Original Article
The expression and functional role of FOX transcription factor FOXJ1 in prostate cancer

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Abstract: As one common malignant tumor in male reproductive-urinary system, the incidence of prostate cancer is increasing rapidly. Recent study revealed the participation of FOX transcription factor family in tumor occurrence and progression. FOXJ1, one member of FOX family, plays an important role in tumor pathogenesis and advancement, although its expression and functional role in prostate cancer has not been illustrated. Both tumor and adjacent tissues were collected from prostate cancer patients. Real-time PCR and Western blot were used to test the FOXJ1 expressions, and to analyze their correlation with pathological feature of cancer. DU145 cells were *in vitro* cultured and were transfected with FOXJ1-overexpressing vectors. MTT assay detected the effect of FOXJ1 on tumor cell proliferation. Western blot quantified MMP-2 and MMP-9 expressions, while Transwell invasion assay determined the impact of FOXJ1 on tumor invasion. Both mRNA and protein levels of FOXJ1 in prostate tumor tissues were significantly depressed compared to adjacent tissues (P<0.05). FOXJ1 expression level was negatively correlated with TNM stage, tumor volume and lymphatic metastasis (P<0.05). The transfection of DU145 cells by FOXJ1-overexpressed vectors inhibited proliferation or invasion abilities, and reduced MMP-2 and MMP-9 expressions (P<0.05 compared to control group). FOXJ1 was down-regulated in prostate cancer tissues, and was closely correlated with pathological features of tumor. FOXJ1 regulated proliferation of prostate cancer cells, and functioned as molecular targets for tumor diagnosis and prognostic prediction via modulating MMP-2 and MMP-9 mediated tumor invasion.

Keywords: FOXJ1, prostate cancer, cell proliferation, tumor invasion

Introduction

With the aging of whole population, the incidence of prostate cancer, which is the major malignant tumor in male reproductive-urinary tract, is rapidly increasing by years [1, 2]. Due to the inconspicuous onset of the disease, most patients were already at terminal stage when receiving primary diagnosis, which severely affected patient survival, life quality and the therapeutic efficacy [3]. The incidence of prostate cancer in China is also increasing in recent years. Due to the low efficiency of diagnosis in early tumor screening, majority patients after confirmed diagnosis were already at advanced stage, who lost the surgical and treatment opportunity, leading to an unfavorable prognosis in most cases [4, 5]. A complicated mechanism is involved in pathogenesis and invasion of prostate cancer, associated

with genetics, environment and biological factors [6]. The inhibition of the development of tumor is critical for improving patients' prognosis. One of important impact factors in prostate cancer is extracellular matrix (ECM) [7]. Once penetrating into the ECM barrier, cancer cells can invade through vascular basal membrane, and enter the blood circulation via blood vessel walls, which alters the body internal enjoinment for tumor invasion and metastasis [8]. Both ECM and basal membrane can be degraded by proteinase, which thus accelerates the progression of tumor. As a group of important proteinase, Matrix metalloproteinase (MMP) plays a significant role in cancer development [9]. Recent studies showed that FOX transcription factor family presented as one novel molecule participating in tumor occurrence and progression [10]. FOX transcription family involves in the signal transduction, and further modu-

lates biological activity of cells [11]. FOX transcription factor family, FOXJ1, on the other hand, performed regulatory function in embryonic development, differentiation and organ formation, and were also related to cell apoptosis and cycle, cilia formation and metabolism [12, 13]. It has been demonstrated that the participation of FOX transcription factor was found in tumor occurrence and progression [14-16]. Across different tumors, FOXJ1 induces or inhibits tumors due to its heterogeneous expression in tumors. For example, FOXJ1 in breast cancer or ovarian cancer may exert down-regulation effect whilst in liver cancer it may have favorable impact [14-16]. So far, the expression of FOXJ1 and its functional mechanism in prostate cancer, however, is still unclear.

Materials and methods

General information

A total of 192 prostate cancer patients who were diagnosed and treated in the department of urology in General Hospital of PLA Rocket Force from January 2014 to August 2015 were recruited in this study for the collection of clinical data. All patients received radical resection during surgery. The average age of patients was 57.2±5.6 years (42 to 71 years). Tissues samples were collected for pathological examination and typing or grading. All patients had confirmed diagnosis of primary prostate cancer by pathology. Normal echocardiography, ECG and chest X ray were performed before the surgery. Resected tumor tissues and adjacent tissues were collected for samples, which were stored at -80°C for frozen storage. This study has been approved by the ethical committee of General Hospital of PLA Rocket Force. All patients included had written consents of this study.

Inclusive and exclusive criteria

Inclusive criteria: (1) Confirmed diagnosis of prostate cancer; (2) Primary tumor without surgery, radio- and chemo-therapy. Exclusive criteria: (1) Received surgery, chemo- or radio-therapy before; (2) Complicated with other diseases, such as infectious disease, malignant tumor, severe liver/kidney disease, pulmonary fibrosis, bone metabolic disorder, secondary renal hypertension, systemic immune disease or cancer complications; (3) Those who were not able to or not willing to cooperate this study, or cannot finish follow-ups.

Clinical information

Patient's information included name, age, family history, body-mass index (BMI), tumor size, tumor type, pathological grade and clinical stage were collected and recorded.

Equipment and reagents

Human prostate cancer cell line DU145 was kept in liquid nitrogen. DMEM medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). PVDF membrane was purchased from Pall Life Sciences (US). EDTA was purchased from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human FOXJ1 monoclonal antibody, anti-human MMP-2 monoclonal antibody, anti-human MMP-9 monoclonal antibody and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling (US). RNA extraction kit, and reverse transcription kit were purchased from Axygen (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). Cell incubator was purchased from Suzhou Instrument (China). Transwell chamber was purchased from Corning (US). Other common reagents were purchased from Sangon (China).

DU145 cell culture and grouping

DU145 cells stored in liquid nitrogen were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 rpm for 3 min, and were re-suspended in 1 ml fresh medium and were removed to 50 ml culture flask which contained 4 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO_2 at 37°C for 24~48 h. DU145 cells were seeded in 6-well plate at 1×10^5 per cm^2 containing 10% FBS and 90% high-glucose DMEM medium (with 100 U/ml penicillin and 100 μg streptomycin). Cells were kept in a humidified chamber with 5% CO_2 at 37°C. Cells at log-phase with 3rd to 8th generation were randomly divided into control group; empty vector group, and FOXJ1 group.

Table 1. Clinical data and pathological features of prostate cancer

	N	%
Age (year)		
≤45	90	46.87
>45	102	53.13
BMI (kg/m^2)		
≤23	97	50.52
>23	95	49.48
Tumor size		
T1 (<2 cm)	41	
T2 (2-5 cm)	54	
T3 (>5 cm)	45	
T4 (Tumor invasion)	52	
Histology grade		
High differentiation	58	30.21
Moderate differentiation	61	31.77
Low differentiation	73	38.02
TNM stage		
I-II	71	36.98
III-IV	121	63.02
Lymph node metastasis		
Yes	78	40.62
No	114	59.38

Transfection of pcDNA3.1-FOXJ1 plasmid into DU145 cells

Trizol reagent was used to extract RNA from DU145 cells. Reverse transcription was performed according to the manual instruction, using primers designed by PrimerPremier6.0 and synthesized by Sangon (China). Primer sequences were: forward 5'-TGCAC GCGTT TCTGC CTGAT-3'; reverse 5'-TATAG CTCTC TGCTC TTGTC-3'. PCR amplification was performed on target genes under the following conditions: 95°C for 2 min, and 50°C for 1 min, followed by 35 cycles each containing 94°C for 30 s, 60°C for 50 s and 72°C for 35 s. PCR products were recycled by gel extraction kit, and were ligated to pcDNA3.1 vector at 3:1 ratio. Ligation was performed at 4°C for 16 h. JM109 competent cells were prepared in LB plate and LB medium, with the transfection of clonal plasmids. The pcDNA3.1-FOXJ1 plasmid was concentrated and quantified. Prostate cancer cells at log growth phase were collected and adjusted to 3×10^6 per ml in 6-well plate. Cells were incubated in a 37°C chamber with 5% CO_2 for 12 h until reaching 70%~80% confluence. 5 μl lipo-

2000 reagent was diluted in 200 μl serum-free DMEM medium for 15 min room temperature incubation. The pcDNA3.1-FOXJ1 and pcDNA3.1 plasmids were mixed in 200 μl serum-free medium for 15 min room temperature incubation. Lipo2000 mixture was then mixed with pcDNA3.1-FOXJ1 dilutions for 30 min room temperature incubation. Serum was removed from cells in 6-well plate, followed by PBS rinsing and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO_2 at 37°C for 6 h, followed by the application of serum-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for FOXJ1 gene expression in tumor tissues and DU145 cells

Trizol reagent was used to extract RNA from tumor or adjacent tissues and DU145 cells. Reverse transcription was performed according to the manual instruction (Axygen, US), using primers designed by Primer6.0 and synthesized by Invitrogen (China). Primer sequences were: FOXJ1-forward, 5'-CATTG ATGGG GATAC C-3'; FOXJ1-reverse, 5'-GCTTC ACAGA AGTCG CAGCT-3'; GAPDH-forward, 5'-ACCAG GTACT TCTGG GTT-3'; GAPDH-reverse, 5'-TAACC AGCCA TGATG GTGTG TT-3'. Real-time PCR was performed under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the baseline. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by $2^{-\Delta\Delta Ct}$ method.

Western blot assay for FOXJ1, MMP-2 and MMP-9 proteins expression

Proteins from tumor or adjacent tissues and DU145 proteins were firstly extracted. In brief, RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA and 1 mM NaVandade) was used to lyse cells on ice for 15~30 min, followed by ultrasound rupture (5 s×4) and centrifugation (4°C, 10000 g, 15 min). Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then

Figure 1. FOXJ1 mRNA expression in prostate cancer and adjacent tissues. *, P<0.05 compared to adjacent tissues.
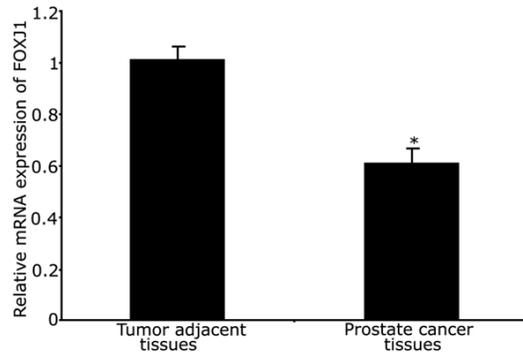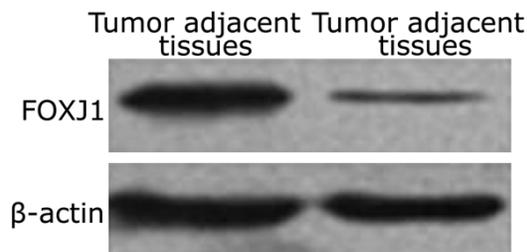

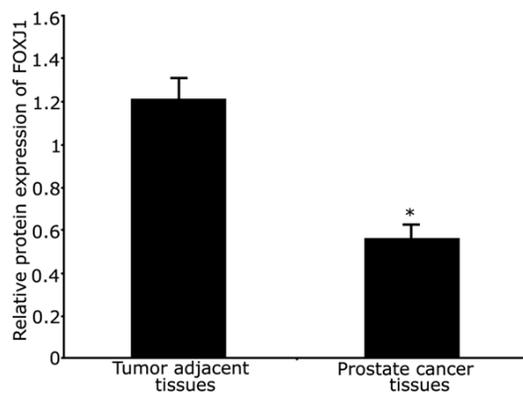**Figure 2.** FOXJ1 protein expression in prostate cancer and adjacent tissues.

Figure 3. FOXJ1 protein expression in prostate cancer and adjacent tissues. *, P<0.05 compared to adjacent tissues.

separated using 10% SDS-PAGE gel, and were transferred to PVDF membrane using semi-dry method (160 mA for 1.5 h). Non-specific background was removed by 5% defatted milk powder at room temperature for 2 h, followed by the addition of anti-FOXJ1 monoclonal antibody (1:1000 dilution), anti-MMP-2 monoclonal anti-

body (1:1500) and anti-MMP-9 monoclonal antibody (1:2000) in 4°C overnight incubation. On the next day, the membrane was rinsed in PBST, and incubated with goat anti-rabbit secondary antibody (1:2000) for 30 min incubation. After PBST rinsing, ECL reagent was used to develop the membrane for 1 min, followed by exposure under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N=4) for statistical analysis.

MTT assay for cell proliferation

DU145 cells at log-phase were digested, counted and seeded into 96-well plate at 3000 cells per well containing DMEM medium with 10% FBS. Cells were then randomly divided into control, empty plasmid group and FOXJ1 group as above mentioned. After 48-hour incubation, 20 μl sterile MTT solution was then added into each test well in triplicates. With 4 h continuous culture, the supernatant was completely removed, with the addition of 150 μl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group. Each experiment was repeated in triplicates for statistical analysis.

Transwell chamber assay for cell invasion

Following the manual instruction, serum-free culture medium was used for 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. 500 μl DMEM culture medium containing 10% FBS was then added into inner and outer surface of the chamber, which contained 100 μl tumor cell suspensions prepared by serum-free culture medium. The chamber was placed in a 24-well plate in triplicates for each group. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber, with the removal of membrane-fixed cells, which were then fixed in cold ethanol and stained by crystal violet. The number of cells at the lower surface of the micro-pore membrane was then counted in triplicates (N=3).

Table 2. Correlation analysis between FOXJ1 expression and clinical features of prostate cancer patients

		Age	BMI	TNM Stage	Tumor volume	Lymph node metastasis	Histology grade
FOXJ1 mRNA	r value	0.018	0.011	-0.678	-0.689	-0.679	0.615
	P	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05
FOXJ1 protein	r value	0.327	0.215	-0.517	-0.728	-0.662	0.562-
	P	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05

de metastasis and Histology grade (treated as a continuous variable).

Results

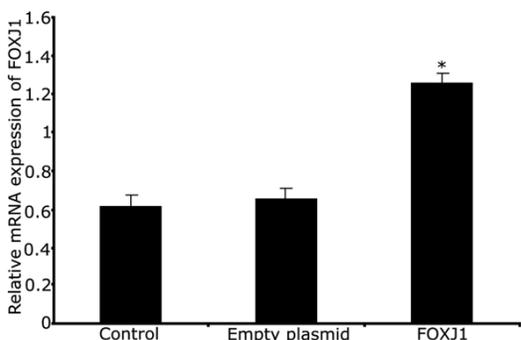*Clinical data and pathological features of prostate cancer*

Both clinical data and pathological features of all prostate cancer patients included in this study were shown in **Table 1**.

FOXJ1 mRNA expression in prostate cancer and tumor adjacent tissues

Real-time PCR was used to test mRNA level of FOXJ1 gene in prostate cancer and adjacent tissues. Results showed significantly decreasing level of FOXJ1 mRNA in prostate cancer tissues ($P < 0.05$ compared to adjacent tissues, **Figure 1**).

FOXJ1 protein expression in prostate cancer and adjacent tissues

Western blot was used to detect FOXJ1 protein expression in prostate cancer and adjacent tissues. Results showed significantly depressed FOXJ1 protein expression in prostate cancer tissues ($P < 0.05$ compared to adjacent tissues, **Figures 2**, **3**).

Correlation analysis between FOXJ1 expression and clinical features of prostate cancer patients

We further analyzed the correlation between FOXJ1 expression and clinical pathological features including age, BMI, tumor volume, TNM stage and metastasis. Results demonstrated that FOXJ1 expression was negatively correlated with TNM stage, tumor volume and lymph node metastasis, positively correlated histology grade, and uncorrelated with age or BMI ($P < 0.05$, **Table 2**).

FOXJ1 expression in prostate cancer DU145 cells

Real-time PCR was used to determine FOXJ1 expression in DU145 cells after plasmid transfection. Results showed significantly elevated FOXJ1 expression in DU145 cells with

Figure 4. FOXJ1 expression in DU145 cells. *, $P < 0.05$ compared to adjacent tissues.
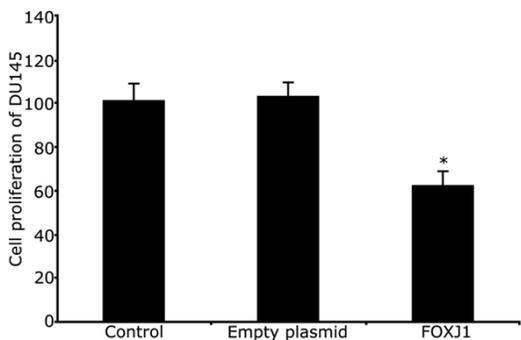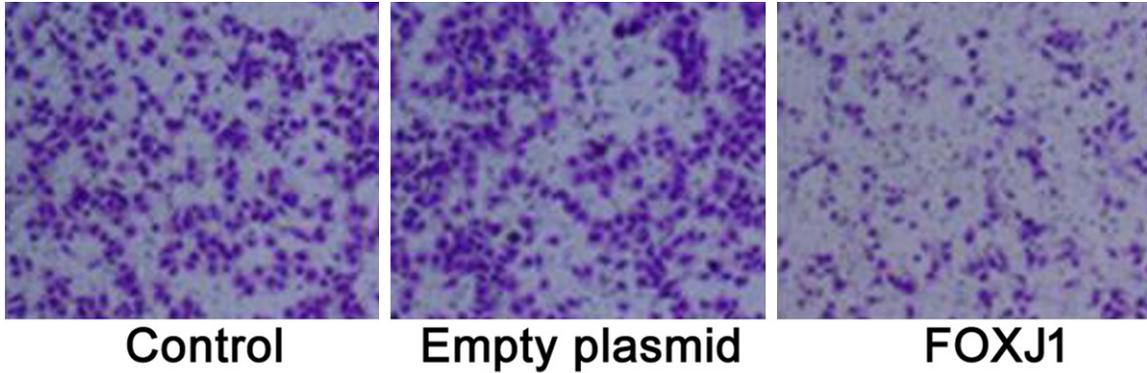

Figure 5. Effects of FOXJ1 on proliferation of prostate cancer cells. *, $P < 0.05$ compared to adjacent tissues.

Statistical processing

All data were presented as mean ± standard deviation (SD), and were analyzed in SPSS19 software. Analysis of variance (ANOVA) was used to analyze between-group differences. Student t-test was used to compare means between two groups. A statistical significance was defined when $P < 0.05$. The Pearson coefficient was used to measure correlations between gene expression patterns and patient Age, BMI, TNM Stage, Tumor volume, Lymph no-

Figure 6. Effects of FOXJ1 on invasion of prostate cancer cells.
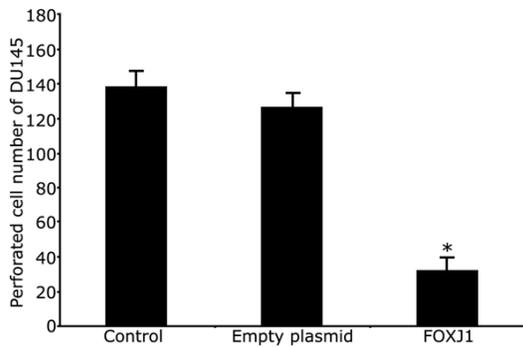
Figure 7. Analysis of FOXJ1 effect on the invasion of prostate cancer cells. *, P<0.05 compared to adjacent tissues.
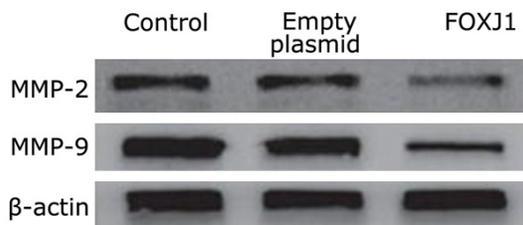

Figure 8. Effects of FOXJ1 on MMP-2 and MMP-9 expression of prostate cancer cells.

pcDNA3.1-FOXJ1 plasmid (P<0.05 compared to control group, **Figure 4**).

Effects of FOXJ1 on prostate cancer cell proliferation

In MTT, the effect of FOXJ1 overexpression on proliferation of prostate cancer DU145 cells was studied. Our data exhibited that DU145 cell proliferation was inhibited after FOXJ1 expression was elevated (P<0.05 compared to control group, **Figure 5**).
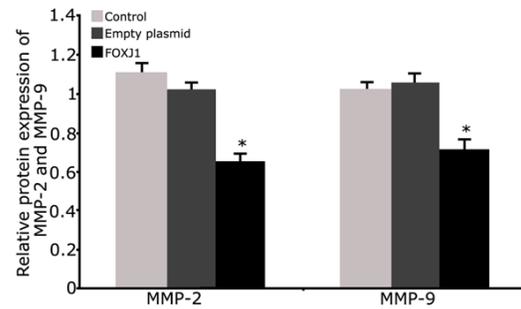

Figure 9. Analysis of effects of FOXJ1 on MMP-2 and MMP-9 expressions in prostate cancer cells. *, P<0.05 compared to control group.

Effects of FOXJ1 on invasion of prostate cancer cells

We then applied Transwell assay to measure the effect of FOXJ1-overexpression on the tumor cell invasion ability of DU145 cells. We found that invasion ability of DU145 cells after over-expression of FOXJ1 gene was remarkably inhibited (P<0.05 compared to control group, **Figures 6** and **7**).

Effects of FOXJ1 on expression of MMP-2 and MMP-9 of prostate cancer cells

In the end, we evaluated the effect of FOXJ1 over-expression on protein levels of MMP-2 and MMP-9 in tumor cells by using Western blotting assay. Results presented reducing levels of MMP-2 and MMP-9 in DU145 cells with elevated FOXJ1 expression (P<0.05 compared to control group, **Figures 8**, **9**).

Discussion

FOX family was firstly discovered in fruit fly with forkhead mutation. There are a multitide of

transcriptional factors in FOX family with similar DNA structures containing a conserved sequence of 110 amino acid residues [17]. FOX has pluripotent functions in multiple signal pathways, embryonic development, cell proliferation, differentiation and apoptosis. FOX family is correlated with various human diseases including premature degeneration of ovary or autism [18]. Recent study showed the involvement of FOX family in the process of malignant tumor metastasis. FOX protein has dual roles as it can competitively bind with promoter region of the same target DNA sequence to affect transcription results. Part of family members even function as tumor suppressor gene, while contribute modulating effect as oncogene in other tumors for mediating various phenotypes of malignant biology of tumor [19, 20]. As one important member of FOX transcription factor family, FOXJ1 participates in immune modulation, nervous system differentiation, genesis of tracheal epithelial and inflammation, in addition to the regulation of oxidative stress and anti-aging effects [21, 22]. The role of FOXJ1 in tumor pathogenesis and progression is unelectable, although its expression and functional role in prostate cancer has not been illustrated. This study revealed significantly decrease of mRNA and protein levels of FOXJ1 in prostate cancer tissues. Our analysis presented that FOXJ1 expression was uncorrelated with age or BMI of patients, but was negatively correlated with TNM stage, tumor volume, and lymph node metastasis, and was positively correlated with histology grade. This result suggested that FOXJ1 might be one tumor suppressor gene of prostate cancer.

We further cultured prostate cancer cell line DU145 *in vitro*, and transfected FOXJ1 into those cells to establish over-expression model, on which the role of FOXJ1 in prostate cancer was discussed. The preliminary data showed the expression of FOXJ1 inhibited the proliferation and invasion of prostate cancer cell line DU145. MMP family exists in the inactive enzymogen form inside various human tissues/organs. They degrade all ECM ingredients except polysaccharides, thus modulate various physio-pathological processes of human body [23]. MMP-2 and MMP-9 in MMP family particularly accelerate tumor proliferation via degrading structures of normal tissues, and cause tissue loosen], thus induced cells predisposed to

metastasis and invasion [24, 25]. This study demonstrated the over-expression of FOXJ1 in prostate cancer DU145 cells, and its inhibitory role for MMP-2 and MMP-9 expression in inhibiting tumor cell invasion.

Conclusion

The down-regulation of FOXJ1 expression in prostate cancer patients is closely correlated with clinical and pathological features. FOXJ1 modulates the proliferation and invasion of prostate cancer cells via regulating MMP-2 and MMP-9 genes, and thus presents as a molecular target for tumor diagnosis and prognostic analysis.

Disclosure of conflict of interest

None.

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