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CMTM3 can affect the transcription activity of androgen receptor and inhibit the expression level of PSA in LNCaP cells

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ABSTRACT

CMTM is a novel family of proteins linking chemokines and TM4SF. Several members of this family are highly expressed in testes and regulate androgen receptor (AR) transcription activity. One member of this family, CMTM3, has the highest expression level in testes and contains one leucine zipper and two LXXLL motifs. As assessed with the dual-luciferase reporter assay, overexpression of CMTM3 represses AR transactivation, while knocking down it can increase AR transactivation. Moreover, CMTM3 inhibits prostate-specific antigen (PSA) expression in LNCaP cells at both mRNA and protein levels with no obvious influence on AR expression. Taken together, CMTM3 may play some roles in the maturation and maintenance of the male reproduction.

CKLF-like MARVEL transmembrane domain containing (CMTM) is a novel family of proteins linking chemokines and transmembrane-4 superfamily (TM4SF). In human, CMTMs are encoded by nine genes, *CKLF* and *CMTM1–8* ; in rat and mouse, *CMTM2* has two counterparts, *Cmtm2a* and *Cmtm2b*. *CKLF* and *CMTM1– 4* form a gene cluster on human chromosome 16q22.1, *CMTM6–8* form another cluster on chromosome 3p22.3, and *CMTM5* is located at 14q11.2 alone [1]. CMTMs play important roles in the immune system and participate in tumorigenesis [2–6]. Several CMTM members are highly expressed in testes and have regulatory effects on androgen receptor (AR) transactivation. Among them, human CMTM2 and mouse Cmtm2b, have been identified as AR co-activators [7]; while mouse Cmtm2a, also named ARR19, is an AR co-repressor [7,8].

It is well known that AR mediates the biological actions of androgens and is necessary during differentiation and maturation of the male reproductive system [9,10]. Prostate-specific antigen (PSA), a serine protease, is regulated at the transcriptional level by AR through androgen response elements in the promoter region of the gene. Since it can be secreted into the seminal fluid, the PSA assay is the primary means of detecting prostate cancer [11] .

This study focuses on *CMTM3* which is highly expressed in human testes [4] as well as tightly linked to *CMTM2*. In addition, CMTM3 protein is rich in hydrophobic amino acids, especially in leucine, some of which are arranged in a typical leucine zipper and two "LXXLL" motifs (where L represents leucine and X represents any amino acid), these special structures are often present in steroid receptor binding proteins, including AR [12] .Therefore, it is reasonable to infer that CMTM3 might have an important role in regulating AR transcription. Consequently, we performed dual-luciferase reporter assays in both overexpression and knockdown systems and found that CMTM3 could repress AR transactivation. Furthermore, in LNCaP cells, CMTM3 inhibited prostate-specific antigen (PSA) expression at mRNA and protein levels without influencing AR expression.

Materials and methods

CMTM3 expression profile. Two panels of Human Multiple Tissue cDNA (Human MTC TM Panel I, Catalog No. 636742; Human MTC TM Panel II, Catalog No. 636743; BD Biosciences Clontech, USA) contain the mixed cDNA libraries from different individuals. Analysis across these human tissues was performed using real-time polymerase chain reaction (PCR), as previously described [4]. The expression level of CMTM3 in skeletal muscle was used as baseline.

Antibodies. The following primary antibodies were used in these experiments: anti-AR monoclonal antibody (sc-7305 Santa Cruz), and PSA antibody (DAKO). IRDye 800 or IRDye 700 conjugated anti-mouse and anti-rabbit IgG antibodies (Rockland) were used as the secondary antibodies. Rabbit anti-CMTM3 antiserum was prepared in our laboratory [4]. For advanced studies, the antibody was purified by immunoaffinity chromatography. The peptides for the rabbit immunization were coupled with CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Then, PBS was used to wash the beads and dilute the antiserum. After rolling overnight at 4 °C, the beads were washed with PBS again, the antibody was eluted with 0.1 M glycine (pH 2.4), then immediately neutralized with 1 M Tris, pH 9.0; finally, the eluted antibody was dialyzed into PBS.

Cell culture. Human prostate cancer cell lines LNCaP and PC-3 were obtained from American Type Culture Collection (Manassas, VA). Cell lines were grown at 37 °C in a humidified incubator with 5% CO_2, and in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 4 mM L-glutamine,

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penicillin (100 U/ml), and streptomycin (100 μg/ml). Routine cell culture procedures were strictly followed to maintain proper cell density and to avoid culturing cells more than six generations after thawing stock vials.

