PTTG1, A novel androgen responsive gene is required for androgen-induced prostate cancer cell growth and invasion

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ABSTRACT

Androgens (AR) play an important role in initiation and progression of prostate cancer. It has been shown that AR exert their effects mainly through the androgen-activated AR which binds to androgen response elements (AREs) in the regulatory regions of target genes to regulate the transcription of androgen-responsive genes, thus, identification of AR downstream target gene is critical to understand androgen function in prostate cancer. In this study, our results showed that androgen treatment of LNCaP cells induced PTTG1 expression, which was blocked by the androgen receptor antagonist, Casodex. Bioinformatics analysis and experiments using PTTG1 promoter deletion mutants showed that the PTTG1 promoter contains a putative androgen response element (ARE), which localizes in the -851 to -836 region of the promoter. Androgen activated androgen receptor (AR) binding to this ARE was confirmed by Chromatin immunoprecipitation (ChIP) assay. Furthermore, Knockdown of PTTG1 expression using short hairpin RNA significantly reduced androgen-induced LNCaP cell growth and invasion. In addition, we showed PTTG1 is highly expressed in metastasis prostate cancer tissue. These results suggest that PTTG1 is a novel downstream target gene of androgen receptor and take part in prostate cancer proliferation and metastasis.

KEYWORDS: PTTG1; Prostate cancer; Androgen responsive gene

INTRODUCTION

Prostate cancer is one of the most frequently diagnosed non-cutaneous cancers and the second leading cause of cancer death in US men⁽¹⁾. Prostate cancer displays clinical, morphological, and biological heterogeneity and its exact etiology remains unclear. One factor that clearly plays a critical role in both the normal prostate function and initiation, progression of prostate cancer is the presence of androgens. Androgens are required for homeostasis of the prostate^(2, 3). Androgens exert their molecular and cellular effects through the androgen receptor (AR), a key regulator of androgen signaling. AR, complexed with coactivator or corepressor proteins, binds to

androgen response elements (AREs) in the regulatory regions of target genes to regulate the transcription of androgen-responsive genes^(4,5). Androgens regulate both normal prostate homeostasis and prostate disease development through regulating expression patterns of androgen responsive gene. Due to the diverse function of various androgen-responsive genes in regulating growth, proliferation and differentiation of prostate cells, the overall balance between the growth inhibitory and stimulatory androgen responsive genes is critical for normal prostate development and prostate cancer development and progression⁽⁶⁻⁸⁾. Therefore, identification and characterization of the genes critical for hormone-stimulated cancer growth could lead to new potential targets and more effective therapies.

The pituitary tumor transforming gene 1(PTTG1) encodes for a securin involved in the regulation of chromatid separation during cell division. Overexpressed PTTG1 disrupts cell division and induces chromosomal instability and, thereby, the cells become vulnerable to the accumulation of more mutations (Significance of Pituitary Tumor Transforming Gene 1 (PTTG1) in Prostate Cancer). Several studies have demonstrated that PTTG1 regulates proliferation and invasion of prostate cancer cells (Pituitary tumor-transforming gene 1 regulates invasion of prostate cancer cells through MMP13; Prostate cancer cell response to paclitaxel is affected by abnormally expressed securin PTTG1; PTTG1 inhibits SMAD3 in prostate cancer cells to promote their proliferation)⁽⁹⁾. Our previous results also showed that transform gene PTTG1 expression increases in response of prostate cancer cell line LNCaP to androgen. However, it is not known whether PTTG1 is directly regulated by androgen through AR. Furthermore, PTTG1 was found overexpressed in several human tumors, such as ovarian cancer, pancreatic cancer, kidney cancer and so on (Significance of Pituitary Tumor Transforming Gene 1 (PTTG1) in Prostate Cancer), while little is known the expression of PTTG1 in human prostate cancer progression.

