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ORIGINAL ARTICLE

Prostate Disease

Methylated CpG dinucleotides in the 5-α reductase 2 gene may explain finasteride resistance in benign prostatic enlargement patients

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The inhibition of 5-α reductase type 2 (SRD5A2) by finasteride is commonly used for the management of urinary obstruction resulting from benign prostatic enlargement (BPE). Certain BPE patients showing no SRD5A2 protein expression are resistant to finasteride therapy. Our previous work showed that methylated cytosine-phosphate-guanine (CpG) islands in the *SRD5A2* gene might account for the absence or reduction of SRD5A2 protein expression. Here, we found that the expression of the SRD5A2 protein was variable and that weak expression of the SRD5A2 protein (scored 0–100) occurred in 10.0% (4/40) of benign adult prostates. We showed that the expression of SRD5A2 was negatively correlated with DNA methyltransferase 1 (DNMT1) expression. *In vitro* SRD5A2-negative BPH-1 cells were resistant to finasteride treatment, and *SRD5A2* was re-expressed in BPH-1 cells when *SRD5A2* was demethylated by 5-Aza-2'-deoxycytidine (5-Aza-CdR) or N-phthalyl-L-tryptophan (RG108). Furthermore, we determined the exact methylation ratios of CpG dinucleotides in a CpG island of SRD5A2 through MassArray quantitative methylation analysis. Ten methylated CpG dinucleotides, including four CpG dinucleotides in the promoter and six CpG dinucleotides in the first exon, were found in a CpG island located from –400 bp to +600 bp in *SRD5A2*, which might lead to the silencing of *SRD5A2* and the absence or reduction of SRD5A2 protein expression. Finasteride cannot exert a therapeutic effect on patients lacking SRD5A2, which may partially account for the resistance to finasteride observed in certain BPE patients.

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INTRODUCTION

Benign prostatic enlargement (BPE) resulting from the histologic condition of benign prostatic hyperplasia (BPH) is one of the most common urological diseases in aging men.[1] BPH typically develops after the age of 40 years and ranges in prevalence from over 50% at 60 years of age to 90% by 85 years of age.[2,3] 5-α reductase type 2 (SRD5A2) inhibitors are most commonly used for the management of BPE.[4,5] The targeted inhibition of SRD5A2 by finasteride leads to a reduction in prostate volume and an improvement in lower urinary tract symptoms (LUTS)[4] and reduces the risk of acute urinary retention or BPE-related surgery.[6–8]

However, certain BPE patients are resistant to finasteride therapy.[9–12] The Medical Therapy of Prostatic Symptoms (MTOPS) research group reported that clinical progression was observed in 10% of BPE patients on long-term finasteride treatment, and further invasive treatments were necessary in 2%.[10] While it would be ideal to not have patients waste time, experience discomfort, or incur the potential cost of therapies that are not efficacious, it is not clear whether by placing these patients on SRD5A2 inhibitor therapy, we are missing a curative window or harming them by promoting the progression of their disease. Therefore, it is very important to distinguish these patients from patients who are sensitive to SRD5A2 inhibitor therapy.

It has been reported that 28%–36.5% of benign prostatic tissues do not express the SRD5A2 protein, and the methylation of the *SRD5A2* gene promoter might account for the absent or reduced expression of the SRD5A2 protein in some adult prostatic tissues.[9,11,13] DNA methylation, which is an epigenetic modification that causes gene silencing, occurs mainly at the C5 position of cytosine-phosphate-guanine (CpG) dinucleotides and is carried out by DNA methyltransferase 1 (DNMT1). DNMT1 is responsible for both copying DNA methylation patterns to the daughter strands during DNA replication and methylating previously unmethylated CpG islands, and DNA methyltransferase 3a (DNMT3a) and DNA methyltransferase 3b (DNMT3b) are the *de novo* methyltransferases that introduce DNA methylation early in development.[14] We speculate that the genetic silencing of the *SRD5A2* gene upon methylation by DNMT1 may be an important determining factor for those patients who are resistant to finasteride. To further investigate the mechanism of the genetic silencing of the *SRD5A2* gene, through database analysis, we located a CpG island in *SRD5A2* that might potentially be methylated between 400 bp upstream of the transcription initiation

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site (TSS) and 600 bp downstream (−400 bp to +600 bp), which encompasses the promoter region and the first exon (TSS to +352 nt). The aim of this study was to determine the specific methylated CpG dinucleotides in the CpG island mentioned above that may partially account for the absent or reduced expression of SRD5A2 in BPE transitional zone tissues and to explore the correlation between the methylation of the CpG island and the expression of the SRD5A2 protein.

PATIENTS AND METHODS

Patients

A total of 64 BPE patients who were not taking SRD5A2 inhibitors were collected and approved by the Institutional Review Board of Beijing Chaoyang Hospital, Capital Medical University (2017-KE-6; Beijing, China). The research was carried out according to the World Medical Association Declaration of Helsinki, and each patient has signed written inform consent. The transition zone of the BPE tissues was obtained from patients who underwent transurethral resection of the prostate (TURP) at Beijing Chaoyang Hospital between January 2014 and December 2018. The mean age of the BPE patients was 72 years, ranging from 58 years to 84 years. Forty paraffin-embedded tissues were evaluated for the expression of SRD5A2 and DNMT1. Fresh BPE tissues from 24 cases selected after the evaluation of the expression of SRD5A2 were used to determine the exact methylated CpG dinucleotides in the sequence from −1009 bp to +922 bp of *SRD5A2* and to explore the correlation between these methylated CpG dinucleotides and the expression of the SRD5A2 protein. It is important to note that all samples were evaluated through routine histological analysis, and no malignancy was detected in any of the transition zones.

Immunohistochemical analysis of SRD5A2 and DNMT1

Immunohistochemistry (IHC) was performed in samples from forty patients as previously described[9] using primary antibodies against SRD5A2 (Novus Biological Inc., Centennial, CO, USA) and DNMT1 (Abcam, Cambridge, UK) following the manufacturer's recommendations at concentrations of 1/1500 and 1/100, respectively. Positive and negative controls were used throughout all the immunostaining protocols. A total of three representative areas from each sample were randomly selected under 40× magnification to assess immunoreactivity by two genitourinary pathologists, and 100 cells selected from the epithelium were manually counted from each representative section. Each individual cell was scored on a 0–3 scale according to the intensity of the staining. Then, a visual score was generated for each sample, which ranged from 0 to 300. A score of 0–100 was defined as weak expression, and a score of 101–300 indicated strong expression.

