Original Research

Hypermethylation-mediated transcriptional repression of TMPRSS2 in androgen receptor-negative prostate cancer cells

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Abstract

Prostate cancer is the most common type of cancer for men in the developed world. Androgen receptor (AR) is very important in prostate cancer progression. TMPRSS2 is an AR signaling downstream gene and closely related to prostate carcinogenesis. DNA methylation is a key mechanism to influence gene expression. Though previous reports have shown that AR signaling plays a critical role in the regulation of TMPRSS2 in prostate cancer, hardly any studies have examined whether the DNA methylation has been involved in the regulation of TMPRSS2. In the present study, we demonstrated that AR-negative prostate cancer (PCa) cells showed low expression levels and hypermethylation of TMPRSS2. In contrast, AR-positive PCa cells displayed high levels and hypomethylation of TMPRSS2. Treatment with the DNA methylation inhibitor 5-Aza-2'-deoxycytidine reversed the low expression levels of TMPRSS2 in the AR-negative PCa cells. Additionally, we found that the level of DNA methyltransferases 1 (DNMT1) was high in AR-negative PCa cells, in which hypermethylation of TMPRSS2 and low expression level of TMPRSS2 were observed. Collectively, these data suggest that the high level of DNMT1 might be the mechanism for the hypermethylation-mediated transcriptional repression of TMPRSS2 in AR-negative PCa cells.

Keywords: TMPRSS2, hypermethylation, androgen receptor, prostate cancer

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Introduction

Prostate cancer (PC) is the most commonly diagnosed male cancer in the developed world and shows an increasing rate in the developing countries.[1] Androgen/androgen receptor (AR) signaling pathway is very crucial in the onset and progression of prostate carcinogenesis.[2,3] Many androgen/AR signaling downstream genes that are involved in prostate tumorigenesis include prostate-specific antigen (PSA),[4] FGF8,[5] CCL4[6] as well as TMPRSS2.[7]

TMPRSS2, a type II transmembrane protease, is predominantly expressed in the prostate gland.[8] Recent studies have shown that TMPRSS2 shows an aberrant subcellular localization in the prostate epithelial cells, which results in the loss of epithelial polarity and then enables the cells to inappropriately gain access to and/or activate some cancer pathway.[8,9] Both the mislocalized expression and the increased expression of TMPRSS2 are believed to contribute to prostate tumor formation and progression. Studies have shown that TMPRSS2 is expressed at high levels in primary and metastatic PC as compared to adjacent normal prostate tissues and associated with a poorly differentiated phenotype.[8,10,11] The role of TMPRSS2 in tumor progression of PCa cells might be through activation of protease-activated receptor 2 (PAR2), which contributes to cancer cell migration and metastasis.[12] Recently, gene fusions of TMPRSS2 promoter regions with the ETS family members (predominantly ERG) have received a great deal of attention.[13–15] Overexpression of ERG has been reported in different stages of PC development.[13–16] Under the TMPRSS2-ERG fusion circumstance the expression of ERG follows the TMPRSS2 expression as it is under the control of TMPRSS2 promoter.[17] Although overexpression of TMPRSS2 or TMPRESS2-ERG fusion are known to influence androgen signaling,[7] little is known about the mechanism that regulates TMPRSS2 expression. On the other hand, DNA methylation is a key mechanism to regulate gene expression, and many important genes are silenced by DNA methylation in PC, such as AR,[18] GSTP1,[19] CD44,[20] microRNA-375,[21] and microRNA-205.[22]

The aim of the present study was to determine whether DNA methylation is involved in the expression of TMPRSS2 and what molecule regulates the DNA methylation of TMPRSS2. Our study provides evidence that high level of

DNA methyltransferases 1 (DNMT1) appears to be the mechanism for the hypermethylation-mediated transcriptional repression of TMPRSS2 in AR-negative PCa cells.

Materials and methods

Cell lines and cell culture

PC-3, DU145, LNCaP, and 22Rv1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). C4-2 cells, a subline of LNCaP cells, were obtained from UroCore (Oklahoma City, OK, USA).

