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TUG1 promotes the development of prostate cancer by regulating RLIM

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Abstract. – OBJECTIVE: The aim of this study was to investigate the specific role of Taurine up-regulated gene 1 (TUG1) in the development of prostate cancer (PCa), and to explore its underlying mechanism.

PATIENTS AND METHODS: The serum level of TUG1 in healthy subjects, benign prostatic hyperplasia (BPH) patients and PCa patients was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between TUG1 expression and clinical indexes of PCa patients was analyzed. TUG1 expression in PCa cells and human normal prostate cells was determined by qRT-PCR as well. Overexpr or knockdown of TUG1 was achieved by mal transfection. Subsequently, the reg o-ry effects of TUG1 on the proliferative an gratory capacities of DU145 cells were acces by Cell Counting Kit-8 (CCK-8) assay, colony fo mation assay and transwell as pectively. An online software was used whether RLIM could be regulated by 1, wh vas further verified by qRT-PCR r RLIM in DU145 cells, the prolife and pacities were also de y, Western blot was conducted eterm tive protein expressions in th F-β1/Smac ay after altering TUG1 e n in DU145 c

RESULTS: T w hly expressed in serum sample PCa p s when compared with heal subjects an H patients. Besides, T expression in patients with Gleas 7 was ignificantly higher than those with ason 4 leanwhile, TUG1 expression in atien as rem kably higher than that of Bl s at th A grey zone (4-10 ng/mg). RO es ind ed that TUG1 might be a l hal nguish PCa patients from BP tients althy subjects. The overexpres n of TUG markedly promoted the proliferative and migratory capacities of DU145 cells. H kdown of TUG1 obtained the opposite results. QRT-PCR confirmed that TUG1 was positively correlated with RLIM at the mRNA level. RLIM knockdown significantly inhibited the proliferative and migratory capacities of DU145 cells. Furthermore, knockdown of TUG1 in DU145 cells markedly down d TGF-β1 and p-Smad2/3, whereas up-regu p-Smad7.

CONCLUSIONS: TUG1 is highly expressed in peripheral blood of PCa patients, which can serve as a potential diagnostic marker for PCa. The expression of promotes the proliferative and migratory capacities of PCa cells. Furthermore, TUG1 promotes the development of PCa by regulating RLIM through the TGF-β1/Smad pathway.

Keywords:
Prostate cancer (PC), TUG1, RLIM, TGF-β1/Smad pathway, Proliferation, Invasion.

Introduction

Prostate cancer (PCa) is a common type of tumor in males with high incidence globally. PCa is the second leading cause of cancer deaths in men in the United States[1]. According to the newly published tumor surveillance data in China, PCa ranks 7th in tumor incidence and 12th in tumor death[2,3]. PCa is a clinically heterogeneous multifactorial disease with an increased incidence. Similar to many solid tumors, PCa patients mainly die from tumor metastasis. It is known that metastasis is a complex process involving changes in the extracellular matrix microenvironment of tumor cell invasion and growth[4]. However, the exact molecular mechanism of PCa remains unclear. Furthermore, clarifying the mechanism of PCa is of great importance for clinical diagnosis, treatment and monitoring.

Long non-coding RNA (lncRNA) is a type of transcript located in the nucleus or cytoplasm with over than 200 nucleotides in length. LncRNA is structurally similar to mRNA; meanwhile, it lacks open reading frame[5]. In 2012, researchers from Stanford Medicine conducted the first large-scale analysis of lncRNA expression in cancer. They performed RNA sequencing in 64 tu-