Plasmids, small interference RNAs (siRNAs) and transient transfection. The plasmids pSG5-AR and mouse mammary tumor virus promoter-luciferase (MMTV-luc) were kindly provided by Dr. C. Chang (Cancer Center, University of Rochester, Rochester, NY) and the SV40-*Renilla* luciferase reporter plasmid (pRL) was bought from Promega Company. The plasmid for mammalian expression and *in vitro* translation of CMTM3 was constructed using pcDNA3.1-myc-his b (−) (Invitrogen) expression vectors. CMTM3 was also subcloned into pEGFP-N1 plasmid (CLONTECH) and designated as pEGFP-CMTM3.

Three double-stranded siRNAs siCMTM3-1 (5′-AGCAGAGCCUUGUCUGUAU-3′), siCMTM3-2 (5′-CAAUGACCCUUCCAAGAAU-3′), and siCMTM3-3 (5′-GCCCUCAUCU ACUUUGCUA-3′) targeting the nucleotides of the CMTM3, were synthesized by Genechem Corporation (Shanghai, China). A control double-stranded siRNA named as non-silencing (5′-UUCUCCGAACGUGUCACGU-3′), which had no sequence homology to any known human genes, was also generated.

Transfection of PC-3 cells was performed by electroporation at 130 V, 20 ms in 2 mm gap cuvettes using an electric pulse generator (Electro Square Porator ECM 830, BTX, San Diego, CA) with indicated plasmids or siRNAs. Transfection efficiency was monitored by pEGFP-N1 plasmid (CLONTECH). Cells with >80% transfection efficiency and >80% viability were used for further experiments.

Real-time reverse transcription (RT) PCR. Total RNA was extracted from the LNCaP cells using Trizol reagent (Life Technologies, Inc.) according to standard protocols. Reverse transcription was then performed with the ThermoSCRIPT™ II cDNA Synthesis System (Invitrogen Life Technologies, USA). The synthesized first-strand cDNA was used as the template for the real-time PCR, which was performed for quantitative analyses of PSA and AR mRNA expression in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). Amplifications were carried out using SYBR Green PCR Master Mix Kit (Applied Biosystems, USA) in a total volume of 20 μl. Template cDNA was denatured at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantification data were analyzed with ABI Prism 7000 SDS Software. The expression levels for target genes were measured as fluorescent signal intensity and normalized to the internal standard gene GAPDH. The experiment was performed three times to achieve reproducibility. The primers used in this study are shown in Table 1.

Luciferase assay. In PC-3 cells, electroporation was performed with the indicated amount of expression plasmids or siRNAs and the reporter MMTV-Luc; pRL was used as an internal control. Total amounts of expression vectors or siRNAs were kept constant by adding appropriate amounts of pcDNA3.1-myc-his b (−) or non-silencing siRNA. Transfected cells were then maintained in RPMI 1640 medium containing 5% charcoal-stripped FBS; and 24 h later, either 10 nM DHT (Sigma) or vehicle were added. Cells were harvested 36 h after hormone addition, and the dual-luciferase reporter 1000 assay system (Promega) was employed to measure the luciferase activity.

Adenovirus, LNCaP cell infection, and hormone stimulation. The adenovirus carrying the CMTM3 gene (Ad-CMTM3) were generated and produced by Vector Gene Technology Company Limited (Beijing, China) and the empty adenovirus (Ad-null) were used in parallel as a negative control. LNCaP cells were seeded at 5000 cells/cm^2 into 6 or 12-well tissue culture dishes, and 24 h later infected with Ad-null or Ad-CMTM3 at the required MOI value, in RPMI 1640 medium containing 5% charcoal-stripped FBS. Twenty-four hours after the infection, either 10 nM DHT (Sigma) or vehicle was added. Cells were harvested 24 h after hormone addition, then, the real-time RT-PCR assays and Western blot analyses were performed to detect the changes of cytoplasmic PSA expression at mRNA and protein levels, respectively. Meanwhile, the supernatants were also collected to measure the concentration of secreted PSA.