Cells and cell culture conditions

The immortalized human prostatic epithelial cell line BPH-1 was obtained from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Rockville, MD, USA) supplemented with 2 mmol l^{-1} L-glutamine, 10% fetal bovine serum (FBS; Gibco, Melbourne, Australia), and 1% penicillin-streptomycin (HyClone, Logan, UT, USA) at 37°C with 5% CO_2. Finasteride (LKT Laboratories, Inc., St. Paul, MN, USA) was added at concentrations of 25 μmol l^{-1}, 50 μmol l^{-1}, and 100 μmol l^{-1} as described in our previous work.[15] The *in vitro* experiments were repeated three times, and each of the different concentrations of finasteride were applied to each sample in triplicate.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA). Semi-quantitative RT-PCR was conducted using 2 × EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China) in a PCR thermocycler (Applied Biosystems, Life Technologies, Melbourne, Australia). The primers were as follows: 5-α reductase type 1 (*SRD5A1*), 5'- GAAACTTGCCAACCTTCGTG -3' (forward), and 5'- CTTACTCCGTATGAACCACCA -3' (reverse); *SRD5A2*, 5'- CTCTTCTGCCTACATTACTTCCA -3' (forward) and 5'- CACCCAAGCTAAA CCGTATGTC -3' (reverse); and *DNMT1*, 5'- GCAAACCACCATCACATCTC -3' (forward) and 5'- GTAACTCTACGTCTCTTCTCATCC -3' (reverse). The reactions were predenatured at 94°C for 5 min, followed by 35 cycles of PCR with denaturing at 94°C for 30 s; annealing at 55°C, 54°C, or 57°C, respectively, for 30 s; and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were subjected to electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and photographed.

Treatment of BPH-1 cells with DNA methyltransferases inhibitors

BPH-1 cells were treated with 10 μmol l^{-1} 5-Aza-2'-deoxycytidine (5-Aza-CdR) or N-Phthalyl-L-tryptophan (RG108; Sigma-Aldrich Inc., St. Louis, MO, USA) for 96 h.[9] The medium was replaced with fresh medium containing 5-Aza-CdR or RG108 every 24 h. 5-Aza-CdR was solubilized in acetic acid (HAC), and RG108 was solubilized in dimethyl sulfoxide (DMSO). BPH-1 cells treated with either DMSO or HAC alone were used as controls. RT-PCR was performed as described above.

Evaluation of CpG islands in the SRD5A2 gene

Four databases (NCBI, UCSC, GeneCopoeia, and Ensembl) were used to comprehensively analyze the *SRD5A2* gene. NCBI showed that the locus of the *SRD5A2* gene was NG_008365, and the first exon was located from the TSS to +352 bp. The GeneCopoeia database indicated that the promoter was located from −1216 bp to +71 bp. We used EMBOSS CpG Plot (EMBL-EBI: http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html) to evaluate the CpG islands in the sequence from −5000 bp to +1000 bp, and a CpG island was found covering the region from −400 bp to +600 bp from the TSS (**Supplementary Figure 1**).

Quantitative methylation analysis

The transition zone of 24 fresh human prostate specimens obtained after TURP was used for the evaluation of methylated CpG dinucleotides. To better detect methylated CpG dinucleotides in the sequence ranging from −400 bp to +600 bp, four pairs of primers were designed to cover a broader sequence from −1009 bp to +922 bp of the *SRD5A2* gene, which contained 73 CpG dinucleotides, to determine methylation ratios (**Supplementary Figure 2** and **Supplementary Table 1**). The mass spectra were collected using MassARRAY Compact MALDI-TOF system (Agena; BioMiao Biological Technology, Beijing, China), and the methylation ratios of the spectra were determined by EpiTYPER software (Agena, San Diego, CA, USA). The details of the MassArray DNA quantitative methylation analysis are shown in **Supplementary Figure 3**.

Cell proliferation assay

Cell proliferation was assessed with MTS in accordance with the manufacturer's instructions (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega, Madison, WI, USA). Briefly, 20 μl of CellTiter 96® AQ_{ueous} One Solution Reagent containing a novel

tetrazolium compound was pipetted into each well of a 96-well assay plate containing the samples in 100 μl of culture medium, and the plate was incubated at 37°C for 2 h in a humidified, 5% CO_2 atmosphere. The tetrazolium compound was bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium, and the absorbance was then recorded at 490 nm using a 96-well plate reader.

Fluorescent flow-cytometric assay

Cell apoptosis and cell necrosis were assessed by flow cytometry (Annexin V-FITC/PI Apoptosis Detection Kit, KeyGen BioTECH, NanJing, China) as per the manufacturer's instructions. The cells were analyzed after adding propidium iodide (PI) to gate out dead cells.

Statistical analyses

Data were presented as mean ± standard deviation (s.d.) and median with interquartile ranges (IQRs). Spearman's rank correlation analysis was performed to analyze the relationship between the immunohistochemical staining of SRD5A2 and DNMT1. Analysis of variance (ANOVA) was used for the analysis of cell proliferation, cell death, and apoptosis. Mann–Whitney *U* analysis and Spearman's rank correlation analysis were performed to analyze the correlation between methylated CpG dinucleotides and the reduced expression of the SRD5A2 protein. All the tests were of two tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Variable SRD5A2 expression in BPE tissues

Obviously, the variable expression of SRD5A2 was detected among the forty BPE specimens, as previously described.[9] SRD5A2 was expressed mostly in epithelial cells, and wide expression of the protein was observed in both the cytoplasm and nucleus. Among the other samples, SRD5A2 expression was limited to the nuclei of the epithelial cells in some cases, whereas in other prostate tissues, SRD5A2 was expressed in both the epithelial and stromal compartments. More interestingly, some prostate samples (4/40, 10.0%) showed no or reduced SRD5A2 protein and were scored from 0 to 100. DNMT1 was mainly expressed in the nuclei of epithelial cells; in some samples, DNMT1 was found in the cytoplasm of epithelial cells (**Figure 1a**). As shown in **Figure 1b**, SRD5A2 was negatively correlated with DNMT1 expression in forty samples (Spearman's rank correlation, $r = -0.340$, $P = 0.032$). To address the role of the variable and heterogeneous expression of SRD5A2 in the development of BPE and resistance to finasteride therapy, we first evaluated the response of SRD5A2-negative BPH-1 cells to finasteride treatment.

