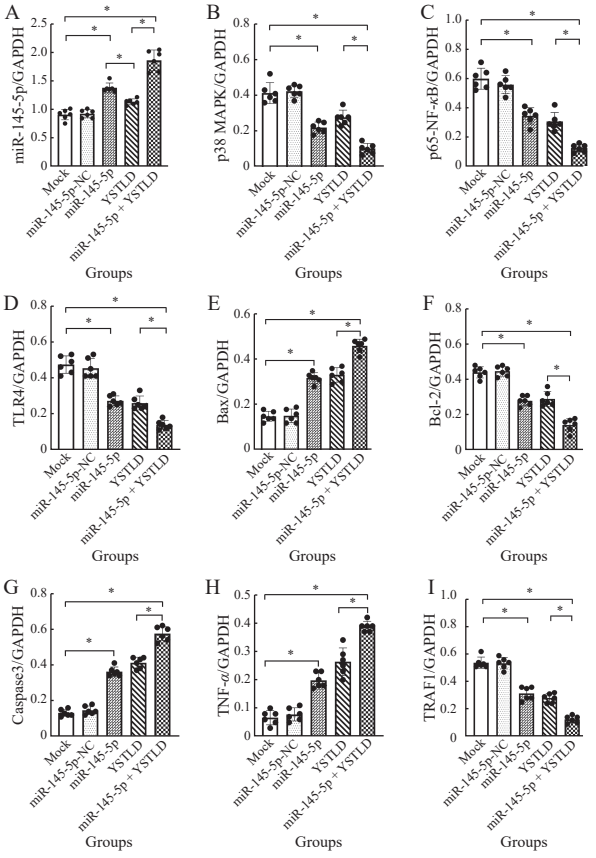

Figure 6 Effects of miR-145-5p and YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and mRNA expression of apoptosis-related genes in prostate cancer PC-3 cells

A – I, the relative expression levels of miR-145-5p, p38 MAPK, p65 NF-κB, TLR4, Bax, Bcl-2, caspase3, TNF-α, and TRAF1 in prostate cancer PC-3 cells of each group. $^*P < 0.05$.

3.7 Effects of YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and protein expression of apoptosis-related genes in prostate cancer PC-3 cells

To determine the effects of serum containing YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and apoptosis-related gene caspase3 protein expression levels, the protein expression of each gene in prostate cancer PC-3 cells treated with serum containing YSTLD for 24 h was detected by Western blot. The results indicated that serum containing YSTLD could up-regulate caspase3 protein expression and down-regulate TLR4, p38 MAPK, p65 NF-κB, and TRAF1 protein expression in prostate cancer PC-3 cells after intervening prostate cancer PC-3 cells. There were significant differences between the Mock group and the miR-145-5p and miR-145-5p + YSTLD groups ($P < 0.05$), and between the YSTLD group and the miR-145-5p + YSTLD group ($P < 0.05$, Figure 7).

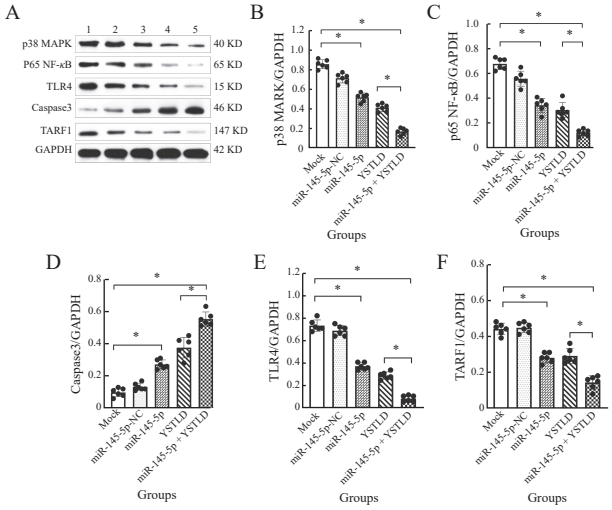

Figure 7 Effects of YSTLD on TLR4/p38 MAPK/NF-κB signaling pathway and protein expression of apoptosis-related genes in prostate cancer PC-3 cells