Western blot analysis and ELISA assay. Cells were washed twice with pre-chilled PBS, pelleted by centrifugation and lysed in lysis buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, with proteinase inhibitor cocktail). After incubation for 30 min on ice, lysates were centrifuged (18,000*g*, 10 min, 4 °C). The supernatants were collected and the protein concentration was measured using a BCA protein assay reagent (Pierce, Rockford, IL), with bovine serum albumin as standard. Total protein (30 μg) was separated on 12.5% SDS–PAGE and then transferred to nitrocellulose membranes (Hybond™, ECLTM, Amersham Pharmacia, UK). After blocked in 5% fat free milk in TBS-T (Tris-buffered saline containing 0.05% Tween 20) for 2 h at room temperature, the membranes were incubated with the appropriate primary antibodies overnight at 4 °C, washed three times with TBS-T, and then incubated with the corresponding IRDye™ 800-labeled IgG secondary antibody (1:10,000) in dark for 1 h at room temperature. Following three additional washes with TBS-T, the IR-fluorophores on the membrane were visualized with the LI-COR Infrared Imaging System (Odyssey, Lincoln, NE). The collected supernatants were centrifuged (1500 rpm, 10 min, at 4 °C) to eliminate the residual cells and debris. Then we employed the Total PSA kit (Abbott Laboratories) to measure the concentration of secreted PSA in the supernatants by the Axsym system, following the manufacturer's instructions.

Table 1
Specific primers used in real-time PCR

	Forward primer	Reverse primer
AR	ATGCTCTACTTCGCCCCTGAT	GGGTGATTTGGAGCCATCC
PSA	TGACCAAGTTCATGCTGTGT	GTCATTTCCAAGGTTCCAAG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATT

Results

CMTM3 has the highest expression level in testes, and possesses one leucine zipper and two LXXLL motifs

Northern blot and real-time PCR analysis of the selected five tissues [1,4] indicated CMTM3 had the highest expression level in testes. To extend its expression profile, we performed real-time PCR analysis using the cDNA libraries of 16 different tissues from human multiple tissue panels. As illustrated in Fig. 1A, across the 16 detected tissues, CMTM3 had the highest expression level in testis, high levels in spleen, lung, placenta, leukocyte and ovary, and moderate levels in prostate and small intestine, which was consistent with previous studies. Then we analyzed its structural characteristics and found that CMTM3 protein was very hydrophobic. More remarkably, leucine alone accounted for 12.1% (22/182) of the total number of residues and formed one leucine zipper (from 30 residues to 51 residues), as well as two "LXXLL" (where L represents leucine and X represents any amino acid) motifs (from 27 residues to 31 residues and from 73 residues to 77 residues). These motifs were often present in transcription factors and steroid receptor binding proteins, including AR [12].

Overexpression of CMTM3 can repress AR transactivation in PC-3 cells

The possible importance of CMTM3 in regulating AR activity was suggested by its high expression level in testes, and the special structural characteristics. Therefore, we examined whether CMTM3 could affect AR transactivation. Both AR and CMTM3 expression plasmids, along with the MMTV-Luc reporter construct, were transiently transfected into PC-3 cells treated with DHT or vehicle. The influence of overexpressed CMTM3 was measured via a luciferase activity assay. As shown in Fig. 2, in the presence of DHT, AR induced the expression of the reporter gene, and overexpressed CMTM3 repressed the androgen-dependent AR transcription activity.

SiCMTM3 screening

In order to further study the function of CMTM3, we designed and synthesized three siRNAs targeting different positions of CMTM3 and identified their abilities to inhibit targeting protein by RT-PCR and real-time RT-PCR. Both assays showed that siCMTM3-3 was the most efficient at inhibiting endogenous CMTM3

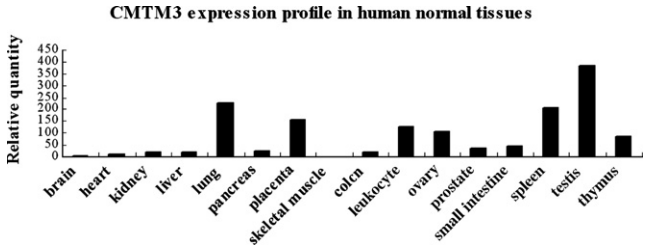

Fig. 1. The expression profile of CMTM3 at mRNA level. Real-time PCR was employed to investigate the expression profile of CMTM3 in 16 normal human tissues. The expression level in skeletal muscle was treated as 1, and GAPDH was used as the internal control. This assay was performed in duplicate with similar results obtained.

