Silencing of URG11 expression inhibits the proliferation and epithelial-mesenchymal transition in benign prostatic hyperplasia cells via the RhoA/ROCK1 pathway

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Abstract. Upregulated gene 11 (URG11) represents a gene upregulated by hepatitis B virus X protein and is involved in the biological processes of multifarious tumors. The present study aimed to investigate the protective effects and regulatory mechanisms of URG11 in benign prostatic hyperplasia (BPH). URG11, Ras homolog family member A (RhoA) and Rho-associated protein kinase 1 (ROCK1) expression was detected in patients with BPH using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Furthermore, URG11 expression was silenced using URG11-targeting small interfering RNAs. In addition, cell viability was determined by performing a Cell Counting Kit-8 assay, and the effect of URG11 on the cell cycle was investigated by flow cytometry. Expression levels of cyclin D1, p27, E-cadherin, N-cadherin, vimentin, RhoA and ROCK1 were investigated by RT-qPCR and western blotting. The results revealed that the expression levels of URG11, RhoA and ROCK1 were enhanced in patients with BPH compared with matched healthy controls. Furthermore, it was demonstrated that transforming growth factor-β (TGF-β) induced the proliferation of BPH-1 cells in vitro, and silencing of URG11 inhibited the effects of TGF-β on BPH-1 cell proliferation and the cell cycle. In addition, silencing of URG11 altered the expression levels of cell cycle-associated genes, epithelial-mesenchymal transition-associated genes, and RhoA and ROCK1 protein levels. Thus, the results of the present study suggest that URG11 may be a potential therapeutic target, which may be important to inhibit the development and progression of prostatic hyperplasia.

Introduction

Benign prostatic hyperplasia (BPH) has become a common and frequently-occurring disease among elderly men worldwide, following the improvements in living standards and the prolongation of average life expectancies (1). Age, growth and testicular function have previously been revealed to affect the pathogenesis of BPH (2); however, its underlying mechanisms remain unclear. In recent years, the role of upregulated gene 11 (URG11; also termed von Willebrand factor C and EGF domain-containing protein) in the pathogenesis of BPH has gained increasing attention.

URG11 was initially identified as an upregulated gene in hepatitis B virus X protein (HBx)-positive HepG2 cells by polymerase chain reaction (PCR)-selected cDNA subtraction, and it has also been demonstrated that URG11 promotes the growth of hepatocellular carcinoma and tumorigenesis (3). URG11 encodes for a 70 kDa protein, which comprises of cysteine-rich and lectin domains (4). A further study revealed that lectin domains are closely associated with cell adhesion, matrix interaction, migration and invasion (5). Furthermore, it has previously been demonstrated that the expression of URG11 is enhanced in liver, thyroid, gastric and bladder cancer (3,4). In addition, a previous study revealed that URG11 is involved in the regulatory processes of cell growth, adhesion, migration and tumor metastasis (6). Furthermore, it has been demonstrated that URG11 is involved in epithelial-mesenchymal transition (EMT) via E-cadherin in the human proximal tubule cells (7). Therefore, URG11 may be an important gene in the progress of tumorigenesis and metastasis. However, the function and mechanism of URG11 in the pathogenesis and development of BPH have not yet been determined.

EMT refers to the transformation process whereby epithelial cells lose their polarity and move freely among the cell matrix with a mesenchymal cell phenotype (8). A previous study has demonstrated that within the patients with BPH, epithelial cells lose E-cadherin protein expression, get vimentin protein expression, which suggests that the development
of BPH may be initiated from the dysregulation of prostate epithelial cells (9). Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that is involved in cell reconstruction processes, such as cell proliferation, apoptosis, differentiation, migration and invasion (10). TGF-β is considered to have an important role in EMT (11). Despite the TGF-β signaling pathway being relatively simple, its regulatory mechanism is highly complex. Currently, the TGF-β signaling pathway and its associations with other signaling pathways have become an important research field.