A, YSTLD on p38 MAPK, p65 NF-κB, TLR4, caspase3, and TRAF1 protein expression in prostate cancer PC-3 cells. B – F, p38 MAPK, p65 NF-κB, caspase3, TLR4, and TRAF1 protein expression levels in prostate cancer PC-3 cells of each group. $^*P < 0.05$.

4 Discussion

In this study, we found that 35 miRNAs, including miR-433, miR-135b, miR-135b, and miR-145-5p, were expressed in prostate cancer PC-3 cells treated with YSTLD using microarray technology. Among them, miR-145-5p demonstrated the most significant difference and the results were consistent in three repeated experiments, suggesting that miR-145-5p acts as a key miRNA of YSTLD against prostate cancer. We further explored its mechanism in a subsequent series of validation experiments.

To clarify the effects of miR-145-5p on the proliferation and migration of prostate cancer PC-3 cells, PC-3 cells overexpressing miR-145-5p were constructed by the lentiviral system. The transfection efficiency was observed to reach over 90% by fluorescence microscopy. The expression of miR-145-5p in PC-3 cells after lentiviral infection was significantly higher than that in the Mock and the miR-145-5p-NC groups by qRT-PCR, suggesting that the prostate cancer PC-3 cell line overexpressing miR-145-5p was successfully constructed. CCK-8 assay was adopted to detect the effects of miR-145-5p overexpression on the proliferation ability of prostate cancer PC-3 cells. The results showed that overexpression of miR-145-5p did not significantly affect the proliferation of PC-3 cells within 72 h, and the proliferation ability of PC-3 cells was inhibited to varying degrees over time after 72 h. Cell scratch assay indicated that miR-145-5p overexpression could significantly inhibit the migration of PC-3 cells. This suggests that miR-145-5p is closely related to prostate cancer development, which plays a tumor suppressor gene role in prostate cancer.