SRD5A2 mRNA was absent in finasteride-resistant BPH-1 cells

In immortalized nontumorigenic BPH-1 cells, *SRD5A1*, not *SRD5A2*, was predominantly expressed (**Figure 2a**). BPH-1 cells were resistant to the therapeutic effect of finasteride, and the treatment of BPH-1 cells with high concentrations of finasteride failed to induce cell death (**Figure 2**). Finasteride promoted the proliferation (**Figure 2b**) and repressed the apoptosis (**Figure 2c**) and necrosis (**Figure 2d**) of BPH-1 cells over an increasing concentration gradient.

Re-expression of SRD5A2 mRNA in BPH-1 cells by 5-Aza-CdR or RG108

SRD5A2 mRNA was detected in BPE tissue but was not expressed in BPH-1 cells or some malignant epithelial cells (**Figure 3a**). To confirm the methylation status of *SRD5A2* and determine whether DNA-demethylating agents can reactivate the *SRD5A2*

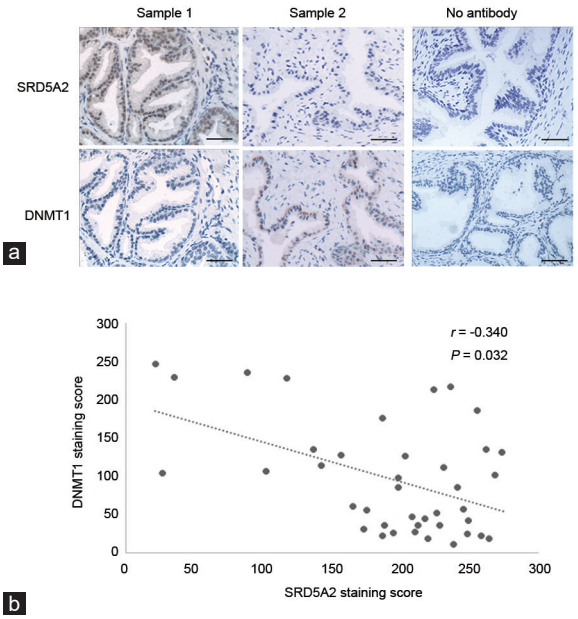


Figure 1: A total of forty BPE specimens were evaluated for immunoreactivity to SRD5A2 and DNMT1. (**a**) SRD5A2 was mostly expressed in epithelial cells and showed broad expression in both the cytoplasm and nuclei. DNMT1 was mainly expressed in the nuclei of epithelial cells. Sample 1: strong SRD5A2 expression with corresponding reduced expression of DNMT1. Sample 2: negative SRD5A2 expression with corresponding strong expression of DNMT1. No antibody: negative control without incubation with a primary antibody. (**b**) SRD5A2 was negatively correlated with DNMT1 expression in forty samples (Spearman's rank correlation, $r = -0.340$, $P = 0.032$). Scale bars = 50 μm. SRD5A2: 5-α reductase type 2; DNMT1: DNA methyltransferase 1.

gene, BPH-1 cells were treated with 5-Aza-CdR and RG108. After the exposure of BPH-1 cells to 5-Aza-CdR and RG108, we found that *SRD5A2* mRNA was re-expressed. These data demonstrate that *SRD5A2* contains CpG dinucleotides that are methylated and that demethylating agents reactivate the expression of *SRD5A2* (**Figure 3b**).

Multiple methylated CpG dinucleotides are associated with the expression of the SRD5A2 protein

To determine the specific methylated CpG dinucleotides in the CpG island ranging from −400 bp to +600 bp of *SRD5A2*, 24 specimens were chosen from our cohort to detect the methylation ratios of the 73 CpG dinucleotides in the sequence ranging from −1009 bp to +922 bp. These specimens were characterized by the expression of the SRD5A2 protein and divided into two groups: ten specimens in the weak-expression group received scores from 0 to 100, and 14 in the strong-expression group received scores from 101 to 300. Our results revealed that 14 methylated CpG dinucleotides were correlated with the expression of the SRD5A2 protein (**Figure 4a** and **5** and **Table 1**). Ten CpG dinucleotides, including four CpG dinucleotides (−235 nt, −74_72_69 nt; "_" indicates that these CpG were detected as clusters) in the promoter region and six CpG dinucleotides (+46 nt, +53_56 nt, and +65_68_70 nt) in the first exon, were located in the CpG island. Four CpG dinucleotides in the first intron (+824_829 nt, +839_842 nt) were located outside of the CpG island. Furthermore, the statistical description and Spearman's rank correlation analysis showed a negative correlation between the methylation ratios of these CpG dinucleotides and the expression of the SRD5A2 protein (**Figure 4b** and **4c**).