PC3/AR cells are PC3 cells that stably overexpress the wild-type AR gene[23]; PC3/neo cells are a control mock-transfected cell line. These cells were maintained in RPMI-1640 or DMEM media supplemented with 10% FBS and streptomycin/penicillin (Life Technologies, Rockville, MD, USA). All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

RNAi

A 21mer sequence (5-GGTGTCACTATGGAGCTCTCA-3) was used to generate the AR short hairpin RNA (shRNA) vector.[24] The AR shRNA plasmid construction, lentiviral production, and lentiviral Infection were performed as previously described.[21]

Real-time PCR

Total RNA were extracted using the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) from PCa cells and treated with DNase (Promega, Madison, WI, USA). Reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

The following primers were used: TMPRSS2 forward 5-TGCGTGGAAAAACCTCTT-3, reverse 5-TGTTCTTG GTCTTGGAGTC-3; β-actin forward 5-CATGTACGTTG CTATCCAGGC-3, reverse 5- CTCCTTAATGTCAC GCACGAT-3. Real-time PCR was performed with a 7500 Real Time PCR System (ABI, Foster City, CA, USA) by using the SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan), according to the manufacturer's directions. The levels of TMPRSS2 gene expression was analyzed by the comparative CT method and normalized to an internal standard, β-actin.

Western blot analysis

PCa cells were prepared for Western blot analysis. Standard Western blotting was performed using anti-TMPRSS2 RabMAb (dilution, 1:2000; #2770-1; Epitomics, Burlingame, CA, USA) and anti-β-actin antibodies (dilution, 1:1000; #4970, Cell signaling, Danver, MA, USA). Anti-rabbit IgG HRP-linked antibody (dilution, 1:2000; #7074; Cell Signaling, Danver, MA, USA) was used as secondary antibody. TMPRSS2 protein expression was normalized to β-actin expression. The proteins were detected using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Bisulfite conversion

PCa Cells were directly subjected to a bisulfite conversion using a bisulfite conversion kit (EZ DNA methylation-direct kit, Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

Bisulfite sequencing PCR

Bisulfite-converted DNAs from PCa Cells were analyzed using a Bisulfite sequencing PCR technique to determine the methylator status of CpG islands within the TMPRSS2 promoter using the following primers: forward primer 5-TGTTTAGAGGTTATAGTGGGTTTTT-3 and reverse primer 5-AATTCCCTACCTAACTCAACCC-3. The PCR products were ligated into the pMD19-T Simple Vector (TaKaRa Biotechnology Co., Ltd., Dalian, China), and a total of 10 colonies were sequenced per PCa cell line by Shanghai Shenggong Biotech (Shanghai, China).

Treatment of cells with demethylating agents

The PC-3 and DU-145 cells were plated in T-25 flasks. Subconfluent cells (60%-70% confluent) were maintained in the medium containing either 5-Aza-2′-deoxycytidine (5-Aza-dC) (5 μM, Sigma-Aldrich, St. Louis, MO, USA) or vehicle (dimethylsulfoxide, DMSO). The cells were treated with fresh 5-Aza-dC with change of media every 24 h and grown for five days.

Nuclear extraction and DNA methyltransferase assay

Nuclear extracts from PCa cells were prepared using the nuclear and cytoplasmic protein extraction kit (BioTeKe Corporation, China) in accordance with manufacturer's protocol. The level of DNA methyltransferase 1 was assessed by DNMT1 Assay Kit (Epigentek, Brooklyn, NY, USA), according to manufacturer's protocol.

Statistical analysis

Data were analyzed using the Prism GraphPad 5 (GraphPad Software, La Jolla, CA, USA) and SPSS 13.0 softwares (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using the Student's *t*-test. Statistical significance was defined as $p < 0.05$. Data are shown as mean ± SEM of at least three independent experiments.

Results

Expression of TMPRSS2 mRNA and protein in PCa cells

To determine whether AR was involved in the control of the expression of TMPRSS2 in PCa cell lines, several AR-positive PCa cell lines (LNCaP, C4-2, 22Rv1) and AR-negative PCa cell lines (PC3 and DU 145) were analyzed by real-time PCR and western blots. The results indicated that TMPRSS2 mRNA were significantly higher in AR-positive PCa cell lines (LNCaP, C4-2,22Rv1) than AR-negative PCa cell lines (PC3 and DU 145) (Figure 1a). In accordance with the mRNA results, a dramatic higher level of TMPRSS2 protein was also seen in AR-positive PCa cell lines (LNCaP, C4-2,22Rv1) (Figure 1b). In addition, PC3/AR cells, which are PC3 cells engineered to express AR,[23] showed an