In the present study, we verified that AR bound the AREs in the promoter region of PTTG1 gene and transcriptionally up-regulated PTTG1 expression. Besides, Functional analysis showed that knock-down PTTG1 expression may attenuate AR-stimulated proliferation and invasion of prostate cancer cells. Furthermore, we

also found PTTG1 highly expressed in metastasis prostate cancer tissues. These results suggest that PTTG1 is a direct target gene of AR and play key role in prostate cancer.

MATERIALS AND METHODS

Materials

Dihydrotestosterone (DHT), propidium iodide were purchased from Sigma (St. Louis, MO). Lipofectamine 2000 and puromycin were purchased from Invitrogen (Carlsbad, CA). Casodex was from AstraZeneca Company. DHT and Casodex were dissolved in 100% ethanol. Anti-PTTG1 (H-160), AR (N-20), normal rabbit IgG and beta-actin (C-11) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein G Sepharose 4 Fast Flow was obtained from GE Healthcare Bio-Sciences AB. The Nitrocellular membrane for Western blot was from Schleicher & Schuell BioScience Inc. (Keene, NH). X-ray films were from Kodak Co. (Rochester, NY).

Cell Cultures

Androgen-sensitive prostate cancer LNCaP cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 Unit/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Routinely prior to DHT or Casodex treatment, LNCaP cells were cultured in phenol red-free RPMI 1640 medium with 5% of steroid-depleted fetal bovine serum (Biochrom) for 48 hours to ensure ligand-free conditions.

Plasmids construction

The deletion mutants of the PTTG1 promoter plasmids were kindly provided by Dr. Ningzhi Xu⁽¹⁰⁾. The region of 1272 to +1 and -822 to +1 were cloned into the pGL3-Basic vector at BglII and KpnI sites. The androgen response element (ARE) contained in the region of -1272/+1 was mutated using site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. The primers used for generating site mutated promoter plasmid were: 5'-G

GGAACGATCAGTTCGCCCGTAATCATAAGCATAAGAGAATG-3' and 5'-CATTCTCTTATGCTTATGATTACGGGGCGAACTGATCG TTCCC-3'. The mutated nucleotides are underlined. To knock down PTTG1 gene, we generated the PTTG1 shRNA vector. This oligonucleotide targeted codons 408-428 in PTTG1 mRNA (CC TCTAGACTTT) and contained a sense and antisense portion separated by a hairpin loop (5'-CAAGAGAACTCTC-3') was cloned into pSilencer 2.0 vector. Two single-strand oligos were synthesized as follows: top-strand oligo: 5'-CACCGTCCTCTAGACTTTGAGAGTTTCAAGAGAACTCTCAAAGTCTAGAGGATTTTTTGAA-3', and bottom-strand oligo: 5'-AAAATTCCAAAAAATCCTCTAGACTTTGAGAGTTCTCTTGAAACTCTCA AAGTCTAGAGGAC-3'. All the plasmids used in our studies were verified by sequencing.

Knockdown of PTTG1

To establish a stable cell line knockdown of PTTG1, LNCaP cells were transfected with p Silencer 2.0 -PTTG1 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Forty eight hours post-transfection, the cell medium was changed to containing 2 µg/ml of puromycin and selected for 3 weeks. The puromycin resistant colonies were pooled and expanded for further analysis. The cells were designated as LNCaP/shPTTG1 for this study. The stable LNCaP/shPTTG1 cells were maintained in RPMI1640 complete medium supplemented with 0.5 µg/ml puromycin. Empty p silencer 2.0 vector was used as a control and was designated as LNCaP/shControl.