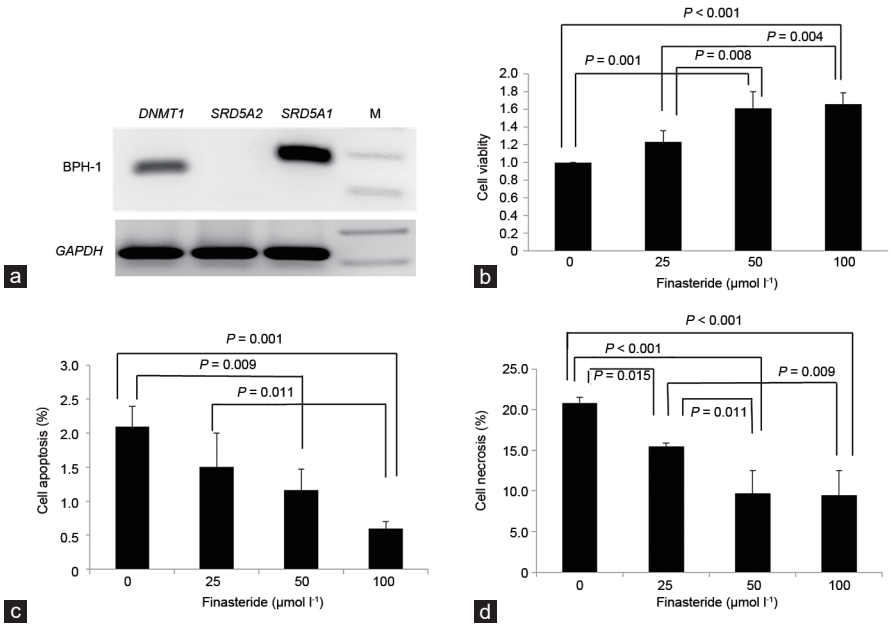


Figure 2: The therapeutic effect of finasteride on SRD5A2-negative BPH-1 cells. (**a**) In BPH-1 cells, positivity for *DNMT1* mRNA and *SRD5A1* mRNA was observed, while *SRD5A2* mRNA was not expressed. "M" represents the molecular weight standard. (**b**) Finasteride promoted the viability of BPH-1 cells, rather than inducing cell death. (**c**) Finasteride repressed BPH-1 cell apoptosis. (**d**) Finasteride inhibited BPH-1 cell necrosis. *DNMT1*: DNA methyltransferase 1; *SRD5A2*: 5-α reductase type 2; *SRD5A1*: 5-α reductase type 1; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

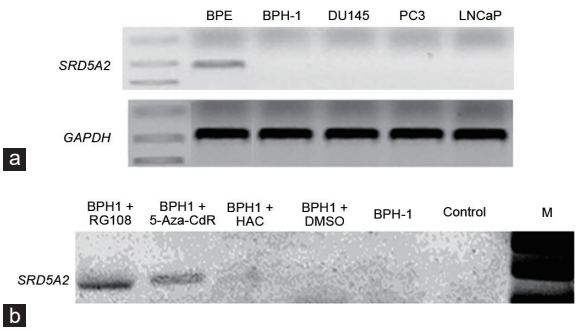


Figure 3: DNA methyltransferases inhibitors could recover the expression of *SRD5A2* mRNA in BPH-1 cells. (**a**) *SRD5A2* mRNA was detected in BPE tissue but was not expressed in BPH-1 cells or some malignant epithelial cells. (**b**) *SRD5A2* mRNA was re-expressed in BPH-1 cells that were treated with 5-Aza-CdR or RG108. "M" represents the molecular weight standard. Control: negative control without the loading of *SRD5A2* mRNA. BPE: benign prostatic enlargement; *SRD5A2*: 5-α reductase type 2; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; 5-Aza-CdR: 5-Aza-2'-deoxycytidine; RG108: N-Phthalyl-L-tryptophan; HAC: acetic acid; DMSO: dimethyl sulfoxide.

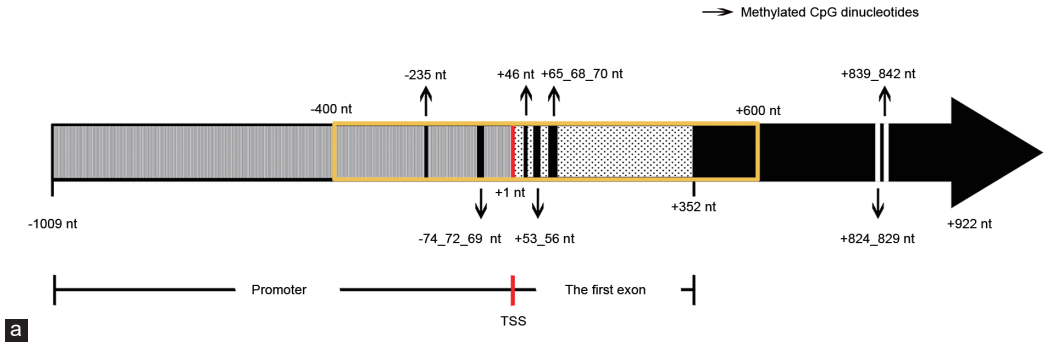
DISCUSSION

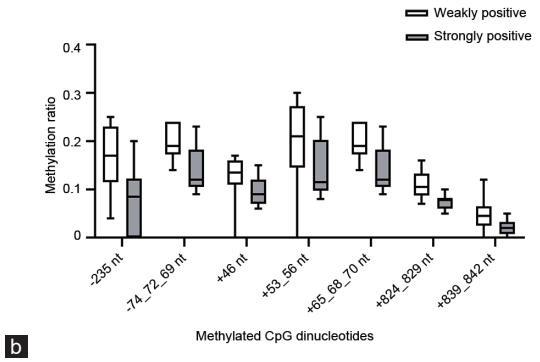
The SRD5A family consists of three enzymes, SRD5A1, SRD5A2, and 5-α reductase type 3 (SRD5A3), which are encoded by distinct genes, *SRD5A1, SRD5A2*, and *SRD5A3,* located on separate chromosomes.[16,17] SRD5A2 has traditionally been viewed as an isozyme that is predominantly expressed in prostate tissue.[18,19] We observed reduced SRD5A2 expression, receiving scores of 0–100, in 10% of BPE tissues, which was lower than the percentage of 29% reported by Niu *et al*.[9] in benign para-cancer tissue obtained from patients undergoing radical prostatectomy. Ge *et al*.[11] reported that up to 36.5% of the transitional zone tissues obtained from TURP did not express the SRD5A2 protein. The ratio of 10% is more consistent with the data reported by MTOPS in patients resistant to finasteride therapy.

Finasteride inhibits dihydrotestosterone (DHT) synthesis by targeting SRD5A2 and induces the apoptosis of benign prostate cell lines or prostate cell lines.[20] In our study, finasteride treatment surprisingly promoted the proliferation of BPH-1 cells in a dose-dependent manner, which is probably explicable. Li *et al*.[21] revealed that autophagy was involved in BPH development. SRD5A2 inhibitor treatment would induce autophagy and decrease the apoptosis of PWR-1E cells. Similarly, castration therapy in individuals with prostatic cancer induces autophagy in cancer cells, which allows some cells to escape from death.[22] Based on this evidence, we hypothesized that an increasing concentration of finasteride might further decrease DHT in SRD5A2-negative BPH-1 cells and contribute to autophagy induction, promoting the proliferation of BPH-1 cells. This may indicate why 10% of BPE patients receiving long-term finasteride treatment show clinical progression. Although our hypothesis remains to be further studied, our findings imply that an absence or reduction of SRD5A2 expression is probably the reason for finasteride resistance in BPE patients.