The Ras protein family of guanosine triphosphates (GTPases) is comprised of numerous subfamilies. Ras family proteins include Rho, Rac1 and Cdc42 (12). Rho acts as a molecular switch in response to cytokines, G-protein coupled receptors, adhesion molecules and growth factor cell surface receptors by cycling between active GTP and inactive guanine diphosphate (13). Rho-associated protein kinase (ROCK)1 and ROCK2 protein kinases of the Rho protein family (14) that regulate actomyosin contractility via phosphorylation of the myosin light chain and inactivation of the myosin-binding subunit of myosin phosphatase (15). Furthermore, Ras homolog family member A (RhoA) and downstream target ROCK1 are involved in numerous biological and pathological processes, including cell adherence, apoptosis, proliferation and migration (16).

In the present study, the expression levels of URG11, RhoA and ROCK1 in patients with BPH, the effect of TGF-β on BPH-1 cells, and the roles of URG11 in BPH-1 cell proliferation, the cell cycle and EMT were investigated. In addition, the role of URG11 in BPH was investigated. The results of the present study suggest that URG11 may be a novel therapeutic target for the treatment of BPH.

Materials and methods

Clinical samples. A total of 5 ml of peripheral blood from 37 male patients (aged from 40-75 years old; mean, 52.3±3.6) with prostatic hyperplasia and the healthy prostate glands were collected from healthy volunteers (aged from 40-75 years old; mean, 49.9±5.2) at The First Affiliated Hospital of Xinxiang Medical University (Xinxiang, China) between July 2015 and June 2016. The participants who were diagnosed as BPH and willing to participate in the present study were enrolled. The exclusion criteria were as follows: Prostate carcinoma, bladder cancer, urethral stricture; cardiovascular, liver, kidney and hematopoietic disease and mental illness. Serum samples were then frozen at −80˚C until further analysis. The sequence of si-NC was 5'-UUCUCCGAACGUUCACG-U-3', and the sequence of si-URG11 was 5'-CAGACGGAUUGCUGACU-U-3'. BPH-1 cells (2x10^4 cells/well) were seeded into 6-well plates and incubated at 37˚C overnight. Following this, cells were transfected with si-NC (50 µM) and si-URG11 (50 µM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 36 h post-transfection, the cells were collected to proceed with the subsequent experiments.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the treated BPH-1 cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized from the extracted RNA using a reverse transcription kit, PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to manufacturer's instructions. The reaction mixture was incubated at 37˚C, 15 min, and reaction was inactivated at 85˚C for 5 sec. Following this, qPCR was performed using SYBR Premix Ex Taq™ (Takara Bio, Inc.,). The PCR thermocycling conditions were as follows: 95˚C for 10 min, 35 cycles at 95˚C for 15 sec and 60˚C for 60 sec. Expression levels were quantified using the 2^-ΔΔCq method (17). The primer sequences are presented in Table I.

Western blotting. Cells were rinsed with PBS (cat. no. 37350; Stemcell Technologies, Inc., Beijing, China) and directly lysed in ice-cold radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology, Beijing, China). Protein samples (20 mg) were then separated by performing 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. Membranes were then blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; cat. no. A0281) at room temperature. After 2 h, membranes were then incubated with the following primary antibodies (all Abcam, Cambridge, UK) at 4˚C overnight: Anti-URG11 [1:600 (3)], anti-cyclin D1 (1:10,000; cat. no. ab134175), anti-cyclin-dependent kinase inhibitor 1B (p27; 1:5,000; cat. no. ab32034), anti-E-cadherin (1:50; cat. no. ab1416), anti-N-cadherin (1:500; cat. no. ab18203), anti-vimentin (1:800; cat. no. ab8978), anti-RhoA (1:5,000; cat. no. ab187027), anti-ROCK1 (cat. no. ab45171, 1:200), anti-prostate specific antigen (PSA; 1:5,000; cat. no. ab182031) and anti-GAPDH (1:5,000; cat. no. ab8245). Following this, the membranes were incubated with an

TGF-β treatment. BPH-1 cells (5x10^4 cells/well) were seeded into 6-well plates with 2 ml serum-free medium and then incubated at 37˚C for 8 h. Following this, cells were treated with 10 ng/ml TGF-β for 24 h.