RT-PCR

Total RNA was isolated with QiagenRNeasy Kit (Qiagen, Valencia, CA) from LNCaP/shPTTG1 or LNCaP/shControl cells and then 2 µg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen) in a reaction volume of 20 µl. 1 µl of cDNA was used as template to amplify PTTG1 mRNA. Primer pairs for the amplification of PTTG1 and GAPDH are as follows: PTTG1 forward, 5'-TGATC-CTTGACGAGGAGAGAG-3', PTTG1 reverse, 5'-GGTGGCAATTCAACATCCAGG-3'; GAPDH forward,

5'-CACCAGGGCTGCTTTTAACTC -3', GAPDH reverse,
5'-GAAGATGGTGATGGGATTTC -3'. PCRs were performed using Platinum Taq (Invitrogen) with a thermal profile of 94 °C for 2 min followed by 22 (GAPDH) or 30 (PTTG1) cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 1 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel and stained with ethidium bromide (Sigma). The gel was analyzed by scanning densitometry on a Kodak Image Station TM. All the measurements were performed in triplicate for each sample and normalized to the internal control gene, GAPDH. Four independent experiments were performed.

Western Blot Analysis

After the designated treatments, cells were lysed in modified radioimmune precipitation assay buffer [50 mM Tris (pH 7.4), 1% Igepal CA-630, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF, 2 mM phenylmethylsulfonylfluoride, 1 mM Na₃VO₄, and protease inhibitor cocktail (Sigma)]. Protein concentration was determined using a BioRad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts (30 µg) of cell lysates were denatured, separated on a 12% SDS-polyacrylamide gels (Promega equipment), and blotted onto a nitrocellulose transfer membrane (Whatman Inc. Florham Park, NJ, USA). Membranes were then blocked in a Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat milk and probed with primary antibody (1:500 dilution) at 4 °C for overnight. The membranes were further probed with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) at room temperature for 2 hours. Immunoreactive bands were visualized by use of an enhanced chemiluminescence method (ECL, Amersham Biosciences, Piscataway, NJ). Detection was performed by exposure to X-ray film (Kodak Co.).

Transfection and Luciferase Assays

Transfections were done using Lipofectamine 2000 transfection reagents (Invitrogen) according to manufacturer instructions. Briefly, LNCaP cells were seeded in 6-multiwell plates and were transfected at 70%–80% of confluence with 2.0 µg of PTTG1 promoter-PGL3 and 0.1µg of PRL-CMV. Reagents were removed 6 h

post-transfection. After 48 h, the cells were harvested for Luciferase activity assay and measured using the Dual Luciferase Reporter (DLR) system (Promega) according to the manufacturer's protocol. Individual Luciferase activities were corrected using the corresponding PRL-CMV vector activities to obtain the relative activity. Values were expressed as multiples of induction relative to the activity of the PGL3 basic vector and represent mean $\pm$ SD of at least four determinations, and the whole experiment was repeated three times.

Chromatin Immunoprecipitation (CHIP) Assay

LNCaP cells were treated with 1 nM DHT or ethanol vehicle in the absence or presence of 10 μ M Casodex for 48 h. ChIP assays were performed as previously described ^(11,12). LNCaP cells were cross-linked with 1% formaldehyde in phosphate-buffered saline for 2 min at room temperature and scraped off the plate following three washes with ice-cold phosphate-buffered saline. Subsequently, cells were lysed with lysis buffer containing proteinase inhibitors (Sigma) and 1 mM dithiothreitol. Cell lysates were sonicated to break down chromatin into fragments 200–500 bp in length. Chromatin was diluted 1:10 and pre-cleared with protein A-agarose beads (Upstate) for 1 h at 4 °C. Approximately 3% of the chromatin from each sample was saved as input. The rest of the chromatin was used for immunoprecipitation with either anti-AR or normal IgG as a control at 4 °C overnight. Immunoprecipitated antibody-protein-DNA complexes were collected by protein A-agarose beads at 4 °C for 1 h and extensively washed. The complex was eluted and reverse cross-linked at 65 °C for 6 h, and then proteinase K was added for further incubation at 45 °C for 1 h. DNA samples were extracted using phenol/chloroform. The primers specific for PTTG1 and PSA promoter region were: PTTG1 forward, 5'-AATAGAAACAGTGTCTTAGCCT-3', PTTG1 reverse, 5'-CAATGTTGGGTCTAAAGAAT-3'; PSA forward, 5'-CTGCCTTTGTCCCCTAGATG-3', PSA reverse, 5'-TATGCTGGAGGCTGGACA AC 3', PCR was performed for 35 cycles with the following cycling conditions: 94 °C, 30 s; 53 °C, 30 s; 72 °C, 1 min. The promoter

region of PTTG1 without containing putative ARE was used as negative control. The primers used for PTTG1 negative control were:

Forward 5' TCT ACT TGG TGA CCA CGC 3' reverse 5' AGA GCC GGC TCA ACT AAC 3'.