In addition, DNMT1 expression was negatively correlated with SRD5A2 immunoreactivity. DNMT1 executes the most crucial function in DNA methylation, which may lead to the methylation of CpG dinucleotides in the transcription factor-binding site area, resulting in the transcriptional silencing of the gene.[23] Therefore, we postulated that the upregulation of DNMT1 promoted the methylation of CpG dinucleotides in the promoter or the first exon, leading to the silencing of *SRD5A2* and reduced expression of the SRD5A2 protein. We confirmed this hypothesis by re-expressing *SRD5A2* mRNA by treating BPH-1 cells with DNMT1 inhibitors, suggesting that further investigation of the methylation status of *SRD5A2* is necessary.

It has been reported that the silencing of *SRD5A2* is significantly correlated with promoter methylation,[9,13,24] although 10% of the cases showing absent or weak expression of the SRD5A2 protein exhibit hypomethylation in the promoter region.[11] In our study, we identified a CpG island from −400 bp to +600 bp, encompassing the





Methylated CpG dinucleotides	SRD5A2 expression	
	Correlation coefficient	*P* value
-235 nt	-0.567**	0.004
-74_72_69 nt	-0.565**	0.004
+46 nt	-0.521**	0.009
+53_56 nt	-0.435*	0.026
+65_68_70 nt	-0.565**	0.004
+824_829 nt	-0.663**	0
+839_842 nt	-0.469*	0.021

"**" means significant correlation when the confidence limit is 99%
"*" means significant correlation when the confidence limit is 95%

c

Figure 4: Multiple methylated CpG dinucleotides are negatively associated with the expression of SRD5A2. (**a**) Schematic diagrams of 14 methylated CpG dinucleotides in the sequence from −1009 bp to +922 bp in *SRD5A2*. (**b**) The methylation ratios of the 14 CpG dinucleotides in the weakly positive group were higher than those in the strongly positive group. (**c**) Spearman's rank correlation between the methylation ratios of seven methylated CpG dinucleotides and the expression of SRD5A2. TSS: transcription start site; SRD5A2: 5-α reductase type 2; CpG: cytosine-phosphate-guanine.

Table 1: Correlation between the expression of the 5-α reductase type 2 (SRD5A2) protein and methylated dinucleotides in *SRD5A2*

CpG dinucleotides	*Weak expression*	*Strong expression*	*P*
−235 nt	0.17 (0.115, 0.23)	0.085 (0, 0.1225)	0.07
−74_72_69 nt	0.19 (0.1725, 0.24)	0.12 (0.105, 0.1825)	0.007
+46 nt	0.135 (0.11, 0.16)	0.09 (0.07, 0.12)	0.012
+53_56 nt	0.21 (0.145, 0.2725)	0.115 (0.0975, 0.2025)	0.03
+65_68_70 nt	0.19 (0.1725, 0.24)	0.12 (0.105, 0.1325)	0.007
+824_829 nt	0.105 (0.0875, 0.1325)	0.08 (0.06, 0.08)	0.001
+839_842 nt	0.045 (0.025, 0.065)	0.02 (0.0075, 0.0325)	0.025

Statistical description and Mann–Whitney *U* analysis contingency table (two-tailed analysis) between the methylation ratios of 14 CpG dinucleotides of *SRD5A2* and the expression of the SRD5A2 protein. The statistical description of 24 BPE cases is presented as median (IQRs), and the methylation ratios of the 14 CpG dinucleotides in the weakly positive group are different from those in the strongly positive group. "_" indicates that these CpG dinucleotides were detected as clusters, and the average methylation ratios of these CpG dinucleotides are shown. SRD5A2: 5-α reductase type 2; CpG: cytosine-phosphate-guanine; IQRs: interquartile ranges; BPE: benign prostatic enlargement

promoter region and the first exon of *SRD5A2*, and 73 methylated CpG dinucleotides were found in a broader sequence from −1009 bp to +922 bp. MassArray DNA methylation analysis was performed to evaluate the methylation ratios of the 73 methylated CpG dinucleotides in 24 BPE specimens, which were divided into two groups according to their IHC scores. The results of methylation analysis showed variable methylation ratios of the 73 CpG dinucleotides in all the 24 specimens, and a negative correlation with the expression of the SRD5A2 protein was confirmed for 14 CpG dinucleotides, including 4 CpG dinucleotides (−235 nt and −74_72_69 nt) in the promoter region of the CpG island, 6 CpG dinucleotides (+46 nt, +53_56 nt, and +65_68_70 nt) in the first exon, and 4 CpG dinucleotides(+824_829 nt and +839_842 nt) in the first intron. For the first time to our knowledge, we have identified the specific methylated CpG dinucleotides in the CpG island from −400 bp to +600 bp in *SRD5A2* that may account for the absent and reduced levels of expression of the SRD5A2 protein, which probably lead to resistance to finasteride treatment in certain BPE patients.

Although it has been established that DNA methylation in the promoter region of *SRD5A2* is negatively correlated with protein expression,[9,11] the methylated CpG dinucleotides in the first exon of *SRD5A2* that were associated with the repressed expression of the SRD5A2 protein were fortunately identified in our study. The downregulation of gene expression resulting from the methylation of CpG islands associated with the first exon is not uncommon. Shivapurkar *et al*.[25] found that the hypermethylation of a CpG island in the first exon was responsible for the silencing of the transcription factor 21 (*TCF21*) gene. Ye *et al*.[26] showed that p16 protein expression tended to be lower when the methylation level of the CpG island in the first exon was increased. Our finding of a correlation between the methylation of first exon and repression of the SRD5A2 protein adds a new element to the understanding of gene silencing due to the methylated promoter, which might provide a complementary explanation for why 10% of cases with hypomethylated promoters show no or weak SRD5A2 protein expression.[11]

The mechanism of DNA methylation has not been elucidated. Age and obesity are common factors in many benign and malignant diseases.[13,27] Ge *et al*.[11] reported that the mean age of patients whose *SRD5A2* sequence was methylated in prostate samples was 73 years, compared to 68.7 years for patients whose *SRD5A2* was unmethylated.