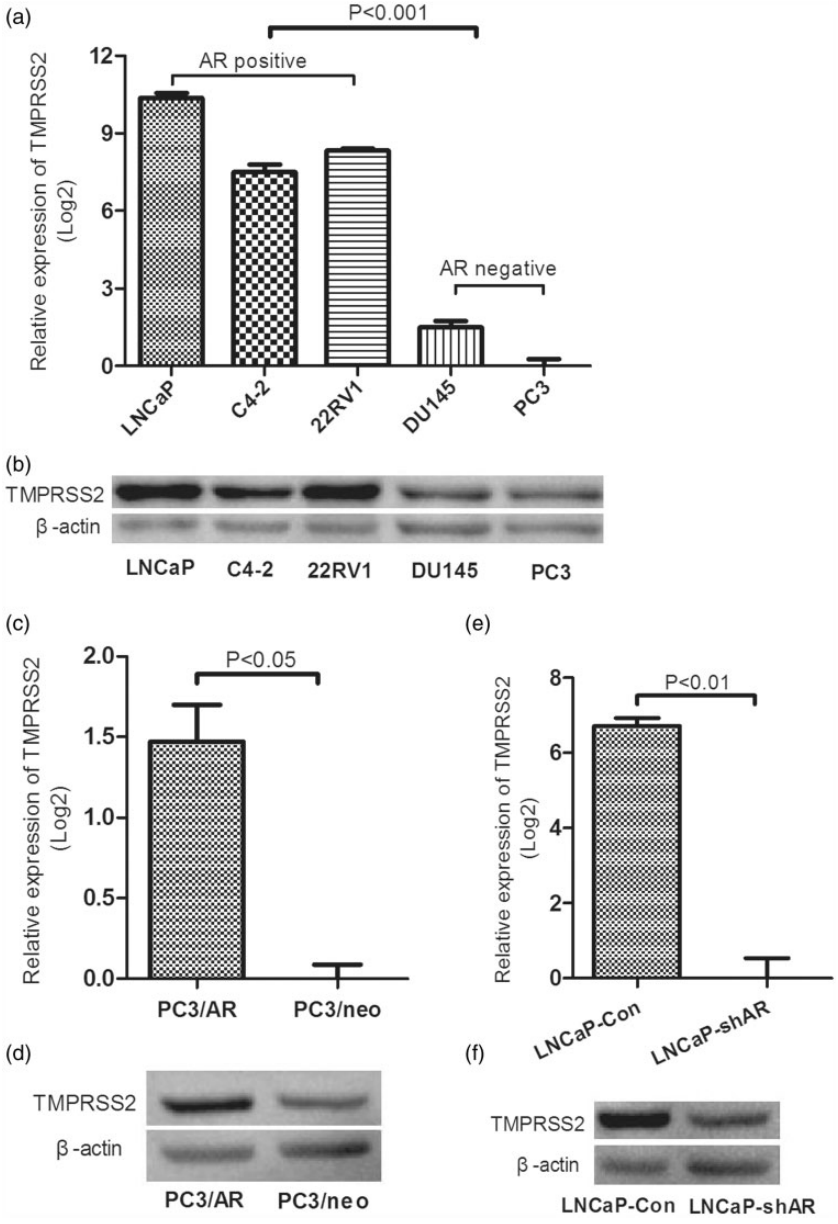

Figure 1 Expression levels of TMPRSS2 in PCa cell lines. Relative mRNA (a) and protein (b) levels of TMPRSS2 in the AR-positive PCa cell lines (LNCaP, C4-2, 2s2Rv1) and AR-negative PCa cell lines (PC3 and DU 145). Relative expression of TMPRSS2 mRNA (c) and protein (d) in PC3/AR cells and PC3/neo cells. Relative mRNA (e) and protein (f) levels of TMPRSS2 in the LNCaP-shAR cells (LNCaP cells were treated with lentivious AR shRNA) and LNCaP-Con cells (scramble control shRNA). Relative expression of TMPRSS2 mRNA in PCa cell lines was determined by qRT-PCR and normalized to β-actin levels. Data are presented as log2 value of TMPRSS2 in PCa cell lines compared to (a) PC3 cells, (c) PC3/neo cells, and (e) LNCaP-shAR cells. Error bars represent standard error of the mean. The protein levels of TMPRSS2 were shown by Western blots, and the β-actin was used as the endogenous normalization control (b, d, f)

increased expression level of TMPRSS2 mRNA and protein as compared to the parental PC3 cells (Figure 1(c) and (d)). In sharp contrast, knocking down of AR by lentivious AR shRNA interference in LNCaP cells resulted in a decreased level of TMPRSS2 mRNA and protein (Figure 1(e) and (f)).