Small interfering (si)RNA interference. Negative control siRNA (si-NC) and URG11 siRNA (si-URG11) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of si-NC was 5'-UUCUCCGAACGUUCACG-U-3', and the sequence of si-URG11 was 5'-CAGACGGAUUGCUGACU-U-3'. BPH-1 cells (2x10^4 cells/well) were seeded into 6-well plates and incubated at 37˚C overnight. Following this, cells were transfected with si-NC (50 µM) and si-URG11 (50 µM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 36 h post-transfection, the cells were collected to proceed with the subsequent experiments.

Cell culture. Human BPH-1 cells (BPH epithelium) and normal prostate gland cells (RWPE-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37˚C and 5% CO₂. Cell morphology was then observed under a microscope (magnification, x40).
Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reaction assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
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<tbody>
<tr>
<td>URG11</td>
<td>Forward: 5'-GCCCTTATGGCAATGGTTGCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGCCCTTGGACAGGAGGCT-3'</td>
</tr>
<tr>
<td>PSA</td>
<td>Forward: 5'-AGGCTACGGTAGGGTGA-3'</td>
</tr>
<tr>
<td>RhoA</td>
<td>Forward: 5'-ACCAGTTCCCAGGAGTTA TGT-3'</td>
</tr>
<tr>
<td>ROCK1</td>
<td>Forward: 5'-ACGTGTAACCCAAGGAGAT GTG-3'</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Forward: 5'-GAACAAACAGATCATCCCGCAA-3'</td>
</tr>
<tr>
<td>p27</td>
<td>Forward: 5'-AACGTCGAGTGTCCTAA CGG-3'</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Forward: 5'-CGGACGATGATGGTCGAAC ACC-3'</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Forward: 5'-CATCTCTCTGATCTGTTAAACC-3'</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Forward: 5'-GAGCTCAGTCACGGGAC  ACC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-ATGTTGGAGGTCTACTGGC-3'</td>
</tr>
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URG11, upregulated gene 11; PSA, prostate specific antigen; RhoA, Ras homolog family member A; ROCK1, Rho-associated protein kinase 1; p27, cyclin-dependent kinase inhibitor 1B.

appropriate horseradish peroxidase-coupled secondary antibody at room temperature for 2 h (1:5,000; cat. no. ab6721, Abcam). Proteins were visualized on membranes using an enhanced chemiluminescent kit (Pierce; Thermo Fisher Scientific, Inc.) on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The data was then analyzed using Quantity One software version 4.6. (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression levels were normalized to GAPDH.

Cell Counting Kit-8 (CCK-8) assay. Cells (2.5x10^4/ml) were collected with complete medium and seeded into 96-well plates in a 200 µl cell suspension. Following the transfection of cells according to the aforementioned protocol, CCK-8 reagent (Beyotime Institute of Biotechnology, Haimen, China; 15 µl/well) was added to the wells and incubated for 3 h at 37°C with 5% CO₂. At days 1, 2, 3, 4 and 5 post-transfection, absorbance was then determined at 450 nm using an Elx800 Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. For statistical analyses between two groups, the Student’s t-test was performed. For multiple comparisons, one-way analysis of variance followed by Turkey’s post hoc test was performed. All statistical analyses were performed using IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA) software, and all data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

URG11, RhoA and ROCK1 are highly expressed in patients with BPH. The results of the present study revealed that URG11 expression was markedly higher in serum from patients with prostatic hyperplasia compared with in the healthy controls (n=37; P=0.0013; Fig. 1A). The expression of RhoA was also significantly upregulated in the serum of patients with BPH compared with the controls (n=37; P=0.0073; Fig. 1B). In addition, ROCK1 expression was also significantly increased in the serum of patients with prostatic hyperplasia compared with healthy controls (n=37; P=0.0011; Fig. 1C). The relative expression levels of URG11, RhoA and ROCK1 were presented in Fig. 1D.