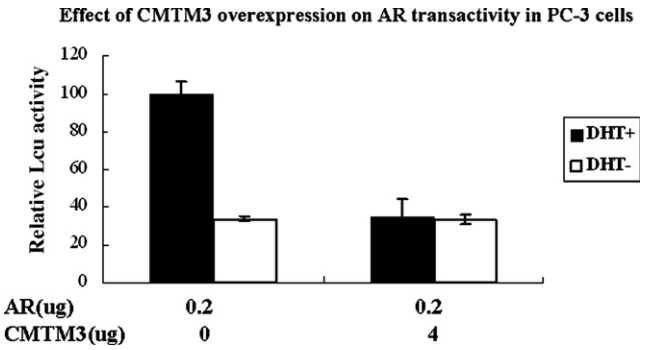

Fig. 2. Overexpressed CMTM3 inhibits the transactivity of AR. PC-3 cells were transiently co-transfected with an AR expression plasmid and a MMTV-Luc reporter, along with CMTM3 encoding plasmid. As participating of CMTM3, AR transactivation decreased. The luciferase activity of AR without a co-regulator in the presence of DHT was set as 100. All values represent means ± SE of at least three independent experiments.

in PC-3 cells (Fig. 3A and B). Thus, it was transfected into PC-3 cells along with a pEGFP-CMTM3 plasmid encoding a fusion protein of CMTM3-GFP to examine its silencing efficacy at protein level. Non-silencing siRNA was used as a negative control. To measure the expression level of CMTM3-GFP, mean fluorescence intensity (MFI) acquired by FACS Calibur flow cytometer (BD Company) was analyzed. CMTM3-GFP expression decreased markedly in the siCMTM3-3 transfected cells. The decrease lasted at least 72 h after transfection (Fig. 3C). We used this siRNA to study the effect of CMTM3 knockdown on AR transcription activity.

Decreasing CMTM3 expression increases AR transactivation in a dose-dependent manner

Increasing amounts of siCMTM3-3 were transiently transfected into PC-3 cells together with an AR expression plasmid and MMTV-Luc reporter construct. The total siRNA amount was kept constant with non-silencing siRNA. As shown in Fig. 3D, siCMTM3 enhanced DHT mediated AR transactivation in a dose-dependent manner.

CMTM3 represses PSA expression without affecting AR levels

To evaluate the effect of CMTM3 in a biologically relevant setting, we tested whether CMTM3 regulated the expression of the PSA gene, an endogenous AR target, in LNCaP cell line, which was currently the only well-characterized prostate cancer cell line that contained a functional AR pathway [13]. Ad-CMTM3 could successfully express exogenous CMTM3 (Fig. 4A), and it dose dependently reduced the endogenous PSA at both mRNA (Fig. 4B) and protein levels (Fig. 4C and D), detected in cell lysate and culture medium. However, CMTM3 did not obviously influence the expression level of AR.

Discussion

Androgen and AR are essential for male germ cell development and reproductive functions, but many of their mechanisms are poorly understood. This study identifies a novel gene, CMTM3, can affect the transactivity of AR. Similar to human CMTM1 and 2, CMTM3 also has the highest expression level in testes at the