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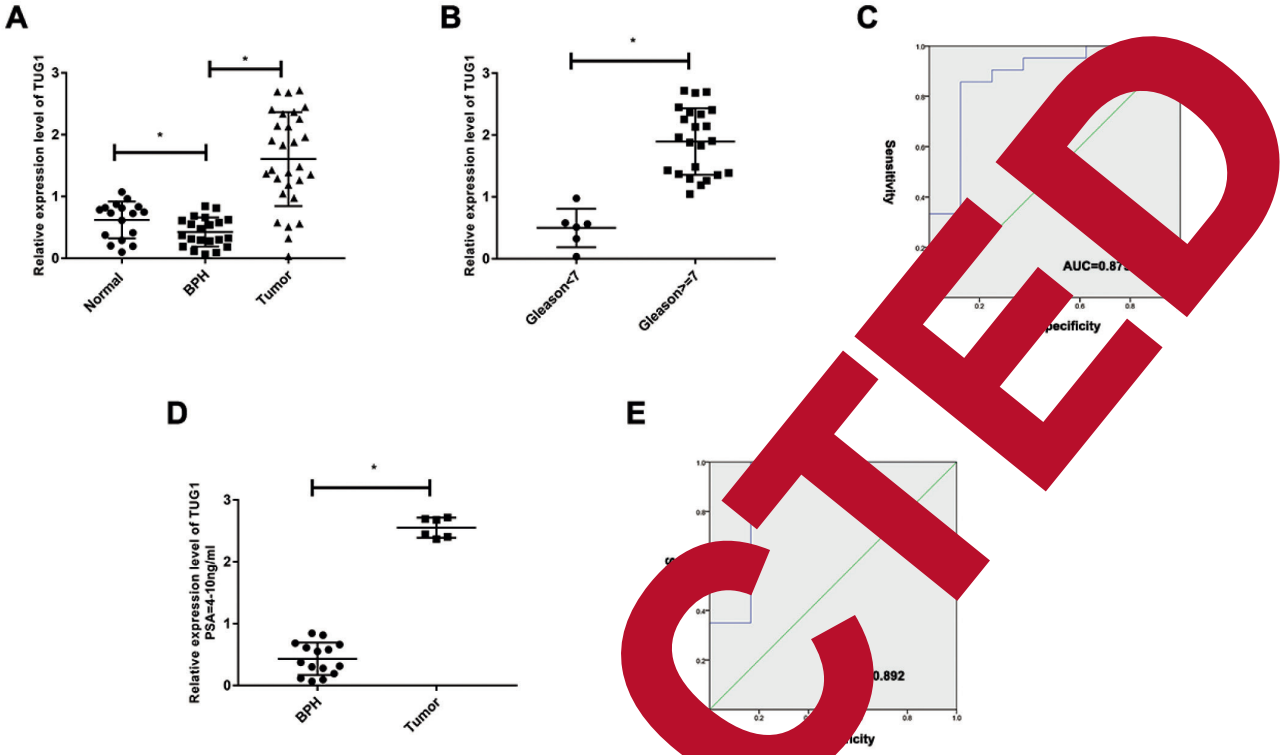


Figure 1. TUG1 was highly expressed in serum ...ients. ***A,*** TUG1 was highly expressed in serum samples of PCa patients when compared with healthy subjects an... TUG1 expression in PCa patients with Gleason ≥ 7 was significantly higher than those with Gleason < 7. ***C,*** ...curves ...ression in PCa patients. The AUC indicated that TUG1 exerted an adequate diagnostic value for differ... ...ng P... ...om controls. ***D,*** TUG1 expression in PCa patients was remarkably higher than those BPH patients at the ...one (4-10 ng/mg). ***E,*** ROC curves for TUG1 expression in BPH and PCa patients. The AUC indicated that TUG1 e... ...n adequate diagnostic value for differentiating PCa patients from BPH patients at PSA grey zone.

mor samples, and found ...ds ... expressed lncRNAs i... ...mo... ...065 known lncRNAs[6]. As is k... ...n to all, ...NA mainly exerts its biologi... ...unctions by ... ...ing the expression of f... ...nes at epige... ...ic, transcriptional an... ...ost-tra... ...tional levels[7,8].

Taurine ...regulated ge... (TUG1) locates at 22q12.2... ...ch exert no prote... ...ding function. TUG1 ... origi... ...y discovered and identified by Ren... ... in st... ...related to retinal development in ... A... ...g all ...er-related lncRNAs, TUG1 i... ...d as a c... ...nogenic lncRNA[10]. Feng ... have ...te... ...t TUG1 promotes the pro-li... ...e and i... ...e capacities of gastric cancer cells ... addition, ...he inhibitory effect of miR-145 on ...ve and invasive capacities of ga... ...cancer ...lls can be reversed by overexpression of TUG1 *in vitro*. Xu et al[12] have suggested ...UG1 is up-regulated in renal cell carcinoma tiss... ...nd cells. Moreover, TUG1 expression is positively correlated with Fuhrman classification and tumor size of renal tumors. Down-regulation of TUG1 inhibits the proliferation, migration and invasion of renal cell carcinoma, whereas induce apoptosis *in vitro*. The aim of this study was to investigate the role of TUG1 in PCa development and its possible mechanism.