Flow Cytometry

LNCaP/shPTTG1 and LNCaP/shControl cells were cultured in ligand-free medium for 2 days and then treated with 1 nM of DHT or ethanol vehicle for 24 hours. Next, cells were trypsinized and washed in ice-cold PBS. Subsequently, cells were fixed by adding drop-wise to ice-cold 70% ethanol. Fixed cells were washed twice in PBS and incubated in PBS containing 100 mg/ml RNase and 10 mg/ml propidium iodide (Sigma) 30 minutes prior to flow cytometric analysis. Stained cells were assayed in a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA).

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] Assay

The MTT assay was performed using Cell Titer 96 (Promega) following the manufacturer's protocol. LNCaP/shPTTG1 and LNCaP/shControl cells were plated in 96-well plate at a density of 2×10^3 cells/well in phenol red free RPMI1640 medium supplemented with 5% of steroid-depleted fetal bovine serum (Biocrom) with or without 1 nM DHT. The cell viability was assayed every day for 7 days. To measure cell viability, dimethyl sulphoxide (DMSO) was added to each well and the plate was further incubated at 37 °C for 1 h. Absorbance at 490 nm was proportional to the number of living cells in a culture. For each time point, the experiment was carried out in triplicate and was repeated at least three times. The data were analyzed using GraphPad Prism (GraphPad software, Inc., San Diego, CA, USA).

Soft Agar Assay

The effect of PTTG1 knockdown on colony formation ability of the LNCaP cells was assessed by soft agar colony formation assay. The assay was done in 6-well plates; in each well, 1.5 ml of 0.5% agar was layered in the bottom followed by 1 ml of 0.35% agar as the top layer. The agar plates were prepared with phenol red-free RPMI1640 medium supplemented with 5% of steroid-depleted fetal bovine serum

(Biochrom) with or without 1 nM DHT. In each well, 5000 cells were plated over the top layer in triplicate. Plates were assessed for size and number of colonies under a Zeiss microscope after 3 weeks of incubation. All experiments were performed at least three times.

Transwell Invasion Assay

1×10^5 cells were seeded into the upper chamber of a transwell insert (Costar, Cambridge, MA) which were precoated with the extracellular matrix (ECM) substitute Matrigel (BD Biosciences) in phenol red free medium. In the lower chamber, phenol red-free RPMI1640 medium supplemented with 5% of steroid-depleted fetal bovine serum (Biochrom) with or without 1 nM DHT was used as the chemoattractant. Then the cells were cultured for an additional 48 hours. Nonmigratory cells were removed from the upper chamber by scraping and the cells remaining on the lower surface of the insert were stained using 0.1% crystal violet and cell number was counted under a microscope. Three independent experiments were performed. In each experiment transwell invasion for each experimental group was measured in triplicate.

Clinical patient cohort

Gene expression data were archived from the respective GEO accession number of GSE21032. Patient characteristics for the prostate cancer cohort were previously published (Cancer Cell. 2010 Jul 13;18(1):11-22. doi: 10.1016/j.ccr.2010.05.026. Epub 2010 Jun 24. Integrative genomic profiling of human prostate cancer.).

Statistical Analysis

All statistical analyses were performed using SPSS software, version 19.0. Mann-Whitney test was used to analyze the original data of human prostate cancer and adjacent normal tissue to evaluate the difference expression of PTTG1. The molecular experiments results are expressed as mean $\pm$ SD. The data were analyzed by ANOVA and Student's t-test, a P value less than 0.05 was considered to be statistically significant.