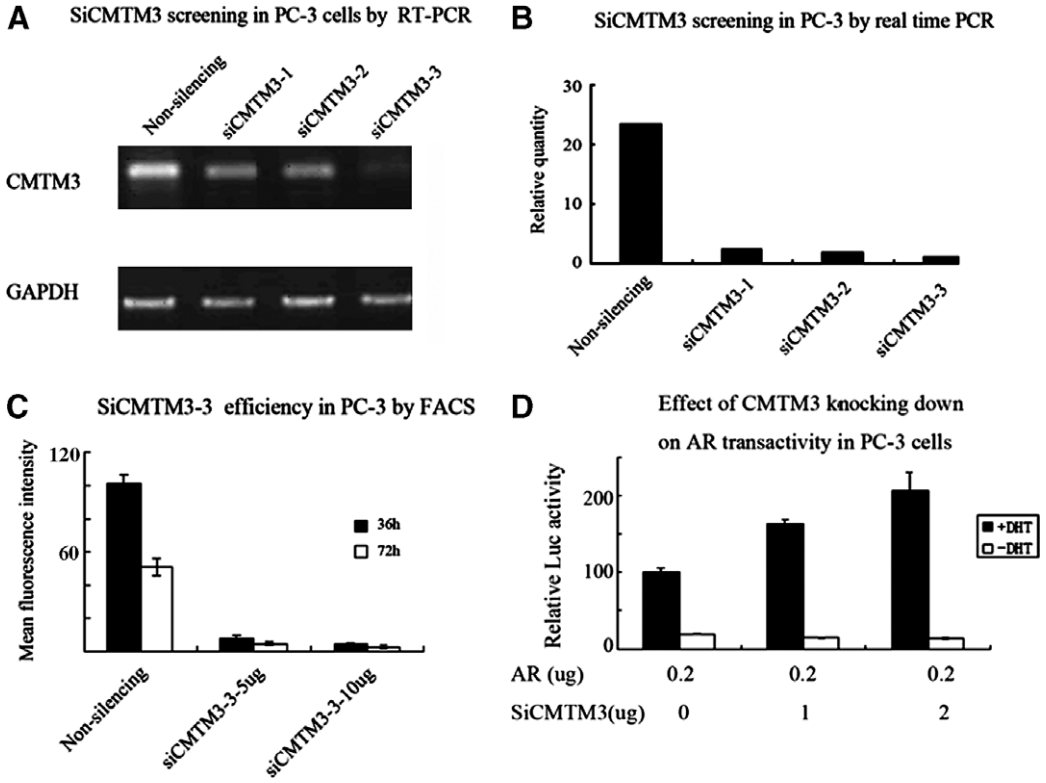

Fig. 3. Decreasing CMTM3 expression dose dependently increases AR transactivation. Three siRNAs targeting different positions of CMTM3 were synthesized and transfected into PC-3 cells; 48 h later, total RNA was collected and RT-PCR (A) and real-time RT-PCR (B) with CMTM3 primers were performed to evaluate their endogenous inhibition efficiency. GAPDH was used as an internal standard. siCMTM3-3 was the most efficient and subsequently co-transfected into PC-3 cells with pEGFP-CMTM3 to examine its efficacy in silencing the exogenous CMTM3. Two doses, 5 and 10 μg of siCMTM3, were used and the expression of GFP-CMTM3 fusion protein was observed by a FACS Calibur flow cytometer and measured as MFI at 36 and 72 h post-transfection (C). Data presented is the average (means ± SE) of triplicate determinations. Subsequently, PC-3 cells were transiently co-transfected with AR expression plasmid and MMTV-Luc reporter, along with increasing amounts of siCMTM3-3 (D). Total amounts of expression vectors were kept constant by adding appropriate amounts of the blank vector. Relative luciferase activity represents the percent of activity level with AR and reporter gene in the presence of the ligand (set as 100). All values represent means ± SE of at least three independent experiments.