OZEN et al. [29] showed that overexpression of miR-145-5p could target junctional adhesion molecule A (JAM-A) and fascin, down-regulate podocalyxin and Serpin El mRNA levels, and up-regulate gamma-actin, transgelin, and recombinant human myosin light chain 9 (MYL9) expression, resulting in reorganization of the actin cytoskeleton and changes in cell morphology, which reduced the formation of actin stress fibers and filaments, thereby inhibiting the motility and migration of prostate cancer PC-3 cells. To investigate the mechanism of miR-145-5p inhibiting the proliferation and migration of prostate cancer PC-3 cells, we adopted qRT-PCR to detect the mRNA levels of TLR4/p38 MAPK/NF-κB and apoptosis-related genes in prostate cancer PC-3 cells overexpressing miR-145-5p. The results indicated that overexpression of miR-145-5p could up-regulate the expression levels of miR-145-5p, caspase3, TNF-α, and Bax mRNA, down-regulate the mRNA expression levels of p38 MAPK, p65 NF-κB, and Bcl-2 in prostate cancer PC-3 cells, up-regulate the expression levels of caspase3 protein, and down-regulate the protein expression levels of TLR4, p38 MAPK, and p65 NF-κB in PC-3 cells. Inflammation is an important predisposing factor for prostate cancer. Our network pharmacological analysis in the early stage also concluded that inflammatory stress acts as an important process in the occurrence and development of prostate cancer. The occurrence and development of prostate cancer are often accompanied by cellular inflammatory factor infiltration, and the increased level of inflammatory signaling factors can also cause pathological changes in the prostate [30, 31]. The TLR4/p38 MAPK/NF-κB signaling pathway is closely related to the inflammatory response, considered to be a common pathway for cell signaling transmission, and plays a key role in the development of diseases such as prostate cancer [32]. P38 MAPK mitogen-activated protein kinase, also known as extracellular signal-regulated kinase, is an important protein of the MAPK signaling pathway and plays an important role in promoting the proliferation and invasion of prostate cancer cells, which can be converted from an inactive state to phosphorylation under various extracellular stimuli such as physiological stress, ultraviolet irradiation, and ionizing radiation. It can activate the p38 signaling pathway after the formation of p-p38, and then regulate inflammatory factors and oxidative stress damage *in vivo*, and participate in the pathological process of tumor cell invasion and migration [33, 34]. NF-κB, a key nuclear transcription factor protein family, was originally discovered and extracted from lymphoid B cells and normally exists as a dimer in a non-activated state, which has been found to have adverse effects of inducing inflammatory responses in the body and tumor microangiogenesis [35]. NF-κB has multiple subunits, of which p65 NF-κB is a star member of the NF-κB protein family, and under the stimulation of adverse factors, it is degraded and separated from a stable trimeric state, and can be activated and transferred to the nucleus, resulting in the expression of various inflammatory factors and mediators. It plays a pivotal role in the regulation of chronic inflammation and an important role in the progression of prostate cancer from inflammation to cancer [36]. Chronic inflammation can directly or indirectly create conditions for the development and progression of prostate cancer. Currently, a large number of experiments have demonstrated that TLR4/p38 MAPK/NF-κB signaling pathway is closely related to the inflammatory response of malignant tumors and the occurrence and development of a variety of diseases, and it also has an important regulatory effect on apoptosis-related biological processes [37]. HEIDER et al. [38] showed that the high expression status of TLR4/p38 MAPK/NF-κB signaling pathway is closely related to the invasiveness of prostate tumors, and directly or indirectly regulates numerous genes thus playing an important part in cell differentiation, proliferation, and apoptosis, with p38 MAPK and p65 NF-κB overexpression in 30% – 50% of prostate cancer patients. CHENG et al. [39] showed that the tumor suppressor gene miR-145-5p played a key part in a complex cellular network, keeping the cells in a balanced state.

TLR4/p38 MAPK/NF-κB signaling pathway is closely related to the biological process and characteristics of tumors, and is activated in tumors including prostate cancer. On one hand, this pathway serves as an important signaling pathway for the development of prostate cancer, and is regulated by various protein factors such as TRAF1 and apoptosis protein (BAX, Bcl-2). On the other hand, miRNAs play a regulatory part mainly by acting at the 3'UTR end of mRNAs to promote mRNA degradation and inhibit their translation. In previous preliminary experimental study [22], we found that miR-145-5p inhibited TLR4/p38 MAPK/NF-κB signaling pathway transduction, and on this basis, we further performed immunoblotting and immunohistochemical experiments. The results showed that YSTLD could inhibit the expression levels of TLR4, p38 MAPK, NF-κB, and TRAF1 in the TLR4/p38 MAPK/NF-κB signaling pathway, which was consistent with miR-145-5p inhibiting TLR4/p38 MAPK/NF-κB signaling pathway transduction. These results indicated that YSTLD could fight against prostate cancer by up-regulating miR-145-5p to inhibit TLR4/p38 MAPK/NF-κB signaling pathway transduction.

The results of this study showed that miR-145-5p could inhibit tumor formation by inhibiting p38 MAPK and p65 NF-κB overexpression, and regulate apoptosis genes such as caspase3, TNF-α, Bax, and Bcl-2 simultaneously. So far, there is no report on the regulation of Bcl-2, Bax, and caspase3 by miR-145-5p in prostate cancer PC-3 cells. Our study indicated that overexpression of miR-145-5p could up-regulate caspase3, TNF-α, and Bax mRNA expression levels in prostate cancer PC-3 cells, and down-regulate Bcl-2 mRNA expression levels, thus exerting an anti-prostate cancer effect.