The TMPRSS2 promoter is hypermethylated in AR-negative PCa cells

To study whether AR expression correlates with the methylator status of TMPRSS2, several AR-positive PCa cell lines and AR-negative PCa cell lines were evaluated by a direct bisulfite sequencing PCR (BSP) assay. Firstly, the TMPRSS2 promoter gene sequence was scanned for potential CpG islands using an online program,[25] and our analysis revealed that the promoter region harbored a CpG island (Figure 2a). Next, genomic DNA in these cell lines were examined for a possible bisulfite conversion and pyrosequenced using BSP. We found that the CpG islands in the TMPRSS2 promoter region showed only a limited methylation (1–33%) in AR-positive PCa cells (LNCaP, C4-2, 22Rv1), but demonstrated a hypermethylation (54–70%) in AR-negative PCa cells (PC3 and DU145) (Figure 2b).

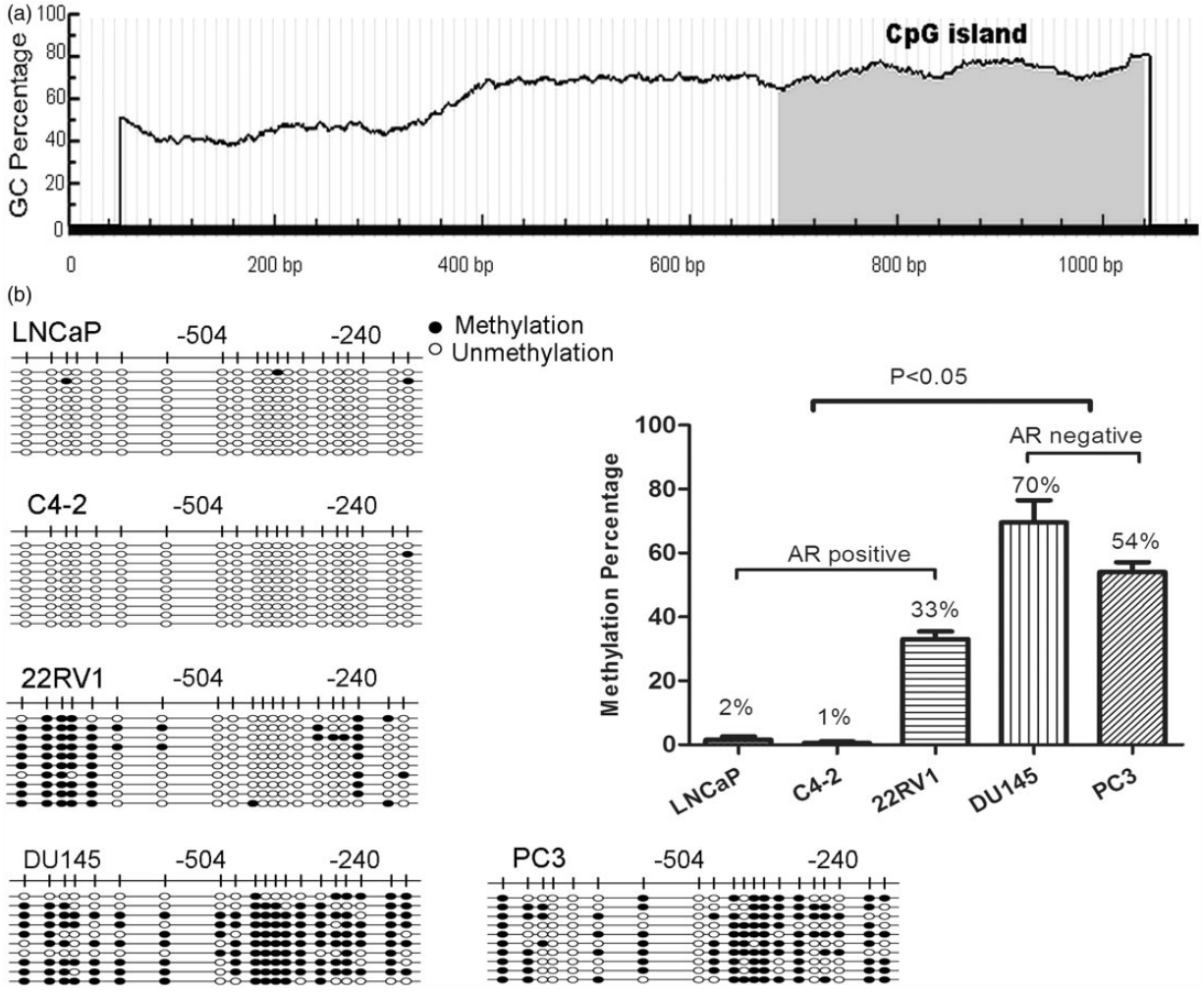

Figure 2 Methylator status of TMPRSS2 CpG island. (a) A CpG island (showed as gray area) is predicted on the 1 kb upstream of TMPRSS2 gene. (b) Schematic summary of 20 CpG sites in the TMPRSS2 promoter region from −504 bp to −240 bp. The results of methylator status are shown in 10 clones from each cell line. Each row of circles represents a single clone, and each circle represents a single DNA methylated or demethylated site. The methylation percentages of each cell line are summarized in the right bar graph. Error bars represent standard error of the mean

Expression of TMPRSS2 is elevated after treatment with 5-Aza-dC in DU145 and PC3 cell lines