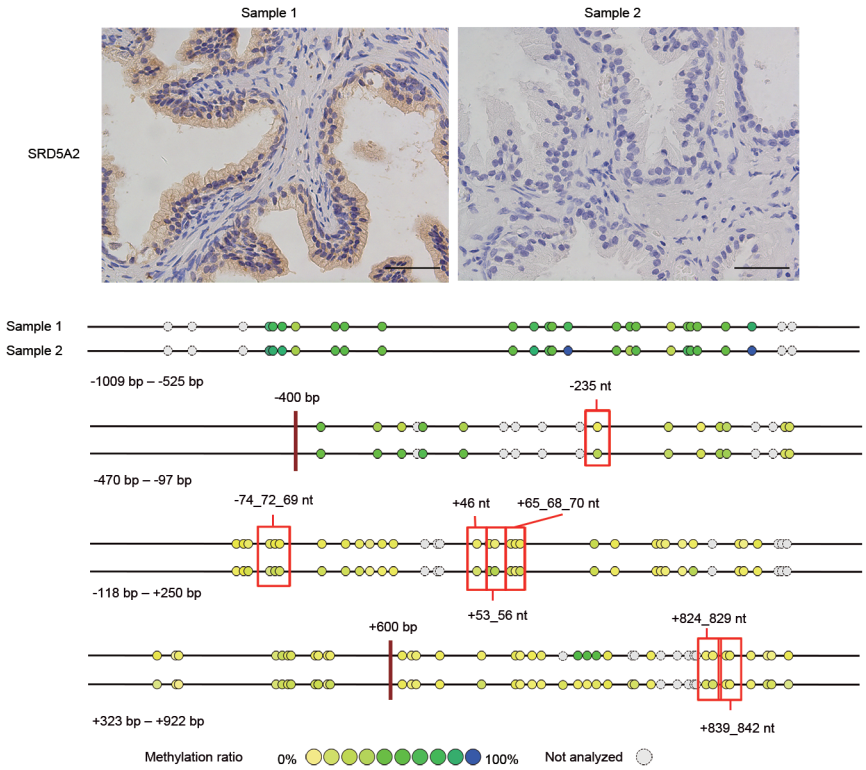


Figure 5: Methylation status of 14 methylated CpG dinucleotides and the corresponding SRD5A2 expression in BPE transitional zone tissues. Sample 1: methylation status of 14 CpG dinucleotides and strong expression of SRD5A2 in fresh BPE tissue. Sample 2: methylation status of 14 CpG dinucleotides and reduced expression of SRD5A2 in fresh BPE tissue. Scale bars = 50 μm. SRD5A2: 5-α reductase type 2; CpG: cytosine-phosphate-guanine; BPE: benign prostatic enlargement.

Moreover, Ge *et al.*[11] verified that the pro-inflammatory mediators tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) upregulate DNMT1 expression through the nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways, respectively, in turn promoting the methylation of *SRD5A2* and ultimately repressing SRD5A2 expression, but the author did not identify the specific methylated CpG dinucleotides of *SRD5A2,* which were reported in our study.

Our study is novel because we evaluated the CpG island extending from −400 bp to +600 bp in *SRD5A2* and identified ten specific methylated CpG dinucleotides in this CpG island, which correlated with the reduced expression of the SRD5A2 protein. We infer that increased expression of DNMT1 may upregulate the methylation ratios of the key CpG dinucleotides in this CpG island, which, in turn, leads to no or reduced SRD5A2 protein expression and gives rise to resistance to finasteride. However, our study has several shortcomings that should be mentioned. First, our sample size (64 cases, including 24 fresh BPE transitional zone tissues) was relatively small, but we still detected a strong correlation between multiple methylated CpG dinucleotides in this CpG island and the reduced expression of the SRD5A2 protein, which is sufficient to support further refining investigations. Second, the clinical significance of the decrease in SRD5A2 for the management of BPE patients requires further investigation. As a result, we are in the process of evaluating whether reduced levels of SRD5A2 are associated with finasteride resistance through the comparison of DNMT1, SRD5A2, cell proliferation, and apoptosis in TURP specimens between finasteride-resistant and finasteride-sensitive BPE patients. If so, our findings could have important implications for the individualized management of BPE and probably chemopreventive strategies for prostate cancer.[28,29] Precise treatments could be provided if we can distinguish finasteride-resistant from finasteride-sensitive BPE patients before a management regime is initiated. More aggressive surgery could be performed immediately, without the need for 6–12 months of diagnostic finasteride treatment, thus avoiding the associated costs and the potential harm caused by long-term ineffective finasteride therapy.

AUTHOR CONTRIBUTIONS

The authors listed below have made substantial contributions to the intellectual content of the paper in the various sections. YNN designed and supervised the study. ZML and DDF contributed to the design, preparation, drafting, statistical analysis, and revision of the manuscript. ZML and ZLL collected prostate tissues and carried out prostate tissue testing. SJ and DDF carried out cell testing. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