Patients and Methods

Sample Collection

Peripheral blood samples were collected from healthy subjects, benign prostatic hyperplasia (BPH) patients at initial diagnosis and PCa patients in the Urinary Surgery of Gansu Provincial Hospital from May 2014 to May 2016. Collected blood samples were immediately preserved in liquid nitrogen for subsequent use. BPH and PCa patients were confirmed by pathological diagnosis. PCa patients did not receive any preoperative treatment. Informed consent was obtained from

enrolled patients and their families before the study. This study was approved by the Ethics Committee of Gansu Provincial Hospital.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

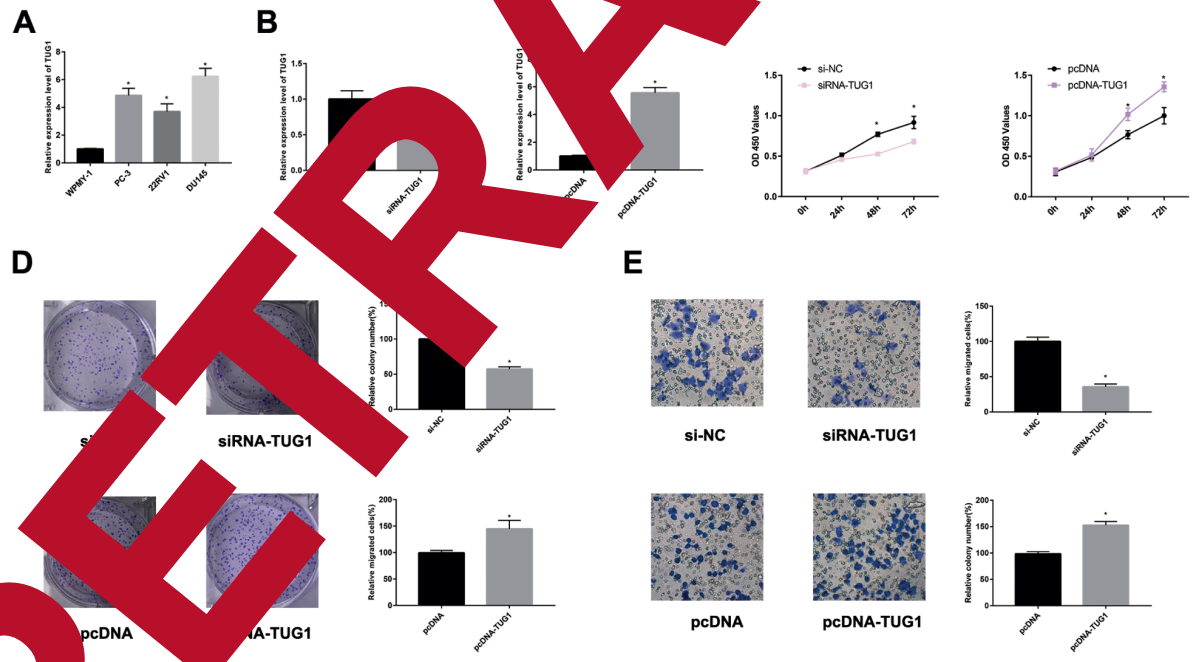
Total RNA was extracted from serum or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). RNAs with 1.8-2.1 of A260/A280 were qualified for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) reaction using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real Time-Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA, USA). Primers used in this study were as follows: TUG1: F: CTGAAGAAAGGCAACATC, R: GTAGGCTACTACAGGATTTG; RLIM: F: AGAGTTGCTGAGACGACTACAG, R: TAGAACGTCTTGCAGATGGCTC; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cell Culture and Transfection

PCa cell lines (PC-3, 22RV1, [illegible]45) and human prostate cell line (WPMY-[illegible] obtained from CellBank, Chinese A[illegible]ademy [illegible]iences. All cells were cultured in R[illegible]well Park [illegible]ri[illegible] Institute-1640 (RPMI-16[illegible] Gibco, Grand [illegible] NY, USA) containin[illegible]% feta[illegible]vine ser[illegible]n (FBS; Gibco, Grand [illegible]d, NY[illegible]SA), an[illegible] main-tained in a 5% C[illegible] [illegible]t 37°C[illegible]r cell transfection, ce[illegible]were [illegible]eded [illegible] 6-well plates at a d[illegible]y of 1×10[illegible] [illegible]ell. Until 60-70% of [illegible]ce, cell trans[illegible]on was performed i[illegible]ict[illegible]dance with Lipofectamine 2000 (Invitrogen, C[illegible]d, CA, USA).