URG11 expression is silenced in BPH-1 cells. BPH-1 cells were cultured in RPMI-1640 medium with 10% FBS at 37°C for 48 h and then observed under a light microscope. Cells grew adhering to the wall of the culture dish, exhibited rectangular or irregular polygon shapes, had large and round nuclei, and grew in close contact and irregular arrangements. These characteristics demonstrated that the cells exhibited typical epithelial cell morphology (Fig. 2A). To further investigate this, the expression levels of PSA in cells were determined. The results demonstrated that the expression level of PSA was significantly enhanced in BPH-1 cells compared with normal prostate gland cells (Fig. 2B and C). Thus, it was confirmed that the obtained cells were BPH-1 cells. Furthermore, the expression level of URG11 in normal prostate gland cells and BPH-1 cells was investigated. The results revealed that the expression levels of URG11 were significantly enhanced in BPH-1 cells compared with normal prostate gland cells (Fig. 2B and C). To further investigate the potential roles of URG11 on BPH-1 cell proliferation and migration, BPH-1 cells were either treated with PBS (control), or transfected with si-NC or si-URG11. RT-qPCR and western blot assays were used to detect the expression level of URG11. The results demonstrated that URG11 expression was significantly suppressed in BPH-1 cells transfected with si-URG11 compared with the si-NC group (Fig. 2D and E; P<0.001). These results revealed that the efficiency and specificity of URG11 knockdown were high.
Decreased expression of URG11 suppresses proliferation and induces cell cycle arrest of BPH-1 cells, regulated by TGF-β. Previous studies have demonstrated that TGF-β enhances cancer progression by inducing EMT (18). To investigate this further, BPH-1 cells were treated with either PBS (control) or TGF-β (5 ng/ml for 8 h), or transfected with si-NC or si-URG11 and treated with TGF-β. RT-qPCR analyses revealed that treatment with TGF-β significantly enhanced URG11 expression, and that transfection with si-URG11 significantly reversed this increase induced by TGF-β (Fig. 3A; P<0.001). The CCK-8 assay results demonstrated that TGF-β significantly increased the proliferation of BPH-1 cells, and transfection with si-URG11 significantly suppressed BPH-1 cell proliferation induced by TGF-β (Fig. 3B; P<0.01 or P<0.001). In addition, the potential mechanism underlying TGF-β and URG11-mediated changes in BPH-1 cell proliferation was
further investigated using flow cytometry. The results revealed that the proportion of cells in the G1 phase was significantly increased in the TGF-β group compared with the control group, and was significantly decreased in si-URG11 + TGF-β group compared with the si-NC + TGF-β group. The proportion of cells in the G2 phase was decreased in the TGF-β group compared with control group, and was significantly increased in the si-URG11 + TGF-β group compared with the si-NC + TGF-β group (Fig. 3C; *P<0.05, **P<0.01, ***P<0.001 vs. control group; ##P<0.01 and ###P<0.001, si-URG11 + TGF-β vs. si-NC + TGF-β, n=5). Flow cytometry was performed to investigate cell-cycle distribution. URG11, upregulated gene 11; si, small interfering RNA; NC, negative control; TGF-β, transforming growth factor-β; OD, optical density. All the experiments were independently repeated at least for 3 times.

Silencing of URG11 downregulates the TGF-β-induced expression levels of cyclin D1, N-cadherin and vimentin, and upregulates the expression levels of p27 and E-cadherin. According to the aforementioned results, silencing of URG11 induced cell cycle arrest. The effect of URG11 on cyclin D1 and p27 expression was investigated further, as these proteins are associated with the cell cycle, and it was revealed that TGF-β enhanced cyclin D1 expression and suppressed p27 expression; however, transfection with si-URG11 reversed these effects in BPH-1 cells (Fig. 4; P<0.01 or P<0.001). In addition, the effect of URG11 on N-cadherin, vimentin and E-cadherin expression levels was investigated, as these proteins are associated with EMT, and it was revealed that treatment with TGF-β enhanced the expression levels of N-cadherin and vimentin, and decreased the expression level of E-cadherin (Fig. 4; P<0.01 or P<0.001). Suppression of URG11 expression via transfection with si-URG11 significantly reversed these effects on BPH-1 cells (Fig. 4; P<0.01 or P<0.001).

Suppression of URG11 expression decreases TGF-β-induced RhoA and ROCK1 expression levels. Studies revealed that RhoA and ROCK1 are downstream targets of TGF-β signaling and are involved in cell proliferation, migration and apoptosis processes (19-22). In the present study, it was revealed that RhoA and ROCK1 expression levels were significantly increased by TGF-β compared with the control group, and
significantly decreased in the si-URG11 + TGF-β group compared with the si-NC + TGF-β group (Fig. 5; P<0.01 or P<0.001).