RESULTS

Androgen Regulates PTTG1 expression in Human Prostate Cancer Cells

Our previous findings demonstrated that PTTG1 was androgen responsive in rat prostate and the human prostate cancer cell line LNCaP. To further clarify whether PTTG1 is directly regulated by androgen through AR and the potential action mechanism of androgen-induced PTTG1 expression in prostate cancer, the androgen sensitive prostate cancer cell line LNCaP was used to measure PTTG1 expression changes after androgen treatment. As shown in Fig 1A, PTTG1 mRNA was up-regulated by DHT, while androgen receptor antagonist Casodex pretreatment blocked DHT induced PTTG1 mRNA up-regulation significantly. Similar change of PTTG1 expression was observed at protein level (Fig. 1B). In addition, we also obtained similar result in the 22Rv1 cell line. (Fig. 1C, D) These data indicate that the androgen stimulatory effect on PTTG1 expression requires the actions of AR.

PTTG1 Gene Promoter Contains a Putative ARE

To clarify that androgen-induced PTTG1 at transcriptional level is mediated through AR, we analyzed the promoter region of PTTG1 gene by using ConSite, a web-based tool for finding *cis*-regulatory elements in genomic sequences ⁽¹³⁾. We found a putative ARE site between -961 to -939 in the promoter region of PTTG1 as shown in Fig. 2A. Based on this finding, we amplified full length (-1272 to +1) or deletion mutant (-822 to +1) of the PTTG1 promoter which were then cloned into PGL3-Basic vector as shown in Fig 2B. These plasmids were transfected into LNCaP cells to measure their transcriptional activity in response to androgen treatment. As a result, the full length promoter exhibited strong luciferase activity in response to 1 nM DHT treatment for 24 hours (Fig. 2C), while 10 μ M of Casodex pretreatment significantly blocked androgen induced transcriptional activity ($P<0.05$). Furthermore, we found that deletion of region -1272 to -822 of the PTTG1 promoter completely abrogated the transcriptional activity. These data provide evidence that the core androgen response element is localized in the region -1272 to -821. This result was consistent with the prediction of ConSite software.

To further confirm that the region of -961 to -939 of the PTTG1 promoter is responsible for androgen stimulated transcriptional activity, 3 critical nucleotides at -942, -944 and -945 were mutated as shown in Fig. 2A. As expected, the mutated promoter construct did not respond to DHT treatment (Fig. 2D). Taken together, these results suggest that PTTG1 promoter region contains functional ARE and indicate the core region is between -961 to -939.

Ligand-Dependent AR Binding to Putative Androgen response element in the PTTG1 Promoter

To confirm that AR mediates androgen-induced PTTG1 transcription *in vivo*, we immunoprecipitated cross-linked chromatin from LNCaP cells with an AR-specific antibody. The precipitated chromatin was then analyzed using a primer pair that amplified a fragment of the PTTG1 promoter which contained an ARE sequence. A fragment of the PTTG1 promoter lacking putative ARE sequence was amplified by a specific primer pair and used as a negative control. A positive control which detects AR binding to PSA promoter was set up as previously described ⁽¹⁴⁾. In Fig. 3A, similar to the positive control (I), the endogenous AR protein binds to the PTTG1 promoter in the presence of DHT (II). Again, Casodex pretreatment interrupted androgen-induced AR binding to the PTTG1 promoter region (III). In contrast, no PCR amplification product was observed in the negative control (IV). We also obtained similar result in the 22Rv1 cell line. (Fig. 3B) Thus, these data suggest that PTTG1 is regulated by AR via direct binding to the PTTG1 promoter in response to androgen treatment.

