# Cytochrome *P*450 1B1 Is Overexpressed and Regulated by Hypomethylation in Prostate Cancer

Takashi Tokizane,[1] Hiroaki Shiina,[2] Mikio Igawa,[2] Hideki Enokida,[1] Shinji Urakami,[1] Toshifumi Kawakami,[1] Tatsuya Ogishima,[1] Steven T. Okino,[1] Long-Cheng Li,[1] Yuichiro Tanaka,[1] Norio Nonomura,[3] Akihiko Okuyama,[3] and Rajvir Dahiya[1]

**Abstract**

**Purpose:** Cytochrome *P*450 1B1 (*CYP1B1*), a dioxin inducible member of the *CYP* supergene family, is overexpressed in various human malignancies including prostate cancer. We hypothesized that promoter/enhancer CpG methylation contributes to the regulation of *CYP1B1* expression in human prostate tissue.

**Experimental Design:** Expression and induction of the *CYP1B1* gene in clinical prostate tissues and prostate cancer cell lines were investigated. The methylation status of the *CYP1B1* gene was analyzed in 175 prostate cancer and 96 benign prostatic hyperplasia samples using methylation-specific PCR (MSP) and bisulfite-modified DNA sequencing. MSP primers covered dioxin response elements (DRE) and Sp1 sites that are important for the expression of CYP1B1.

**Results:** Expressions of CYP1B1 mRNA and protein were increased in prostate cancer. The aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (ARNT) heterodimer complex activates gene transcription by binding to the DREs of CYP1B1. In prostate cancer cells, CYP1B1 mRNA was induced by 2,3,7,8-tetrachlorodigenzo-*p*-dioxin (TCDD) and/or demethylation agent (5-aza-2-deoxycytidine). There was no change in the expressions of AhR and ARNT. Methylation of promoter/enhancer regions was significantly higher in benign prostatic hyperplasia compared with prostate cancer. MSP-positive patients had significantly lower risk for prostate cancer as compared with MSP-negative patients. There was no correlation between CYP1B1 methylation status and clinicopathologic features.

**Conclusions:** CYP1B1 is overexpressed in prostate cancer and regulated by hypomethylation of its promoter/enhancer region. This is the first report about CYP1B1 regulation in human clinical prostate samples showing that hypomethylation of the *CYP1B1* gene may play an important role in prostate cancer.

Prostate cancer is one of the most common cancers in men in the world and it is a commonly diagnosed neoplasm as a result of increased availability of prostate specific antigen testing (1). The prostate is an androgen-dependent organ and contains several enzymes involved in the metabolism of steroid hormones, including androgen and estrogen. In addition, these enzymes metabolize a variety of drugs as well as dietary and environmental factors that may be activated to potent carcinogenic compounds. In this regard, some epidemiologic studies suggest a positive association between exposure to polycyclic aromatic hydrocarbons and increased risk of prostate cancer (2).

Cytochrome *P*450s are a multigene family of constitutively expressed and inducible enzymes involved in the oxidative metabolic activation and deactivation of carcinogens and cancer therapeutics (3, 4). Cytochrome *P*450 1B1 (*CYP1B1*) is a member of the *CYP1* gene family and one of the major enzymes involved in the hydroxylation of estrogens and activation of potential carcinogens. The metabolites of CYP1B1 are known to induce prostate cancer in an experimental animal model (5, 6). Given that expression of CYP1B1 is increased in a wide range of human tumors (7–9), it may be potentially important in prostate tumor development and progression (10). Additionally, CYP1B1 expression may serve as a potential target for anticancer drugs and may be an important tumor biomarker (3).

The upstream regulatory region of the human *CYP1B1* gene has several positive regulatory elements within the promoter region. The core promoter region is located between nucleotides (nt) −164 and +25 relative to the transcription initiation site. This region contains a TATA-like box (TTAAAA) from nt −33 to −28, and two Sp1 binding sites (GGGCGG) starting at nt −84 and −68 on the antisense strand (11, 12). The Sp1 site

**Authors' Affiliations:** [1]Department of Urology, Veterans Affairs Medical Center and University of California, San Francisco, California; [2]Department of Urology, Shimane University, Izumo, Japan; and [3]Department of Urology, Osaka University Graduate School of Medicine, Suita, Japan

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**Requests for reprints:** Rajvir Dahiya, Urology Research Center, University of California San Francisco and Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121. Phone: 415-450-6964; Fax: 415-750-6639; E-mail: rdahiya@urol.ucsf.edu.