Discussion

BPH is one of the most prevalent diseases of the urinary system that affects elderly men. The major clinical manifestations of BPH include frequent urination, urgent urination and dysuria (23). Currently, population aging is a major public health problem, particularly in China. With the aging population, the incidence of BPH is expected to increase further and thus seriously affect the quality of life of older men, and may even result in the emergence of serious complications (24,25).

URG11 is an HBx-upregulated gene and regulates HBx-mediated hepatocarcinoma development (26). A previous study demonstrated that silencing of URG11 expression suppresses gastric cancer cell proliferation, adhesion, invasion and metastasis abilities, and prevents cell transition from G1 phase to S phase in vitro (6). Furthermore, previous studies have revealed that knockdown of URG11 suppresses lung cancer proliferation and invasion via β-catenin (27) and induces EMT via E-cadherin and β-catenin; and overexpression of URG11 enhances hepatocellular carcinoma growth (4). These results suggest that URG11 may regulate tumor progression via regulation of EMT and the β-catenin signaling pathway. However, the function and underlying mechanisms of URG11 in BPH have not yet been determined. In the present study, it was revealed that the expression of URG11 was significantly enhanced in patients with BPH compared with matched controls, and that decreased URG11 expression suppressed BPH-1 cell proliferation and induced...
cycle arrest via regulation of cyclin D1 and p27 expression levels.

EMT is an essential early step and a critical process in tumor metastasis (28). During EMT, tumor cells lose their epithelial characteristics, including cell-cell adhesion and polarity, and obtain mesenchymal-like phenotypes (29). Furthermore, phenotypic markers commonly change during EMT, for example, the epithelial cell phenotypic marker E-cadherin is downregulated and mesenchymal cell phenotypic markers, N-cadherin and vimentin, are upregulated post-EMT (30). Previous studies have demonstrated that URG11 is associated with E-cadherin expression (31,32). E-cadherin, an epithelial cell adhesion protein, is important for the formation and maintenance of embryonic epithelial cells (33). In addition, E-cadherin is important for the maintenance of structural integrity and cell polarity (34). In the present study, it was demonstrated that suppression of URG11 expression significantly enhances E-cadherin expression, and significantly suppresses the expression of N-cadherin and vimentin. TGF-β is a multifunctional protein of the TGF-β protein superfamily and regulates the growth, differentiation, apoptosis and immunity of numerous cell types (35). Previous studies have demonstrated that TGF-β functions as an inducer of EMT and is closely associated with cancer progression, angiogenesis, pathological staging and prognosis (18,36). Thus, the results suggested that silencing of URG11 suppresses EMT progress in BPH.

Rho proteins have GTPase activity and are involved in cell division and proliferation (37). RhoA, an important member of the Rho family, regulates numerous downstream genes, alters actin structure and morphology and regulates cell proliferation, apoptosis, metastasis and other biological processes (38). A recent study demonstrated that ROCK, an important RhoA regulator, is associated with cell apoptosis, EMT, migration and metastasis (39). In the present study, it was revealed that knockdown of URG11 suppresses TGF-β-induced RhoA and ROCK1 expression levels, indicating that URG11 regulates BPH progression via the RhoA/ROCK1 signaling pathway.

In conclusion, the results of the present study revealed that URG11, RhoA and ROCK1 expression levels were enhanced in patients with PBH. Additionally, suppression of URG11 expression inhibited TGF-β-regulated BPH-1 cell proliferation and induced cell cycle arrest via the regulation of cell cycle-associated genes (cyclin D1 and p27). Furthermore, URG11 knockdown suppressed TGF-β-mediated EMT of BPH-1 cells. In addition, silencing of URG11 expression significantly decreased RhoA and ROCK1 expression levels regulated by TGF-β. Therefore, URG11 may represent a novel prognostic marker and candidate drug target for the treatment of BPH.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
GZ wrote the main manuscript. FZ, GH and ZL performed the experiments. GZ and JL designed the study. QY and ZHL performed data analysis. GZ, FZ and JL contributed to manuscript revisions and all authors reviewed the manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (Xinxiang, China), and written informed consent was obtained from all participants.

Consent for publication
Not applicable.
Competing interests

The authors declare that they have no competing interests.

References