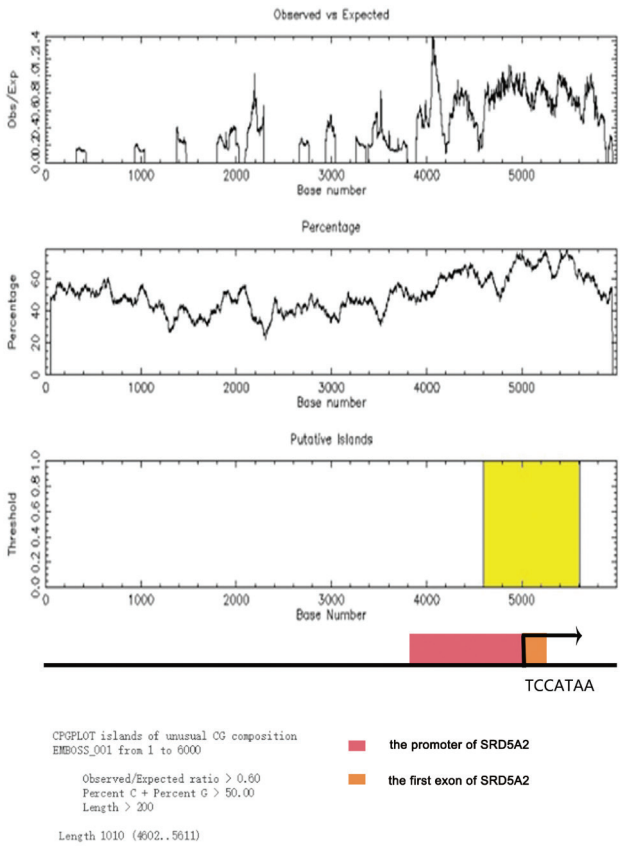
REFERENCES

1 Pagano E, Laudato M, Griffo M, Capasso R. Phytotherapy of benign prostatic hyperplasia. A minireview. *Phytother Res* 2014; 28: 949–55.
2 Roehrborn CG. Pathology of benign prostatic hyperplasia. *Int J Impot Res* 2008; 20: S11–8.
3 Bushman W. Etiology, epidemiology, and natural history of benign prostatic hyperplasia. *Urol Clin North Am* 2009; 36: 403–15.
4 Parsons JK, Schenk JM, Arnold KB, Messer K, Till C, *et al*. Finasteride reduces the risk of incident clinical benign prostatic hyperplasia. *Eur Urol* 2012; 62: 234–41.
5 Cindolo L, Pirozzi L, Fanizza C, Romero M, Tubaro A, *et al*. Drug adherence and clinical outcomes for patients under pharmacological therapy for lower urinary tract symptoms related to benign prostatic hyperplasia: population-based cohort study. *Eur Urol* 2015; 68: 418–25.
6 Yang XJ, Lecksell K, Short K, Gottesman J, Peterson L, *et al*. Does long-term finasteride therapy affect the histologic features of benign prostatic tissue and prostate cancer on needle biopsy? PLESS study group. Proscar long-term efficacy and safety study. *Urology* 1999; 53: 696–700.
7 Bruskewitz R, Girman CJ, Fowler J, Rigby OF, Sullivan M, *et al*. Effect of finasteride on bother and other health-related quality of life aspects associated with benign prostatic hyperplasia. PLESS study group. Proscar long-term efficacy and safety study. *Urology* 1999; 54: 670–8.
8 Babaian RJ, Miyashita H, Evans RB, Ramirez EI. The distribution of prostate specific antigen in men without clinical or pathological evidence of prostate cancer: relationship to gland volume and age. *J Urol* 1992; 147: 837–40.
9 Niu Y, Ge R, Hu L, Diaz C, Wang Z, *et al*. Reduced levels of 5-α reductase 2 in adult prostate tissue and implications for BPH therapy. *Prostate* 2011; 71: 1317–24.
10 McConnell JD, Roehrborn CG, Bautista OM, Andriole GL Jr, Dixon CM, *et al*. The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. *N Engl J Med* 2003; 349: 2387–98.
11 Ge R, Wang Z, Bechis SK, Otsetov AG, Hua S, *et al*. DNA methyl transferase 1 reduces expression of SRD5A2 in the aging adult prostate. *Am J Pathol* 2015; 185: 870–82.
12 McConnell JD, Bruskewitz R, Walsh P, Andriole G, Lieber M, *et al*. The effect of finasteride on the risk of acute urinary retention and the need for surgical treatment among men with benign prostatic hyperplasia. Finasteride long-term efficacy and safety study group. *N Engl J Med* 1998; 338: 557–63.
13 Kang PM, Kim YJ, Seo WT, Kang SH, Kim TS, *et al*. Correlation between 5-α reductase type 2 protein expression and methylation of 5-α reductase type 2 promotor gene of benign prostatic hyperplasia. *World J Urol* 2019; 37: 709–18.
14 Uysal F, Akkoyunlu G, Ozturk S. Dynamic expression of DNA methyltransferases (DNMTs) in oocytes and early embryos. *Biochimie* 2015; 116: 103–13.
15 Wang K, Jin S, Fan D, Wang M, Xing N, *et al*. Anti-proliferative activities of finasteride in benign prostate epithelial cells require stromal fibroblasts and *c-Jun* gene. *PLoS One* 2017; 12: e0172233.
16 Gu X, Na R, Huang T, Wang L, Tao S, *et al*. SRD5A1 and SRD5A2 are associated with treatment for benign prostatic hyperplasia with the combination of 5α-reductase inhibitors and α-adrenergic receptor antagonists. *J Urol* 2013; 190: 615–9.
17 Titus MA, Li Y, Kozyreva OG, Maher V, Godoy A, *et al*. 5α-reductase type 3 enzyme in benign and malignant prostate. *Prostate* 2014; 74: 235–49.
18 Wang K, Fan DD, Jin S, Xing NZ, Niu YN. Differential expression of 5-alpha reductase isozymes in the prostate and its clinical implications. *Asian J Androl* 2014; 16: 274–9.
19 Salam MT, Ursin G, Skinner EC, Dessissa T, Reichardt JK. Associations between polymorphisms in the steroid 5-alpha reductase type II (*SRD5A2*) gene and benign prostatic hyperplasia and prostate cancer. *Urol Oncol* 2005; 23: 246–53.
20 Das K, Lorena PD, Ng LK, Lim D, Shen L, *et al*. Differential expression of steroid 5alpha-reductase isozymes and association with disease severity and angiogenic genes predict their biological role in prostate cancer. *Endocr Relat Cancer* 2010; 17: 757–70.
21 Li M, Yang X, Wang H, Xu E, Xi Z. Inhibition of androgen induces autophagy in benign prostate epithelial cells. *Int J Urol* 2014; 21: 195–9.
22 Chhipa RR, Wu Y, Ip C. AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. *Cell Signal* 2011; 23: 1466–72.
23 Christmann M, Kaina B. Epigenetic regulation of DNA repair genes and implications for tumor therapy. *Mutat Res* 2019; 780: 15–28.
24 Dobosy JR, Roberts JL, Fu VX, Jarrard DF. The expanding role of epigenetics in the development, diagnosis and treatment of prostate cancer and benign prostatic hyperplasia. *J Urol* 2007; 177: 822–31.
25 Shivapurkar N, Stastny V, Xie Y, Prinsen C, Frenkel E, *et al*. Differential methylation of a short CpG-rich sequence within exon 1 of *TCF21* gene: a promising cancer biomarker assay. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 995–1000.
26 Ye X, Mo M, Xu S, Yang Q, Wu M, *et al*. The hypermethylation of *p16* gene exon 1 and exon 2: potential biomarkers for colorectal cancer and are associated with cancer pathological staging. *BMC Cancer* 2018; 18: 1023.
27 Bechis SK, Otsetov AG, Ge R, Wang Z, Vangel MG, *et al*. Age and obesity promote methylation and suppression of 5α-reductase 2: implications for personalized therapy of benign prostatic hyperplasia. *J Urol* 2015; 194: 1031–7.
28 Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, *et al*. The influence of finasteride on the development of prostate cancer. *N Engl J Med* 2003; 349: 215–24.
29 Redman MW, Tangen CM, Goodman PJ, Lucia MS, Coltman CA, *et al*. Finasteride does not increase the risk of high-grade prostate cancer: a bias-adjusted modeling approach. *Cancer Prev Res (Phila)* 2008; 1: 174–81.