To verify whether the promoter hypermethylation was involved in the low expression level of TMPRSS2, a demethylating agent 5-Aza-dC was added to the cell cultures. After five days of treatment of PC-3 and DU145 cells with 5-Aza-dC, higher expression of TMPRSS2 was observed by real time PCR and Western blot analyses (Figure 3).

Expression level of DNMT1 is higher in AR-negative than AR-positive PCas

As the DNMT1 is considered to be the key enzyme affecting promoter methylation status and is abundant in tumor cells and tissues,[26] we examined the level of DNMT1 in PCa cell lines through an ELISA-like reaction. These assays revealed that the level of DNMT1 was higher in AR-negative PCa cells (DU145, PC3) than in AR-positive PCa cells (LNCaP, C4-2, 22Rv) (Figure 4).

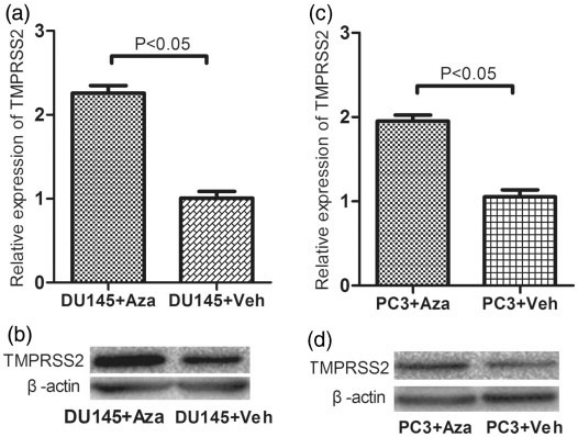

Figure 3 Relative expression levels of TMPRSS2 in PC-3 and DU145 cell lines after treatment with 5-Aza-dC. After five days of treatment of DU145/PC3 cells with 5 μM 5-Aza-dC (Aza) or vehicle (Veh), TMPRSS2 mRNA level (a, c) and protein level (b, d) were analyzed. Relative expression of TMPRSS2 mRNA (a, c) was determined by qRT-PCR and corrected to β-actin levels, values mean fold-change in 5-Aza-dC-treated lines normalized to vehicle-control lines. Error bars represent standard error of the mean. β-actin was used as the endogenous normalization control in Western blots (b, d). Note that treatment with 5-Aza-dC causes an increase in expression of TMPRSS2 in these AR-negative Pca cells

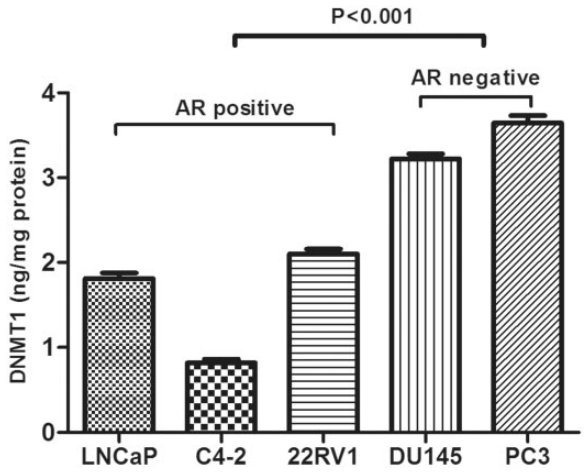

Figure 4 Analysis of DNMT1 in AR-positive and AR-negative PCa cell lines. The expression of DNMT1 level (as measured by an ELISA-based DNMT1 assay) is shown. AR-positive PCa cells include LNCaP, C4-2, and 22RV1 cells; AR-negative PCa cells include PC3 and DU145 cells