C[illegible]unting Kit-8 (CC[illegible]-8)

[illegible]ansfected DU145 cells for 48 h were first se-[illegible]d into 96-we[illegible]tes at a density of 2×10⁴ cells [illegible]vell. After c[illegible]uring for 0 h, 24 h, 48 h and 7[illegible]espectiv[illegible]Cell Counting Kit-8 (CCK-8; Doj[illegible]ies, Kumamoto, Japan) reagent



Fig[illegible]omoted proliferative and migratory capacities of PCa cells. *A,* TUG1 expression in PCa cell lines (PC-2[illegible]V1, [illegible]) and human prostate cell line (WPMY-1). ***B,*** TUG1 expression in DU145 cells after transfection of siR-[illegible]TUG1 or pcDNA-TUG1 detected by qRT-PCR. ***C,*** CCK-8 assay showed that TUG1 overexpression remarkably promoted [illegible]oliferation of DU145 cells. TUG1 knockdown inhibited cell proliferation. ***D,*** Colony formation assay showed that TUG1 o[illegible]ession significantly promoted the proliferation of DU145 cells. TUG1 knockdown inhibited cell proliferation. ***E,*** The trans[illegible]assay showed that TUG1 overexpression markedly promoted the migration of DU145 cells. TUG1 knockdown inhibited cell migration (Magnification × 40).