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has been shown to bind the transcription factor Sp1 causing high rates of transcription. The enhancer region located from nt −1,022 to −835 contains several dioxin response elements (DRE) which are involved in transcriptional activation by polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodigenzo-*p*-dioxin (TCDD; refs. 11–13). Induction of the *CYP1B1* gene is regulated by the aryl hydrocarbon receptor (AhR) signaling pathway to which the polycyclic aromatic hydrocarbons bind with high affinity. In the absence of ligand, AhR is present in the cytoplasm and on binding, the AhR/ligand complex translocates to the nucleus where it forms a heterodimeric complex with the AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer binds to DREs, resulting in enhanced expression of *CYP1* genes (4, 14). In a recent report, AhR has been also identified as a target gene of the Wnt/β-catenin pathway in prostate cancer cells (15).

Tumors are often characterized by an imbalance in cytosine methylation having both hypermethylation and hypomethylation of various regions of the genome. DNA methylation can inactivate genes and suppress gene expression directly by interfering with the binding of transcription factors or indirectly by attracting methylated DNA binding factors that recruit histone deacetylases and precipitate an inactive chromatin structure (16–18). In this regard, CpG island methylation has been shown to be important in the transcriptional silencing of many genes. In contrast, hypomethylation is generally observed in unique sequences, including transcription control sequences that are often subject to cancer-associated hypomethylation (17, 19). Hypomethylation has been implicated in the activation of oncogenes, such as c-*myc* (20), and may also contribute to chromosomal instability (21). The high frequency of cancer-linked DNA hypomethylation is consistent with its proposed role in cancer formation or tumor progression. The relationship between DNA hypomethylation and tumorigenesis is important in light of the interest in cancer therapies to decrease DNA methylation.

Currently, the mechanisms controlling transcription of the *CYP1B1* gene are not fully understood. In this study, we investigated the expression of the *CYP1B1* gene and the epigenetic mechanisms (DNA hypomethylation) involved in overexpression of CYP1B1 in prostate cancer.

## Materials and Methods

***Clinical samples.*** A total of 175 newly diagnosed prostate cancer tissues from radical prostatectomy and 96 pathologically proven benign prostatic hyperplasia (BPH) samples from transurethral resection were obtained from Shimane University Hospital (Izumo, Japan). The clinicopathologic characteristics are summarized in Table 1. All tumors were graded according to the general rule for Clinical and Pathologic Studies on prostate cancer by the Japanese Urological Association and the Japanese Society of Pathology (22). Our routine strategy to diagnose prostate cancer included serum prostate-specific antigen level, transrectal ultrasonography, color Doppler ultrasonography, and magnetic resonance imaging, which enabled us to accurately localize the prostate cancer before radical prostatectomy (23).

***Tissue preparations.*** After radical prostatectomy, the normal and cancer regions of each prostate were identified. Half of each prostate sample (normal and cancer) was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax for histopathologic evaluation. The remaining half of each prostate sample was immediately frozen and stored at −80 °C for RNA extraction. The BPH tissues obtained from other patients were samples that had been fixed in formalin and embedded in paraffin. All samples were microscopically dissected and DNA was isolated for methylation analysis. In BPH samples, high-grade prostatic intraepithelial neoplasia and cancer were ruled out by microscopic analysis.

**Table 1.** Clinical characteristics of prostate cancer and BPH

| | |
|---|---|
| Prostate cancer | |
| Total number of patients | 175 |
| Mean age ± SD (y) | 68.6 ± 7.3 |
| pT stage | |
| $pT_1$ | 0 |
| $pT_2$ | 117 |
| $pT_3$ | 55 |
| $pT_4$ | 3 |
| Gleason score | |
| ≤6 | 96 |
| 7 | 44 |
| ≥8 | 35 |
| Preoperative serum PSA | |
| <4 | 20 |
| 4-10 | 81 |
| >10 | 74 |
| Benign prostatic hyperplasia | |
| Total number of patients | 96 |
| Mean age ± SD (y) | 73.6 ± 5.6 |

***Cell culture.*** The human prostate cancer cell lines DU145, DUPro, LNCaP, and PC-3 were obtained from the American Type Culture Collection (Manassas, VA) and the ND-1 cell line was developed in our laboratory (24). All prostate cancer cell lines were maintained in RPMI 1640 with L-glutamine and sodium pyruvate. The ND-1 cell line was grown in McCoy's 5A media with L-glutamine. FCS (10%) was present in the media and cells were maintained in a humidified atmosphere of 5% $CO_2$/95% air at 37°C.

***Isolation of DNA and RNA from clinical prostate samples.*** Genomic DNA was extracted from prostate cancer and control prostate samples obtained by microdissection using a Qiagen kit (Qiagen, Valencia, CA; ref. 25). Total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati, OH) and the RNA pellet obtained after isopropanol and ethanol precipitation was dried and resuspended in 50 μL of RNase-free water. All RNA samples were treated with DNase (Wako Pure Chemical, Osaka, Japan) and stored in aliquots at −80 °C until reverse transcribed. The concentrations of DNA and RNA were determined with a spectrophotometer and their integrity was assessed by gel electrophoresis.