Discussion

In this study, we have demonstrated that AR is positively associated with the expression of TMPRSS2. In addition, AR-negative PCa cells have high level of DNMT1 and hypermethylation-mediated transcriptional repression of TMPRSS2. In contrast, these results are exactly reversed in AR-positive PCa cells. Hence, we propose that the AR is negatively correlated with the DNMT1 level, which results in hypermethylation or hypomethylation of the TMPRSS2 promoter in AR-negative PCa cells or AR-positive PCa cells, respectively. The differential methylation-mediated transcription then may be one mechanism for the different expression of TMPRSS2 in AR-positive and AR-negative PCa cells. Our findings suggest that beyond androgen/AR signaling, DNA methylation may be another mechanism for the regulation of TMPRSS2 expression in PCa cells.

Our results are consistent with previous studies that TMPRSS2 is positively regulated by androgen/AR signaling,[7,8,10,11] that is, in AR-positive PCa cells, expression of TMPRSS2 is high, whereas in AR-negative PCa cells, the level of TMPRSS2 is low. Knockdown of AR in LNCaP cells downregulates the level of TMPRSS2, while overexpression of AR in PC3 cells upregulates the level of TMPRSS2.

These findings support the notion that the androgen/AR signaling is an important mechanism for the expression of TMPRSS2. However, it is not known whether there are other mechanisms to regulate the expression of TMPRSS2. Given that the DNA hypermethylation is a key mechanism for the repression of gene expression, we have further studied whether the low expression level of TMPRSS2 in AR-negative PCa cells is due to the effect of hypermethylation. Our data indicate that the TMPRSS2 promoter shows a hypermethylation status in AR-negative PCa cell lines. In contrast, the TMPRSS2 promoter displays a hypomethylator status in AR-positive PCa cells. This differential methylation patterns in AR-negative and AR-positive cells appear to control expression of TMPRSS2. This hypothesis is further supported by our finding that the demethylating agent 5-Aza-dC reverses the low expression level of TMPRSS2 to a higher level in AR-negative PCa cells that show a hypermethylation pattern.

Establishment and maintenance of methylation patterns are mediated by a family of DNA methyltransferase enzymes (DNMTs).[27,28] Although there are multiple DNMT isoforms, such as DNMT1, DNMT2, DNMT3a, and DNMT3b,[28] Dnmt1 is responsible for the maintenance of the established DNA methylation patterns and is abundant in tumor cells and tissues.[26] DNMT2 has no enzyme activity.[28] DNMT3A and DNMT3B act as ''de novo'' enzymes during embryogenesis and gametogenesis.[26,28] It is well-known that DNMT overexpression causes aberrant hypermethylation, which results in genes suppression in various types of cancer cells.[29–32] In the present study, we have demonstrated that AR-negative PCa cells, with a hypermethylator status of the TMPRSS2, express high level of DNMT1. Because our previous study shows that expression levels of DNMT3A and DNMT3B are not high in AR-negative PCa cells,[21] we propose that elevated level of DNMT1 may be the cause for the hypermethylation phenotype in AR-negative PCa cells.

It should be pointed out that although the present study demonstrates a direct relationship between the TMPRSS2 gene methylation status and its expression levels or AR levels, the data are limited to cell lines and lack of examination of the hypothesis in prostate tumors from human patients. Future experiments should be conducted to validate this hypothesis directly in clinical samples.

In conclusion, the present study has shown that beyond androgen/AR signaling, the DNA methylation is another mechanism that regulates the expression of TMPRSS2 in PC cells. In particular, high level of DNMT1 may be the mechanism for the hypermethylation-mediated transcriptional repression of TMPRSS2 in AR-negative PCa cells.

Author contributions: MC and YC contributed equally to this work. All authors participated in the design, interpretation of the studies, and analysis of the data. MC, YC, and WQG performed the research, analyzed the data, and wrote the paper; NW, WL, and PL assisted with animal experiments and provided problem solving for technical difficulties. All authors have approved the final version of the manuscript.

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