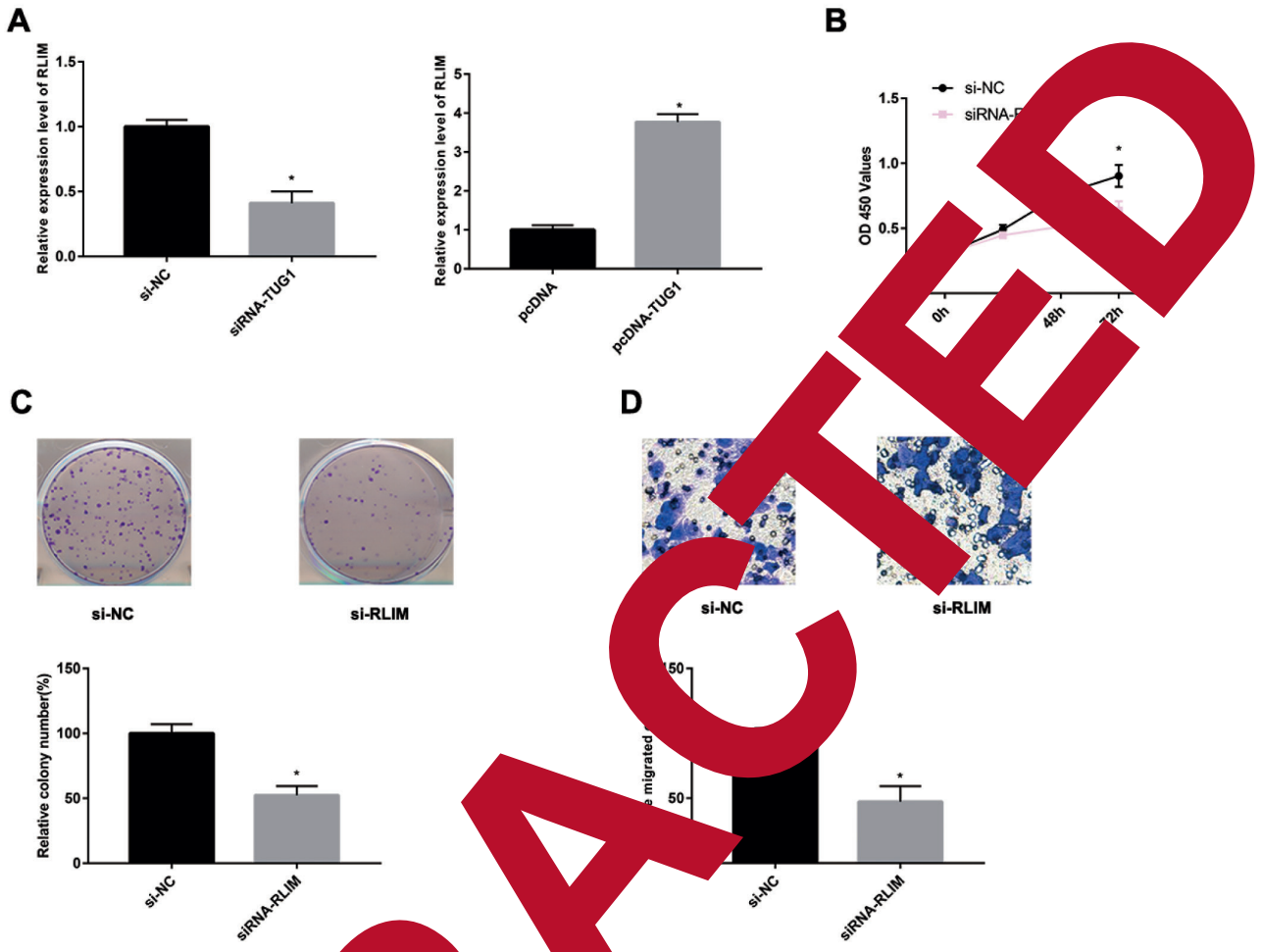


Figure 3. TUG1 up-regulated R... expre... ***A,*** TUG1 ... expression up-regulated RLIM expression, whereas TUG1 knockdown down-regulated RL... xpressio... DU145 cells. ***B,*** CCK-8 assay showed that RLIM knockdown significantly inhibited the proliferation of ... cells. ... ion assay showed that RLIM knockdown remarkably inhibited the proliferation of DU145 ...lls. ... owed that RLIM knockdown markedly inhibited the migration of DU145 cells (Magnificati... 40).

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...nsfec... DU... ells for 48 h were first se-ed... to 6-w... es at a density of 1000 cells per ... l. Subsequ...ntly, cells were cultured with co... m for two weeks. Culture me-... was re...aced once and twice at the first and ...ond week, respectively. Until colony forma-... cells were washed with Phosphate-Buffe-red ...e (PBS; Gibco, Grand Island, NY, USA) twice and fixed with 4% paraformaldehyde for 30 min. After washing with PBS, cells were then stained with 0.1% crystal violet staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Finally, colonies were observed and captured using a microscope.

Transwell Assay

DU145 cells were re-suspended in serum-free medium at a dose of 1×10^5/mL. The transwell chamber was first placed in a 24-well plate. 100 μL of cell suspension was added to the upper chamber, while 600 μL of medium containing 10% FBS was added to the lower chamber. 48 hours later, cells were fixed with 4% paraformaldehyde for 15 min until chamber removal. After

that, cells were stained with 0.1% crystal violet for 5 min. Subsequently, the inner layer cells were carefully removed. Three fields were randomly selected for each sample. Finally, the amount of penetrating cells was calculated.

Western Blot

The protein sample was first extracted in cells, and the concentration of total protein was determined by the bicinchoninic acid (BCA) protein determination kit (Pierce, Waltham, MA, USA). Extracted protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot was conducted according to standard procedures. Primary antibodies and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. Receiver Operating Characteristic (ROC) curves were introduced to analyze the diagnostic value of TUG1 in PCa. $p<0.05$ was considered statistically significant.

Results

TUG1 was Highly Expressed in Serum of PCa Patients

The serum level of TUG1 in healthy subjects, BPH patients and PCa patients was detected by qRT-PCR. The results showed that TUG1 was highly expressed in serum samples of PCa patients when compared with healthy subjects and BPH patients (Figure 1A). The curve indicated that PCa patients could be distinguished from healthy subjects based on the level of TUG1 ($p<0.05$, AUC=0.75, Figure 1C). Subsequently, the correlation analysis was conducted to explore the relationship between TUG1 expression with age, PSA level (10 ng/mL as the board line), Gleason score (7 as the board line), tumor stage, lymph node metastasis and distant metastasis of PCa patients (Table I). The data revealed that TUG1 expression was only correlated with Gleason score of PCa patients more than other indicators. Subsequent results demonstrated that TUG1 expression in PCa patients with Gleason ≥ 7 was markedly higher than with Gleason < 7 (Figure 1B). No significant difference in TUG1 expression was found between PCa patients with PSA higher and lower than 10 ng/mg. However, TUG1 expression in PCa patients was remarkably higher than BPH patients at the PSA grey zone (4-10 ng/mg; Figure 1D). Based on ROC curve analyses, TUG1 might serve as an important diagnostic hallmark for PCa

Table I. The correlation between lncRNA level and clinicopathological characteristics in patients with PCa.