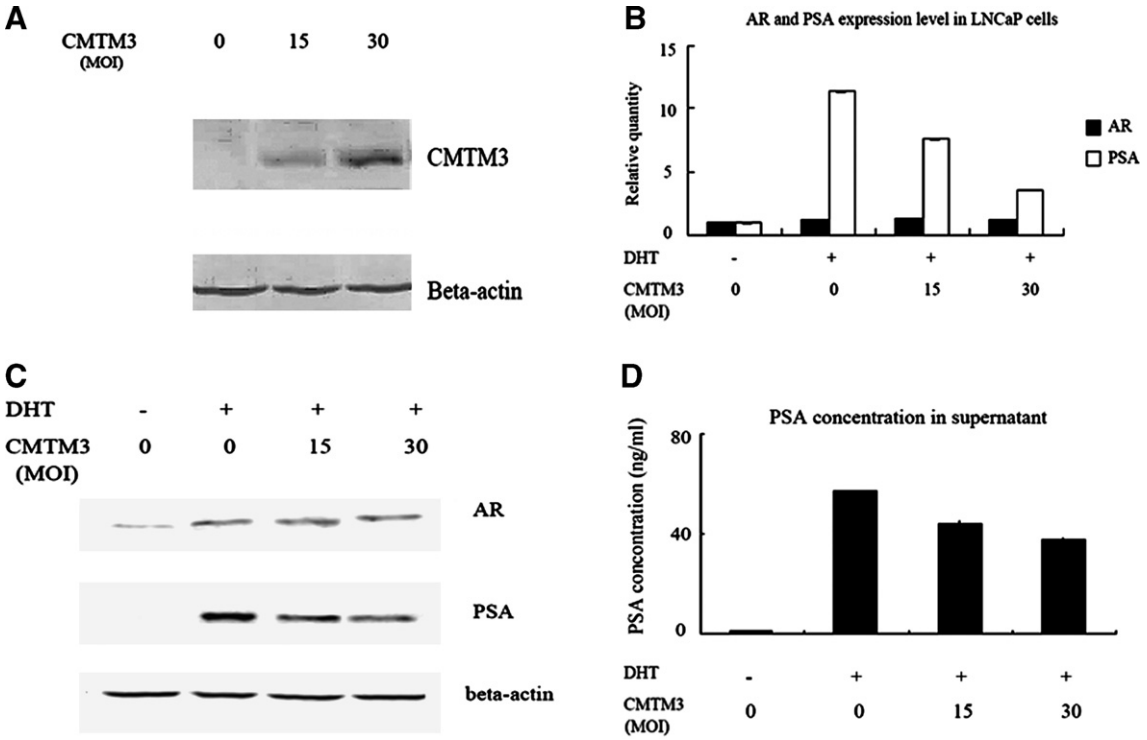

Fig. 4. CMTM3 represses the expression of PSA without obviously affecting AR in LNCaP cells. (A) Ad-CMTM3 infection of LNCaP cells with increasing MOI values (MOI = 15, 30) leaded to increasing CMTM3 expression, beta-actin was probed as an internal standard. After hormone starvation and subsequent stimulation, PSA expression in the infected LNCaP cells was analyzed by Western blotting (B) and real-time RT-PCR (C). The expression level of PSA was inversely related to the infection doses of Ad-CMTM3 at both mRNA (C) and protein (B) levels, while AR was hardly affected. Expression levels of AR and PSA in LNCaP cells infected with Ad-null was set as 1 in real-time RT-PCR; beta-actin was probed as an internal control for Western blotting. (D) Cell culture medium was examined for total PSA using ELISA. Secreted PSA concentration was also decreased by CMTM3 overexpression. All of the above experiments were performed at least three times, and all values represent means ± SE.

mRNA level. Furthermore, CMTM3 represses AR transactivation and has inhibitory effects on PSA expression in LNCaP cells at both mRNA and protein levels without affecting AR expression. Interestingly, human *CMTM2* is tightly linked to *CMTM3* in chromosome and shares similar structural characteristics as well as expression pattern, while mouse Cmtm2a (ARR19) interacts directly with AR to inhibit its transactivation by co-translocating into the nucleus in the presence of androgen and recruiting HDAC [8]. Further experiments are necessary to elucidate the mechanisms by which CMTM3 affects AR transactivation.

CMTM3 is a leucine-rich protein containing one putative leucine zipper and two "LXXLL" AR binding motifs. Proteins such as L7 [14] and JEM-1 [15] containing a leucine zipper-like motif, and other proteins such as NRIF3 [16] and p160 family [17] possessing "LXXLL" motifs have been previously reported to function as co-regulators of nuclear receptors. The detailed mechanism by which CMTM3 interacts with AR still requires further studies, so it is essential to construct mutated forms of CMTM3, such as deletion of LXXLL and/or LZ motifs, to identify the critical structures for its functions.

CMTM3 may play an integral role in the development and progression of prostate cancer. AR is involved in prostate cancer [18] and this study shows CMTM3 is a novel gene that can affect the transactivation of AR and consequently inhibit PSA expression. Moreover, CMTM3 is located on chromosome 16q22, which is usually deleted in prostate cancer [19]. Therefore, it will be interesting to study CMTM3 expression levels in prostate cancer, and to determine the influence of CMTM3 on the growth of prostate cancer cells. These future studies will help understand the mechanisms underlying the development and progress of prostate cancer.

In conclusion, CMTM3 is highly expressed in testes and can repress AR transactivation. In particular, the expression pattern of CMTM3 in male reproductive organs postulates its role in AR-regulated male reproduction. Further studies of CMTM3, for example, with null mutants via knockout mice, may provide strong insight into the physiological functions of CMTM3 in the male reproductive system.

Acknowledgments

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