Inhibition of PTTG1 Expression Attenuated Androgen-induced Prostate Cancer Cell Growth and Invasion

To further demonstrate that PTTG1 is functionally relevant to androgen, LNCaP/shPTTG1 and LNCaP/shControl cells were cultured in medium with or without DHT and cell growth were monitored by MTT assay. As shown in Fig. 4A, LNCaP cells with PTTG1 knockdown grew slower than control cells did in the presence of androgen. In agreement with the result from the MTT assay, knockdown

PTTG1 in LNCaP cells with showed a decreased ability to form colonies in soft agar relative to empty vector-transfected control cells (Fig. 4B). In a further cell-cycle analysis, LNCaP cells with PTTG1 knockdown shows a decreased ability of proliferation relative to control cells (Fig.4C). Matrigel invasion assays of PTTG1-knocking down LNCaP cells also showed decreased invasion (Fig.4D). These results suggest that PTTG1 expression may contribute to androgen-mediated effects on cancer cell growth and invasion.

Identification of PTTG1 expression in prostate cancer tissues

We utilized the RNA levels of PTTG1 reported in the GSE21032 public dataset from 370 prostate cancer and adjacent normal tissues to evaluate the difference expression of PTTG1 between prostate cancer and normal tissues or primary and metastasis tumor tissues, respectively. As a result, we found PTTG1 expression in metastasis tissues was significantly higher than in primary tumor tissues ($P<0.001$, Fig. 5A). However, there was no difference between prostate cancer and normal tissue ($P=0.07$, Fig. 5B). This result confirms that PTTG1 participating in human prostate cancer metastasis.

DISCUSSION

Identification and characterization of androgen responsive genes is essential for understanding the molecular mechanisms underlying androgen-induced prostate cancer cell survival, proliferation, and differentiation. Previously, using cDNA microarrays, we identified PTTG1 as an androgen-regulated gene in rat prostate. We also found that PTTG1 expression is androgen responsive in human prostate cancer LNCaP cells and its up-regulation correlated with high grade of prostate cancer⁽¹⁵⁾. In the current study, we describe the effects and potential action mechanism of the novel androgen responsive gene, PTTG1, on the regulation of androgen-induced prostate cancer cell growth and invasion. Furthermore, we found PTTG1 highly expressed in metastasis prostate tissue.

The AR, a member of the steroid nuclear receptor superfamily^(16, 17), in the basal state, is bound to heat shock proteins and other proteins in a conformation that prevents DNA binding. Binding to androgens induces a conformational change in the

AR that leads to dissociation from the heat-shock proteins. The ligand-induced conformational change facilitates the formation of AR homodimer complexes that can then bind to androgen response elements (AREs) in the promoter regions of target genes to regulate gene expression ⁽¹⁷⁾. The typical AREs contain a core sequence of GGTACAnnnTGTTCT and require at least three out of four guanines. However, there are a variety of ARE consensus sequences found in multiple AR-regulated genes ⁽¹⁸⁾. Since our finding (Fig. 1) that androgen-induced PTTG1 expression in LNCaP cells was significantly interrupted by AR antagonist Casodex suggested that PTTG1 may be directly regulated by androgen through AR, we thus analyzed the PTTG1 promoter using ConSite, a web-based tool ⁽¹²⁾ for finding cis-regulatory elements in genomic sequences and found a putative ARE from position -961 to -939 which had very similar sequence homology to the core sequence (GGAACGATCAGTTCGTTCTTAA) in the promoter region of PTTG1 (Fig. 2A). Luciferase reporter assay further verified that the region -1272 to -821 which contains the putative ARE has androgen dependent transcriptional activity. ChIP analyses confirmed direct binding of AR to this ARE in an androgen-dependent manner. These data clearly show that PTTG1 is an androgen regulated gene in LNCaP cells. Androgen regulation of PTTG1 is directly mediated through AR and is reversible with anti-androgen, casodex. Since Casodex only partly blocked androgen-stimulated PTTG1 expression (Fig.1), it is possible that other unknown factors also participate in the AR-mediated regulation of PTTG1 expression in prostate cancer cells. Several studies have focused on PTTG1 regulation in different cancer. Zhang et al. described mTOR signaling can promote PTTG1 expression through AKT1 activation in pituitary tumor (mTOR promotes pituitary tumor development through activation of PTTG1). In addition, Tong et al. found microRNA-494 inhibited PTTG1 translation in cervical cancer, suggesting that besides transcriptional regulation, post-transcriptional regulation also play crucial role in PTTG1 expression (MiRNA-494 inhibits metastasis of cervical cancer through Pttg1). Thus, we will focus on whether those factors affected AR-mediated regulation PTTG1 expression in prostate cancer progression regulation in the future.

Particularly, LNCaP cells express a mutant AR T877A which occurs frequently in prostate cancer and represents a hot mutation spot⁽¹⁹⁾. This mutation has been thought to not only broaden the ligand spectrum of the receptor but to affect many other aspects of AR function⁽²⁰⁻²²⁾. PTTG1 is a transforming gene with various functions in human cancers. It is also a global transcription factor, which exerts transcriptional activity either by directly binding to DNA or by interacting with proteins, including p53, one of the most important tumor suppressors⁽²³⁻²⁵⁾. Our previous report shows that exogenously overexpressed PTTG1 is sufficient to promote LNCaP cell growth in vitro and in vivo^(14, 15). Based on above observation that AR directly up-regulates PTTG1 expression, we next demonstrated whether PTTG1 contributes to androgen-mediated effects on cancer cell growth and invasion. Our data show that PTTG1 knockdown diminished androgen-induced LNCaP cell proliferation and invasion (Fig. 3), this further confirmed the conclusion that PTTG1 is a downstream target gene of AR. Our previous studies show that PTTG1 overexpression correlates with high grade prostate cancer, and PTTG1 expression levels were even higher in AR negative and castration-resistant prostate cancer cell lines PC3 and DU145^(14, 15) than in androgen-dependent, AR positive LNCaP cells, suggesting that other mechanisms responsible for regulating PTTG1 expression exist in these AR negative and castration-resistant prostate cancer cell lines.

Most of studies in prostate cancer focused on PTTG1 mechanism in prostate cancer cells or animal models. Little has known about PTTG1 in human prostate tumor tissue. We utilized *in silico* approaches to analyze online microarray dataset including totally 370 case and control samples, and found PTTG1 highly expressed in metastasis prostate cancer tissues rather than in primary tumor tissues. Our previous study has shown that the protein level of PTTG1 is higher than adjacent tissues (Significance of Pituitary Tumor Transforming Gene 1 (PTTG1) in Prostate Cancer). Interestingly, our results showed no significant of PTTG1 RNA expression level between prostate tumor tissues and adjacent normal tissue. This result isn't in contradictory with our previous study. This phenomenon indicated that, despite transcriptional regulation, the mechanism of PTTG1 regulation may also associate

with post-translational modification. Thus, we will focus on PTTG1 post-translational modification in prostate cancer progression regulation in the future.

In conclusion, our results shed light on functional and mechanism AR induced PTTG1 expression in prostate cancer progression. Furthermore, the present study also provides evidence to the PTTG1 expression pattern in prostate cancer.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation of China [grant numbers 30772487, 81502391]. We would like to thank Dr. Ningzhi Xu for his gift of the PTTG1 promoter plasmids. Special thanks to Dr. Laura E. Pascal (University of Pittsburgh) for editing this manuscript.

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FIGURE LEGENDS

Fig.1 Regulation of PTTG1 by androgen in prostate cancer LNCaP and 22Rv1 cells is dependent on androgen receptor. A, semi-quantitative RT-PCR analysis of PTTG1 mRNA expression in LNCaP cells upon DHT treatment alone or in combination with androgen receptor antagonist Casodex. LNCaP cells were cultured in ligand-free RPMI 1640 medium for 24 hours and then treated with 1 nM DHT or ethanol vehicle for additional 16 hours in the presence or absence of 10 uM Casodex pre-treatment 2 hours prior to DHT administration. B, Western blot analysis of PTTG1 expression in LNCaP cells with identical treatment with A. C, semi-quantitative RT-PCR analysis of PTTG1 mRNA expression in 22Rv1 cells upon DHT treatment alone or in combination with androgen receptor antagonist Casodex. 22Rv1 cells were cultured in ligand-free RPMI 1640 medium for 24 hours and then treated with 1 nM DHT or ethanol vehicle for additional 16 hours in the presence or absence of 10 uM Casodex pre-treatment 2 hours prior to DHT administration. D, Western blot analysis of PTTG1 expression in 22Rv1 cells with identical treatment with C.