Characteristics	No	*p*
Age (year) <70	13	0.84
≥70	16	
PSA (ng/mL) ≤10	6	0.62
>10	23	
Gleason score <7	6	
≥7	23	0.02*
Tumor stage T2	11	0.38
T3-T4	18	
Lymph node metastasis N0	16	0.29
N1	13	
Development of metastasis M0	9	0.67
M1	20	

No, number of patients; *$p<0.05$.

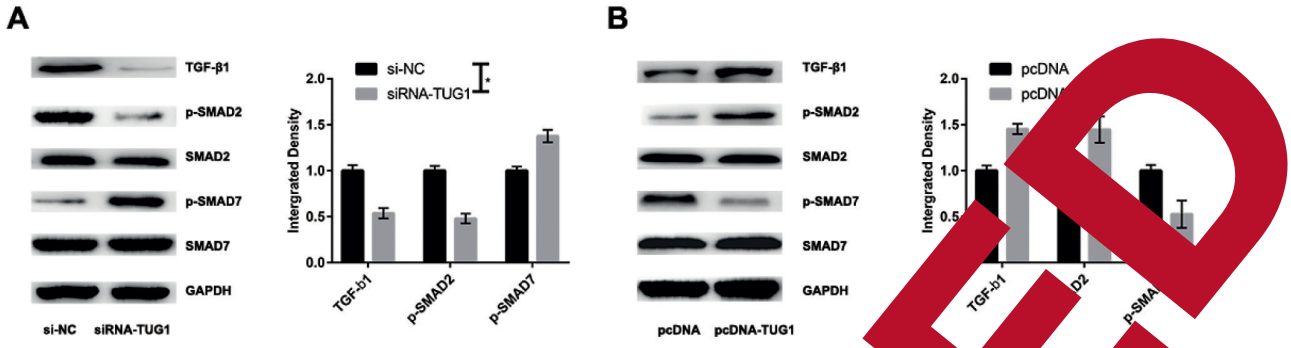

Figure 4. TUG1 activated TGF-β1/Smad pathway in PCa. ***A,*** Protein expressions of TGF-β1, p-Smad2, SMAD2, p-Smad7 and Smad7 in DU145 cells after TUG1 knockdown. ***B,*** Protein expressions of TGF-β1, p-Smad2, SMAD2, p-Smad7 and Smad7 in DU145 cells after TUG1 overexpression.

and BPH at the PSA level of 4-10 ng/mg (p<0.05, AUC=0.892, Figure 1E).

TUG1 Promoted Proliferative and Migratory Capacities of PCa Cells

To further explore the biological functions of TUG1 in PCa *in vitro*, we first detected TUG1 expression in PCa cell lines and normal prostate cell line. The results indicated that TUG1 was highly expressed in PC-3, 22RV1 and DU145 cells when compared with WPMY-1 cells (Figure 2A). In particular, DU145 cells expressed the highest level of TUG1, which were utilized for the following experiments. Subsequently, transfection efficacies of siRNA-TUG1 and pcDNA-TUG1 in DU145 cells were verified by qRT-PCR (Figure 2B). CCK-8 and colony formation assay indicated that transfection of pcDNA-TUG1 significantly increased the proliferative capacities of DU145 cells (Figure 2C and 2D). Similarly, transwell assay demonstrated that the migratory capacities of DU145 was remarkably elevated after TUG1 overexpression (Figure 2E). TUG1 knockdown obtained opposite results as significantly inhibited proliferative and migratory capacities of DU145 cells.

TUG1 Regulated RLIM Expression

Starbase predicted that TUG1 was capable of regulating RLIM expression. To verify this prediction, we detected RLIM expression after knockdown or overexpression of TUG1 in DU145 cells. qRT-PCR data demonstrated that TUG1 was positively correlated with RLIM at mRNA level (Figure 3A). Interestingly, RLIM showed similar regulatory effects on the proliferative and migratory capacities of DU145 cells as TUG1. In brief, RLIM knockdown in DU145 cells significantly inhibited the proliferative and migratory capacities of DU145 cells (Figure 3B-3D).

TUG1 Activated TGF-β1/Smad Pathway in PCa

Previous studies have confirmed that RLIM can regulate the TGF-β1/Smad pathway in PCa. Hence, we speculated that TUG1 might also influence the activation of the TGF-β1/Smad pathway. Western blot was conducted to determine protein expression of relative genes in the TGF-β1/Smad pathway after altering TUG1 expression in DU145 cells. The results indicated that knockdown of TUG1 markedly down-regulated TGF-β1 and p-Smad2, whereas up-regulated p-Smad7 (Figure 4A). However, the overexpression of TUG1 obtained the opposite results.

Discussion

In recent years, lncRNA has become a research hotspot in life sciences and molecular biology. Some studies have demonstrated the significant functions of lncRNAs in the occurrence and progression of malignant tumors. Meanwhile, some lncRNAs may be used as molecular markers for tumor diagnosis and new therapeutic targets for tumor treatment[13,14].

Prostate cancer antigen 3 (PCA3) is only expressed in prostate tissues with good tissue specificity. In 1999, lncRNA PCA3 was found to be highly expressed in PCa tissues[15]. PCA3 may be involved in promoting the proliferation of PCa cells by regulating androgen receptor signaling pathway[16]. At present, researchers have shown that the level of PCA3 in urine exhibits high sensitivity and specificity for the diagnosis of PCa.

This allows a new approach to the non-invasive diagnosis of PCa[17,18]. Currently, digital rectal examination combined with PSA examination is recognized as an important method for early screening of PCa. PSA detection has multiple advantages in the diagnosis of PCa, including high sensitivity and relatively low specificity. However, PSA grey zone seriously restricts the diagnostic efficacy. When PSA is between 4-10 ng/ml (PSA grey zone), the detection rate of PCa is only about 25%[19]. In this work, the serum level of TUG1 in healthy subjects, BPH patients and PCa patients was detected. It was found that PCa patients could be effectively identified based on TUG1 expression even in PSA grey zone. The above results indicated the crucial role of TUG1 in PCa development, which also provided a novel direction for PCa diagnosis. However, its specific mechanism still remained unknown.

Starbase website predicted that TUG1 could regulate the expression of RLIM. In this study, RLIM knockdown significantly inhibited the proliferative and migratory abilities of DU145 cells, which were consistent after TUG1 knockdown. Through literature review, it was found that RLIM could regulate the TGF-β/Smad signaling pathway[20]. Cytosolic Smad is a key substrate for TGF-β receptor, which is capable of mediating the transfer of TGF-β signal from the cytoplasm to the nucleus. Meanwhile, it can specifically regulate the expression of TGF-β target genes[21]. Smad can be classified into the following three types according to their functions. The first is receptor-regulated Smad (R-Smad), the second type which are activated by different receptors, including BMP-activated Smad1, Smad5 and Smad8 and TGF-β/Activin-activated Smad2 and Smad3. The second type is common Smad (Co-Smad), namely Smad4. The last type is inhibitory Smad (I-Smad), including Smad6 and Smad7, which are capable of suppressing TGF-β and BMP pathway. The TGF-β/Smad pathway plays a complex and diverse role in cancers. In the early stages of cancer, TGF-β acts as a tumor suppressor gene. However, during the process of cancer development, TGF-β gradually transforms into an oncogene. In normal cells, TGF-β can inhibit cell proliferation, induce differentiation and apoptosis by blocking cell cycle release to S phase. However, abnormal expressions or functional defects of TGF-β in tumor cells stimulate metastasis and growth of tumors. This may overwhelm the inhibitory effects of TGF-β on cell growth. In this work, we detected the protein expressions of relative genes in the TGF-β/Smad pathway by Western blot. It is found that TGF-β1 and p-Smad2 were significantly up-regulated, while p-Smad7 was down-regulated in DU145 cells after TUG1 overexpression. However, TUG1 knockdown obtained the opposite results.

Conclusions

We first found that TUG1 is highly expressed in peripheral blood of patients, which can serve as a potential diagnostic marker for PCa. The overexpression of TUG1 promotes the proliferative and migratory capacities of PCa cells. Furthermore, TUG1 promotes the development of PCa by regulating RLIM through the TGF-β1/Smad pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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