***Expression of the CYP1B1 gene in prostate cancer cell lines and clinical prostate samples by reverse transcription-PCR.*** Total RNA from cancer cell lines was isolated using an extraction kit (RNeasy Mini kit; Qiagen). Reverse transcription-PCR (RT-PCR) was preformed using a TITANIUM One-Step RT-PCR Kit according to the protocol of the manufacturer (BD Biosciences Clontech, Palo Alto, CA). In prostate cancer cell lines, primer pairs L-1B1_RT, AhR_RT, and ARNT_RT were used to amplify human CYP1B1, AhR, and ARNT, respectively. Primer sequences, RT-PCR conditions (annealing temperatures and cycle numbers), and product sizes are described in Table 2. The glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene was used as an internal control and its primer pair was L-G3PDH (Table 2).

The cDNA from clinical prostate samples was prepared by reverse transcription (Promega Corp., Madison, WI) using DNase-treated RNA as a template. For RT-PCR of CYP1B1 and G3PDH, the primer pairs

**Table 2.** Primer sequences for RT-PCR and MSP

| Primer sets | Sense primer | Antisense primer | Anneal temperature, °C (no. cycles) | Product size (bp) |
|---|---|---|---|---|
| *RT-PCR for clinical prostate samples* | | | | |
| S-1B1_RT | AACGTACCGGCACTATCAC | ACGACCTGATCCAATTCTGC | 55 (40) | 137 |
| S-G3PDH | CAATGACCCCTTCATTGACC | TGGAAGATGATGGGATTT | 55 (40) | 135 |
| *RT-PCR for prostate cancer cell lines* | | | | |
| L-1B1_RT | TACCGGCACTATCACTGACATCTTCG | GGTGAGCCAGGATGGAGATGAAGAGAA | 65 (35) | 510 |
| AhR_RT | CCACAGCAACAGCTGTGTCAGAAGATG | CGGATGATGAAGTGGCTGAAGATGTGT | 65 (35) | 600 |
| ARNT_RT | GCTGGGAGATCAGAGCAACAGCTACAA | TGTTTCTTTCCAGAGGGACTGCTCACA | 65 (35) | 588 |
| L-G3PDH | TCCCATCACCATCTTCCA | CATCACGCCACAGTTTCC | 60 (35) | 376 |
| *MSP* | | | | |
| Pan1 | GGTTTTTTTATAAAGGGAGGGTTT | ACTCCAATCATATCCCTAAAC | 50 (45) | 308 |
| MS1 | GTAGGCGCGATTGTGCGTGC | TCTCACGACACCTAACACGCG | 64 (3), 62 (3), 60 (3), 58 (31) | 118 |
| US1 | TTTGGTAGGTGTGATTGTGTGTT | CTTCTCACAACACCTAACACACAA | 60 (40) | 125 |
| Pan2 | GTTTAGGGATATGATTGGAGT | ACTAAAAAAAACCTAAAAAAAACTAAC | 50 (45) | 189 |
| MS2 | CGTACGTAAAGTTTAGTTTCGTAC | GAAACGAAACCCGAACGAACGAAACG | 56 (3), 54 (3), 53 (3), 52 (31) | 126 |
| US2 | GGTGTATGTAAAGTTTAGTTTTGTATG | ACTAACAAAACAAAACAAAACCCAAACAAAACA | 58 (40) | 134 |
| MS3 | TCGATTTTTTAGAAGCGGCGTACG | GAAACGAAACCCGAACGAAACG | 62 (3), 60 (3), 58 (3), 56 (31) | 144 |
| US3 | AGTTGATTTTTTAGAAGTGGTGTATG | ACTAACAAAACAAAACCCAAACAAAACA | 58 (40) | 152 |
| Pan5 | GATTGGAGGTGGTTGTGATGAAG | CCACTCCCACTCCAAAATCAAAAC | 50 (45) | 256 |
| MS5 | AGTTTCGAGGTCGGTCGGTGC | AACAACGCGACCTAACAAAACG | 59 (38) | 106 |
| US5 | GTAGAGTTTTGAGGTTGGTTGGTGT | AAAACAACACAACCTAACAAAACAA | 61 (32) | 112 |

NOTE: Underline indicates the CpG sites within the primer sequence.

S-1B1_RT and S-G3PDH were used in the analysis of clinical prostate samples (Table 2).

RT-PCR products were electrophoresed in 2.0% agarose gels with ethidium bromide. The expression level of these genes was evaluated by Image J software (http://rsb.info.nih.gov/ij/) and the area under the curve was calculated. