Abstract. Krüppel-like factor 4 (KLF4) is a transcription factor and putative tumor suppressor. However, little is known about its role in the progression of prostate cancer. The aim of the present study was to examine the expression and potential role of KLF4 in prostate cancer. KLF4 and E-cadherin expression in 60 prostate cancer tissues and 60 benign prostatic hyperplasia tissues was characterized by immunohistochemistry. The levels of KLF4 expression in prostate cancer cells were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis. LNCaP cells were transduced with lentivirus to induce KLF4 overexpression. The effects of KLF4 overexpression on proliferation, cell cycle and migration were determined by MTT, flow cytometry, wound healing and Transwell migration assays. KLF4 was identified to be primarily expressed in the cytoplasm of non-tumor prostate tissues. The percentage of KLF4+ tissues among prostate cancer tissues (16.67%) was significantly lower compared with that of non-tumor tissues (84.67%; P<0.05). Downregulated KLF4 expression was associated with higher stage, positive lymph node metastasis and higher Gleason scores of prostate cancer (all P<0.05). Induction of KLF4 overexpression significantly inhibited the proliferation, wound healing and migration of LNCaP cells and induced their cell cycle arrest at S phase. Furthermore, E-cadherin expression was downregulated in prostate cancer tissues and KLF4 overexpression enhanced the levels of E-cadherin expression in LNCaP cells. In conclusion, downregulated KLF4 expression was associated with aggressiveness of prostate cancer, and KLF4 overexpression inhibited the proliferation, wound healing and migration of prostate cancer cells by inducing cell cycle arrest and E-cadherin expression.

Introduction

Prostate cancer is one of the most common malignancies and is the second leading cause of male cancer-associated mortality in the USA (1). Notably, many patients with prostate cancer are diagnosed at the point where their cancers have progressed to advanced stages or distant metastasis (2). Currently, the pathogenesis of prostate cancer is not fully understood, particularly the factors that contribute to the proliferation and metastasis of prostate cancer (3,4). Therefore, the identification of novel diagnostic biomarkers and therapeutic targets will be important for the management of prostate cancer.

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor (5), and is expressed mainly in differentiated epithelial cells in several organs, including the skin (6), lungs (7) and the gastrointestinal tract (8). KLF4 regulates numerous biological processes, including growth, development, differentiation and apoptosis (9-11). Previous studies have indicated that KLF4 negatively regulates cell cycling of epithelial cells, but promotes self-renewal of embryonic stem cells (12-14). KLF4 expression is downregulated in malignant tumors, including colonic adenocarcinomas, and esophageal, gastric, pancreatic and prostate cancers (15-21). These findings suggest that KLF4 may be a suppressor of tumor development and progression. A recent study has identified that KLF4 expression is positively correlated with E-cadherin in breast cancer, and associated with inhibiting metastasis (22). However, the precise role of KLF4 and its mechanisms in the proliferation and migration of prostate cancer have not been clarified.

In the present study, the expression of KLF4 and E-cadherin was examined in prostate cancer and non-tumor tissues and cell lines, and the effect of KLF4 overexpression on the proliferation and migration, as well as E-cadherin expression level, of prostate cancer cells was evaluated. Our findings indicated that KLF4 and E-cadherin expression were downregulated in prostate cancer tissues and that KLF4 overexpression inhibited proliferation and migration, but enhanced E-cadherin expression level, in prostate cancer cells.

Materials and methods

Human prostate cancer and benign prostate hyperplasia tissue specimens. Sixty male patients with prostate cancer, aged between 50 and 67 years old, with an average age of 64.3±6.3 years, and sixty age-matched male patients with
benign prostate hyperplasia, aged between 54 and 65 years old, with an average age of 62.7±4.8 years, were recruited in the Department of Urology, The Affiliated Hospital of Zunyi Medical College (Zunyi, China) between January 2013 and December 2016. Prostate tissues were collected when patients underwent radical prostate resection. Patients with prostate cancer were diagnosed by biopsied tissue pathology and the cancers were staged according to the prostate cancer staging standards of the Union for International Cancer Control (23). The aggressiveness of the cancers was graded using the Gleason score (23). Patients were excluded if they received chemotherapy or radiotherapy or had recently had an infectious disease or severe chronic disease. Written informed consent was obtained from individual patients and the experimental protocol was approved by the Ethics Committee of Zunyi Medical College. The demographic and clinical characteristics of all patients are presented in Table I.

Cells and culture. Human androgen-independent prostate cancer cell lines, PC-3, VCaP, DU145 and LNCaP, and non-tumor prostate RWPE-1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 1 mM sodium pyruvate, 0.1 M nonessential amino acids, 2 mM L-glutamine, and a vitamin solution, as well as 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 5% CO2. Individual cell lines were identified for their authenticity by short tandem repeat-polymerase chain reaction (PCR) using the PowerPlex 16 HS system (Promega Corporation, Madison, WI, USA).

Transduction with lentivirus. LNCaP cells (3x10^5 cells/well) were cultured in 24-well plates overnight and transduced with the lentivirus PSE2609 for expression of KLF4-enhanced red fluorescent protein (ERFP) or PMT194 for expression of ERFP (Shanghai Sunbio Medical Biotechnology Co., Ltd., Shanghai, China) at a multiplicity of infection of 50 in RPMI-1640 for 8 h at 37°C, as previously described (24). Four days later, the stably expressing LNCaP-KLF4-ERFP and LNCaP-ERFP cells were characterized by fluorescence microscopy and >95% of cells were ERFP positive.

Immunohistochemistry. The levels of KLF4 expression in individual tissue samples were determined by immunohistochemistry. Briefly, fresh prostate cancer and BPH tissues were fixed in 4% paraformaldehyde for >24 h at room temperature. After dehydration with an alcohol gradient, the tissue specimens were paraffin-embedded. The paraffin-embedded prostate tissue sections (5-µm thick) were dewaxed, rehydrated and subjected to antigen retrieval. After being washed, the sections were treated with 0.3% hydrogen peroxide in methanol and blocked with 3% normal goat sera (Sijiqing Biological Engineering Materials Co., Ltd.). Subsequently, the sections were incubated with rabbit polyclonal antibodies against human KLF4 (1:500 dilution; ab106629; Abcam, Cambridge, MA, USA) and mouse monoclonal antibody against human E-cadherin (1:500 dilution; sc-71009; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. The sections were incubated with biotinylated rabbit anti-mouse IgG or mouse anti-rabbit IgG (1:200 dilution; sc-516248 or sc-516102; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature and the bound biotinylated antibodies were detected using the Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA). The tissues were stained with diamobenzidine tetrahydrochloride at room temperature for 5 min, and counterstained with hematoxylin at room temperature for 2 min, followed by observation under a light microscope. The reddish-brown anti-KLF4 staining in the nuclei and cytoplasm was semi-quantitatively scored by two investigators in a blind manner (25). The expression of KLF4 or E-cadherin protein was reviewed and scored according to the following grading system: Staining intensity was categorized as negative (-), weak (+), moderate (+++) or strong (+++). The percentage of positive staining was categorized as no staining (--), < 10% of tumor cells stained (+), 10-40% (++), 40-70% (+++) and > 70% (+++). To simultaneously gauge the staining intensity and extent, the average values for the intensity in each section were multiplied by the average values for the percentage area stained in each section to derive a composite score: Histoscore = intensity x area. A sample with a histoscore of ≥0.4 was defined as a positive staining of KLF4 or E-cadherin.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from different groups of prostate cancer cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (2 µg of each) was reverse transcribed into cDNA using the First Strand cDNA Synthesis kit (Promega Corporation). Subsequently, the relative levels of KLF4 and β-actin mRNA transcripts were determined by qPCR using the QuantiTect SYBR-Green RT-PCR kit (Qiagen, Inc., Valencia, CA, USA) and specific primers. The PCR reactions were performed in duplicate at 95°C for 15 sec and subjected to 45 cycles of 95°C for 5 sec and 60°C for 30 sec. The sequences of primers were: Sense, 5'-ACCTACA CAAAGATGGTCCC-3', and antisense 5'-CCA GTC ACA GTG CCC-3' for β-actin (476 bp). The data were normalized to β-actin and analyzed using the 2^ΔΔCt method (26).

Wound healing assay. LNCaP-KLF4-ERFP and LNCaP-ERFP cells (5x10^5 cells/well) were cultured in duplicate in 6-well plates in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO2 overnight. When the cells reached at 90% confluency, the cells were scratched with a yellow tip. After being washed, the cells were cultured for 96 h, and photographed daily under a light microscope.

Western blot analysis. The relative levels of KLF4 protein expression were determined by western blotting. Briefly, the different groups of cells were harvested and lysed in RIPA lysis buffer containing 2 mM phenylmethanesulfonyl fluoride for 30 min, followed by centrifugation (4°C, 15,000 x g for 15 min). After quantification of protein concentration using a Protein Assay Kit II (cat. no. 5000002; Bio-Rad Laboratories, Inc., Hercules, CA, USA), individual cell lysates (50 µg/lane)
were separated by SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% fat-free dry milk at 4°C overnight in TBST and incubated with anti-KLF4 (1:1,000 dilution), anti-E-cadherin (1:1,000 dilution) and mouse monoclonal anti-β-actin (1:1,000; sc-517582; Santa Cruz Biotechnology) at room temperature for 1 h. The bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG (1:2,000 dilution, sc-516142, Santa Cruz Biotechnology, Inc.) and visualized using enhanced chemiluminescence reagent. The relative levels of KLF4 or E-cadherin to β-actin in individual samples were determined by densitometric analysis using Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA).

**MTT assay.** LNCaP-KLF4-ERFP and LNCaP-ERFP cells (1x10^3 cells/well) were cultured in triplicate 96-well plates for up to 4 days and the proliferation of individual groups of cells was determined daily by MTT assay (27). Briefly, during the last 4 h of culture, 25 µl MTT was added to each well and the generated formazan in individual wells was dissolved in 150 µl of DMSO at 37°C for 10 min. Absorbance was then measured at 490 nm in a microplate reader.

**Flow cytometry.** LNCaP-KLF4-ERFP and LNCaP-ERFP cells were harvested, fixed with cold 70% ethanol at 4°C overnight, digested with RNase A (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stained with propidium iodide (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. The cell cycling status of individual groups of cells was characterized by flow cytometry on a flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using the ModFit program (version 3, BD Biosciences) (28).

**Transwell migration assay.** The impact of KLF4 overexpression on the migration of LNCaP cells was examined by Transwell migration assay (29,30). Briefly, individual groups of cells (2x10^5 cells/well) were cultured in triplicate in 5% FBS RPMI-1640 medium in the upper chambers of 24-well Transwell plates (8.0 µm pore size polycarbonate membrane). The bottom chambers were filled with 20% FBS RPMI-1640 medium. After culture for 24 h, the cells on the surface of the upper chambers were removed by scrubbing with a cotton swab. The cells on the bottom surface of the upper chambers were fixed with 4% paraformaldehyde at room temperature for 30 min, air-dried and stained with Giemsa (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min, followed by photoimaging. The migrated cells in five different fields (magnification, x400) of each well were counted under a light microscope in a blinded manner.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean. Groups were compared by Student's t-test, or analysis of variance and post hoc Bonferroni correction when comparing multiple parameters. Categorical data were analyzed using the chi-square test. Statistical analysis was performed using SPSS version 21 (IBM Corp, Armonk, NY, USA). *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**Downregulated KLF4 expression is associated with the aggressiveness of prostate cancer.** To determine the expression and potential role of KLF4 in the development of prostate cancer, the expression of KLF4 in 60 prostate cancer and 60 benign prostate hyperplasia tissues was characterized by immunohistochemistry. KLF4 expression was detected in the cytoplasm of the majority of non-tumor tissue cells, but only in a small number of tumor tissue cells (Fig. 1A and B). The percentage (16.67%) of positive anti-KLF4 staining in prostate cancer was significantly lower compared with benign prostate cancer.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of cases</th>
<th>Number of KLF4+ cases</th>
<th>KLF4+ rate (%)</th>
<th>P-value</th>
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<td>4</td>
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</tr>
<tr>
<td>≥65</td>
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<td>6</td>
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<td></td>
</tr>
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<td></td>
</tr>
<tr>
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<td>3</td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td>T2</td>
<td>25</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>T3</td>
<td>20</td>
<td>2</td>
<td>10.00</td>
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</tr>
<tr>
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<td></td>
</tr>
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<tr>
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</tr>
<tr>
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<td>3</td>
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KLF4, Krüppel-like factor 4.
hyperplasia tissues (81.67%; P<0.05). Stratification analysis indicated that KLF4 expression was significantly associated with lower stage, negative lymph node metastasis and lower Gleason score (P<0.05 for all), but not with age in this population (Table I). Similarly, analysis of KLF4 expression indicated that the levels of KLF4 mRNA transcripts in prostate cancer PC-3, DU145, LNCaP and VCaP cells were significantly lower compared with the non-tumor prostate RWPE-1 cells (P<0.05; Fig. 1C). Furthermore, western blot revealed low or undetectable KLF4 expression in prostate cancer cells, compared with that in the non-tumor prostate RWPE-1 cells (Fig. 1D and E). These data indicated that downregulated KLF4 expression was inversely associated with the aggressiveness of prostate cancer.

**KLF4 overexpression inhibits the proliferation of LNCaP cells.** To explore the role of KLF4 in the development of prostate cancer, LNCaP cells were transduced with lentivirus for KLF4 expression and the levels of KLF4 expression were determined by immunofluorescent assay. The parent LNCaP cells did not express KLF4. Subsequently, the proliferation of LNCaP-KLF4-ERFP and control LNCaP-ERFP cells was determined longitudinally by MTT assays. (A) Immunofluorescent analysis of KFL4 expression. (B) Parent prostate cancer cells serve as a control group. (C) MTT analysis of LNCaP-KLF4-ERFP and LNCaP-ERFP cell proliferation. Data are representative images or expressed as the mean ± standard error of the mean of each group of cells from three separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs. LNCaP-ERFP cells. KLF4, Krüppel-like factor 4; ERFP, enhanced red fluorescent protein.

KLF4 overexpression induces cell cycle arrest at S phase in LNCaP cells. A previous study has demonstrated that KLF4 negatively regulates cell cycling (12). To understand the mechanisms underlying the inhibitory effect of KLF4 overexpression on the proliferation of LNCaP cells, the cell cycling
status of LNCaP-KLF4-ERFP and LNCaP-ERFP cells was determined by flow cytometry (Fig. 3A and B). The percentage of LNCaP-KLF4-ERFP cells at G0/G1 phase was significantly lower compared with LNCaP-ERFP cells (62.78±2.84 vs. 69.64±3.75%; P=0.007; Fig. 3C), while the percentage of LNCaP-KLF4-ERFP cells at S phase was significantly higher compared with that in LNCaP-ERFP cells (23.19±0.91 vs. 13.97±0.73%; P=0.02). These results indicated that KLF4 overexpression induced cell cycle arrest at S phase in LNCaP cells.

KLF4 overexpression attenuates wound healing and migration of LNCaP cells. The impact of KLF4 overexpression on migration of LNCaP cells was determined by wound healing and Transwell migration assays. As indicated in Fig. 4A-C, the number of migrated LNCaP-KLF4-ERFP cells was significantly lower compared with that of LNCaP-ERFP cells at 24 h post-culture (P<0.05). Similarly, the gap width of the wounded LNCaP-KLF4-ERFP monolayer cells was significantly larger compared with that in the LNCaP-ERFP cells at 48 (P<0.05), 72 (P<0.01) and 96 h (P<0.001) post-wounding (Fig. 4D and E). These results indicated that KLF4 overexpression inhibited the wound healing and migration of LNCaP cells in vitro.

KLF4 overexpression enhances E-cadherin expression in LNCaP cells. Previous studies have indicated that KLF4 and E-cadherin exhibit a similar expression pattern in nasopharyngeal carcinoma, and KLF4 enhances E-cadherin expression to inhibit the metastasis of breast cancer (30,31). To determine whether KLF4 and E-cadherin expression were expressed in a similar manner in prostate cancer, the expression of E-cadherin in 60 prostate cancer and 60 benign prostate
hyperplasia tissues was characterized by immunohistochemistry. The percentage of anti-E-cadherin staining in prostate cancer tissues was significantly lower compared with that of benign non-tumor prostate tissues in this population (30.0 vs. 88.33%；P<0.01). The levels of E-cadherin expression in prostate cancer tissues were markedly lower compared with benign prostate hyperplasia tissues in this population (Fig. 5A). By contrast, western blot analysis revealed that the relative levels of E-cadherin expression in LNCaP-KLF4-ERFP cells were significantly higher compared with in LNCaP-ERFP cells (P<0.05; Fig. 5B). Thus, KLF4 overexpression increased E-cadherin expression, which is likely to contribute to the inhibition of migration of LNCaP-KLF4 cells.

Discussion

KLF4 is expressed in various tissues, including epithelial cells, vascular smooth muscle cells and monocytes/macrophages (32,33), but its expression is commonly downregulated in several types of malignant tumor (18,24,34,35). Notably, KLF4 expression is upregulated in breast cancer (36). In the present study, it was identified that KLF4 expression was downregulated in prostate cancer tissues, which was consistent with previous observations (24,37). Furthermore, downregulated KLF4 expression was associated with higher tumor stage, positive lymph node metastasis and higher Gleason scores. Hence, downregulated KLF4 expression may be associated with poor prognosis in patients with prostate cancer (24).

Similarly, KLF4 expression was decreased in several prostate cancer cell lines. The downregulation of KLF4 expression in prostate cancer may be due to hypermethylation of the KLF4 promoter during the development and progression of prostate cancer (38). This would support the notion that KLF4 acts as an oncogene or tumor suppressor, depending on its molecular environment (35) and KLF4 may be a suppressor of prostate cancer. The current findings add to previous observations that loss of KLF4 expression in primary hepatocellular carcinoma is very common, and KLF4 overexpression activates Smad7 and blocks TGF-β signaling, which is one of the key mechanisms by which KLF4 effectively inhibits liver cancer cell migration and invasion (39). The inverse association of KLF4 expression with the aggressiveness of prostate cancer suggests that KLF4 may be valuable for evaluating the pathological degree of prostate cancer.

To understand the role of KLF4 in the proliferation and migration of prostate cancer cells, KLF4 overexpression was induced in human prostate cancer LNCaP cells. It was identified that KLF4 overexpression inhibited the proliferation of LNCaP cells and induced their cell cycling arrest at S phase. These results were consistent with previous observations in colon cancer and breast cancer cells (15,36). A previous study demonstrated that KLF4 acts as a CSC pluripotency regulator in breast cancer, as blocking TGF-β signaling suppressed its expression and decreased lung metastasis (40). This mechanism may be involved in the regulation of proliferation and migration of prostate cancer cells. The inhibition of cell

Figure 5. KLF4 expression is associated with E-cadherin expression in prostate cancer. (A) E-cadherin expression in prostate cancer tissues, and (B) non-tumor prostate tissues was characterized by immunohistochemistry (magnification, x400). E-cadherin expression was low or absent in prostate cancer tissues and positive in non-cancer prostate tissues. The levels of E-cadherin and KLF4 expression in LNCaP-KLF4-ERFP and LNCaP-ERFP cells were (C) determined by western blot and (D) quantitatively analyzed. Data are representative images or expressed as the mean ± standard error of the mean of each group of cells from three separate experiments.*P<0.05 vs. LNCaP-ERFP cells. KLF4, Krüppel-like factor 4.
cycling by KLF4 may be associated with its role in promoting the expression of cell cycling inhibitors, while inhibiting the expression of pro-cell cycling transition genes (12). In future studies, our group hopes to investigate how KLF4 regulates the expression of cell cycling regulators.

Tumor metastasis is a risk factor of prostate cancer-associated mortality. In the present study, it was identified that downregulated KLF4 expression was associated with lymph node metastasis in this population. Furthermore, it was identified that KLF4 overexpression significantly inhibited the wound healing and migration of LNCaP cells. Similarly, downregulated E-cadherin expression was detected in prostate cancer tissues and KLF4 overexpression enhanced the levels of E-cadherin expression in LNCaP cells. These data indicate that KLF4 expression is positively associated with E-cadherin expression in prostate cancer tissues (30). Given that increased levels of E-cadherin expression are negatively associated with epithelial mesenchymal transition (EMT), KLF4 overexpression may inhibit the EMT process in LNCaP cells. The current data were consistent with previous studies, in which it was reported that downregulated KLF4 expression has a potential prognostic value for lymph node metastasis (12,29). A previous study demonstrated that KLF4 inhibits the EMT process in prostate cancer cells by downregulating Slug expression (41). Hence, a therapeutic strategy to increase KLF4 expression may be valuable for inhibiting the proliferation and migration of prostate cancer. KLF4 is a multifunctional regulator of malignant tumors and stem cell differentiation. Our group is interested in exploring the potential roles and mechanisms underlying the action of KLF4 further, including its interaction with SOX2, Myc, or its crosstalk with TGF-β signaling during the progression and metastasis of prostate cancer.

In summary, the current results indicated that downregulated KLF4 expression was associated with aggressiveness of prostate cancer in this population. Similarly, downregulated E-cadherin expression was detected in prostate cancer tissues. KLF4 overexpression inhibited the proliferation, wound healing and migration of LNCaP cells, which was associated with enhanced E-cadherin expression. These results extend previous findings and support the notion that KLF4 acts as a tumor suppressor in prostate cancer. The current findings suggest that KLF4 expression may be valuable for evaluating the aggressiveness of prostate cancer. A therapeutic strategy to increase the KLF4 expression may be valuable for the management of prostate cancer. The present study was limited by a relatively small sample size and a lack of detailed mechanisms underlying the action of KFL4 further, including its interaction with SOX2, Myc, or its crosstalk with TGF-β signaling during the progression and metastasis of prostate cancer.

Acknowledgements

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Availability of data and materials

The datasets used during this present study are available from the corresponding author on reasonable request.

Authors’ contributions

NZ designed the project and wrote the manuscript. PS analyzed the data. XIL collected prostate cancer tissue and non-cancer prostate tissue specimens for immunohistochemistry. XUL and JX performed the experiments. LX designed and guided the project. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from individual patients and the experimental protocol was approved by the Ethics Committee of Zunyi Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

References