5 Conclusion

In this study, we further investigated the mechanism of YSTLD inhibiting TLR4/p38 MAPK/NF-κB signaling pathway against prostate cancer by up-regulating miR-145-5p based on previous studies, suggesting that YSTLD can promote prostate cancer PC-3 cell apoptosis by up-regulating the expression level of miR-145-5p and inhibiting TLR4/p38 MAPK/NF-κB signaling pathway, which may be an important mechanism of YSTLD's anti-prostate cancer effectiveness.

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Competing interests

The authors declare no conflict of interest.

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益肾通癃汤通过上调 miR-145-5p 抑制 TLR4/p38 MAPK/NF-κB 信号通路抗前列腺癌作用机制研究

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【摘要】目的 探讨益肾通癃汤(YSTLD)通过上调 miR-145-5p 抑制 TLR4/p38 MAPK/NF-κB 信号通路抗前列腺癌的作用机制。**方法** 借助 miRNA 芯片技术检测 YSTLD 处理前列腺癌 PC-3 细胞后的 miRNA 表达谱的变化，筛选 miRNA 芯片结果中差异显著的 miRNA，并通过实时荧光定量聚合酶链反应(qRT-PCR)进行验证。慢病毒转染 miR-145-5p 入前列腺癌 PC-3 细胞，CCK8 法及划痕实验检测 miR-145-5p 对前列腺癌 PC-3 细胞增殖与迁移作用；qRT-PCR 法及 Wstern blot 法检测 miR-145-5p 对 TLR4/p38 MAPK/NF-κB 信号通路及凋亡相关基因 caspase3、TNF-α、Bax、Bcl-2 表达的影响；qRT-PCR 及 Wstern blot 法检测 YSTLD 含药血清对 miR-145-5p、TLR4/p38 MAPK/NF-κB 信号通路及凋亡相关基因 caspase3、TNF-α、Bax、Bcl-2 表达的影响。**结果** miRNA 基因芯片检测发现 YSTLD 处理前列腺癌 PC-3 细胞后存在 35 种 miRNA 表达水平与对照组存在显著差异，其中以 miR-145-5p 差异最为显著，同时 qRT-PCR 验证发现 YSTLD 处理的 PC-3 细胞中的 miR-145-5p 水平显著高于 DMSO 对照组($P < 0.05$)。慢病毒转染 miR-145-5p 入前列腺癌 PC-3 细胞后，发现 miR-145-5p 能抑制前列腺癌 PC-3 细胞的增殖与迁移。过表达 miR-145-5p 可上调前列腺癌 PC-3 细胞 caspase3、TNF-α 及 Bax mRNA 表达水平，下调 p38 MAPK、p65 NF-κB 及 Bcl-2 的 mRNA 表达水平($P < 0.05$)，同时上调前列腺癌 PC-3 细胞 caspase3 蛋白表达水平，下调 TLR4、p38 MAPK、p65 NF-κB 的蛋白表达水平($P < 0.05$)；YSTLD 含药血清在干预前列腺癌 PC-3 细胞后，可上调前列腺癌 PC-3 细胞 caspase3、TNF-α 及 Bax mRNA 表达水平，下调 p38 MAPK、p65 NF-κB、Bcl-2、TRAF1 的 mRNA 表达水平($P < 0.05$)，同时上调前列腺癌 PC-3 细胞 caspase3 蛋白表达水平，下调 TLR4、p38 MARK、p65 NF-κB、TRAF1 的蛋白表达水平($P < 0.05$)。**结论** YSTLD 可通过上调 miR-145-5p 的表达水平，抑制 TLR4/p38 MAPK/NF-κB 信号通路促进前列腺癌 PC-3 细胞凋亡，这可能是 YSTLD 抗前列腺癌的重要机制。

【关键词】 前列腺癌；益肾通癃汤；基因芯片技术；miR-145-5p；TLR4/p38 MAPK/NF-κB 信号通路