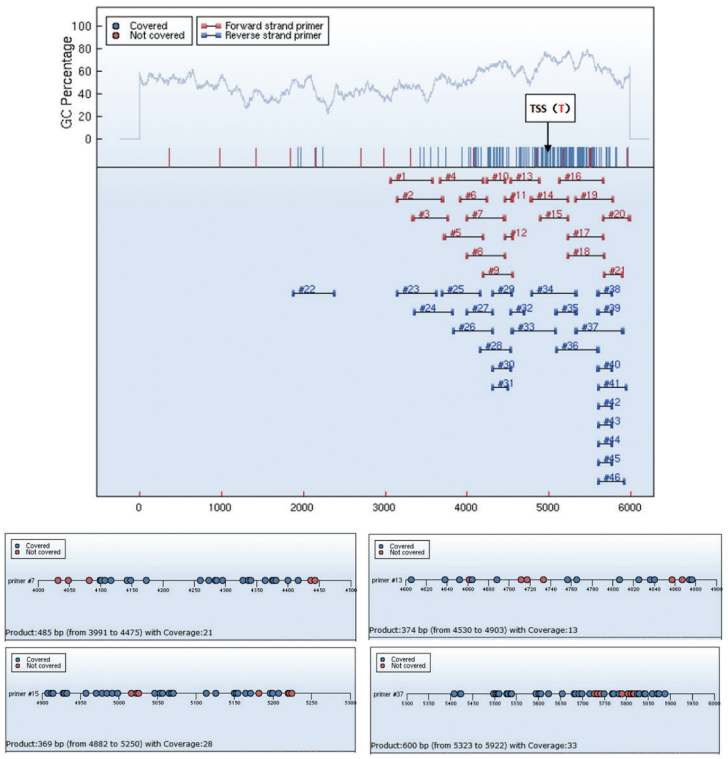
Supplementary Table 1: The primers listed were designed to cover the sequence from –1009 bp to +922 bp in 5-α reductase type 2

Sequence	*Direction*	*Primer*
SRD5A2/–1009_525	F	5'-GGAGGAGGTAGTTAAGAAAGTTTGG-3'
SRD5A2–1009_–525	R	5'-CTCAACATCAATACCAACTCTACCC-3'
SRD5A2/–470_–97	F	5'-GTTAGGATGGTTTAGGGTTTAAGGA-3'
SRD5A2/–470_–97	R	5'-CAATACCCCTTTCTCAAAAATACAA-3'
SRD5A2/–118_+250	F	5'-TATTTTTGAGAAAGGGGTATTGTTG-3'
SRD5A2/–118_+250	R	5'-AAAAACAACTCCTACAAAAACCAAA-3
SRD5A2/+323_+922	F	5'-GAAGTTTGGATTTGGGTTTTATTTA-3'
SRD5A2/+323_+922	R	5'-AAACCTCTTCTACCTACATTACTTCCA-3'

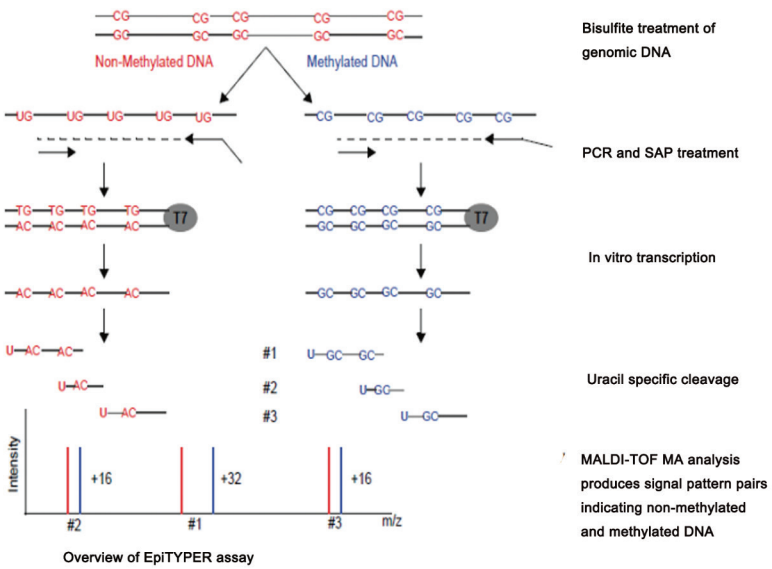
SRD5A2: 5-α reductase type 2



Supplementary Figure 1: Schematic representation of the strategy for detecting CpG islands in the region from –5000 bp to +1000 bp in *SRD5A2*. Calculations using EMBOS suggested that a CpG island was located from –400 bp to +600 bp. %GC dinucleotide values and observed/expected ratios of CpGs were calculated as suggested. *SRD5A2*: 5-α reductase type 2; CpG: cytosine-phosphate-guanine.



Supplementary Figure 2: Schematic representation of the detected CpG dinucleotides in the –1009 bp to +922 bp region of SRD5A2. The binding sites of four pairs of designed primers are indicated with arrows. Four diagrams show the detected CpG dinucleotides. The blue dots show CpG dinucleotides that could be detected and methylated, and the red dots show CpG dinucleotides that could not be detected because they were located in segments that were too short or too long in relation to the detection limit for MassArray mass spectra. SRD5A2: 5-α reductase type 2; CpG: cytosine-phosphate-guanine.



Supplementary Figure 3: Schematic representation of the MassArray quantitative methylation analysis. The methylation ratios of single CpG dinucleotides could be quantified in the segments of 100–700 bp each time. Because RNaseA specifically cuts at U'3 termini in RNA sequences, if there was no thymine between two or more CpG dinucleotides in the corresponding DNA sequences, the average methylation ratios of these CpG dinucleotides detected in clusters are shown. The segments detected were between 5 and 22 nt outside of the limits of the MassArray mass spectra, for which the detection range was between 1500 and 7000 Da. CpG: cytosine-phosphate-guanine.