Fig.2 Identification of an androgen response element in the PTTG1 gene promoter. A, sequence of the PTTG1 gene promoter region containing putative ARE. The ARE region is underlined and the nucleotides with substitution mutations are indicated. B, schematic of PTTG1 promoter deletion constructs. C, quantitation of Luciferase transcriptional activity of the different PTTG1 promoter deletion constructs in LNCaP cells. D, quantitation of luciferase transcriptional activity of wild type and ARE

mutant of PTTG1 promoter in LNCaP cells. Results are representative of three separate experiments, bar indicates mean $\pm$ SD.

Fig. 3 ChIP analysis of AR binding to PTTG1 promoter. LNCaP and 22Rv1 cells were treated for 16 hours with either vehicle control or 1 nM DHT or in combination with androgen receptor antagonist Casodex and ChIP assays were performed with anti-AR antibody. A, B (I) ChIP analysis of AR binding to PSA promoter in the absence of Casodex. (II) ChIP analysis of AR binding to the fragment of the PTTG1 promoter containing putative ARE in the absence of Casodex. (III) ChIP analysis of AR binding to the fragment of the PTTG1 promoter containing putative ARE in the presence of Casodex. (IV) ChIP analysis of AR binding to the fragment of the PTTG1 promoter lacking putative ARE sequence in the absence of Casodex.

Fig. 4 Effects of PTTG1 knockdown on LNCaP cell growth, cell cycle and invasion ability. A, cell viability measured by MTT assay in the presence of DHT. LNCaP/shControl and LNCaP/shPTTG1 cells were cultured in ligand-free RPMI 1640 medium in the absence or presence of 1 nM DHT on six-well plates in triplicate. The results presented here are representative of three independent experiments. B, colony suppression by knockdown of PTTG1 in LNCaP cells in the presence of DHT. One set of representative results is shown here (left panel). Quantification of colony numbers in per low power field (right panel). Colonies bigger than 1 mm in diameter were counted. Each result is the mean of three independent experiments. C, cell-cycle distribution of LNCaP cells with knockdown of PTTG1 following DHT or ethanol vehicle treatment. Cells were stained with Propidium iodide, and subjected to cell-cycle analysis. D, suppression of androgen induced invasion ability of LNCaP cell by knockdown of PTTG1. Transwell invasion assay was performed in LNCaP/shControl and LNCaP/shPTTG1 cells in the absence or presence of 1 nM DHT. Quantification of cell numbers is shown in right panel. A representative result of three independent experiments is shown. Error bar represents mean $\pm$ SD. *, A P value < 0.05 was generated using an unpaired t test in GraphPad Prism.

Fig. 5 RNA level of PTTG1 transcripts in human prostate cancer samples. (A) The RNA levels of PTTG1 expression in metastasis tissues was significantly higher than in primary tumor tissues ($P<0.001$) included in the GSE21032 public dataset.. (B) There was no difference between prostate cancer and normal tissues ($P=0.07$) included in the GSE21032 public dataset.

Figure 1

Figure 2

(A)

CCTAAGACAGATTGATGGTATAATAGAAACAG
TGTCTTAGCCTGAAGTATTGAGGATAGTCCTAAGA
-961 Predicted site -939
TTAGGGAACGATCAGTTCGTTCTTAATCATAAGCA
Mat C C G
TAAGAGAATGACTCAAACGCTGCTGTTTGATCTAC
CTGTACTTACATTTCTTCTTAATTTTAAATTCCTTC
TGC TAGTATTCTTTAGACCCAACATGGTTGTT

(B)

(C)

(D)

Figure 3

Figure 4

(A)

(B)

(C)

(D)

Figure 5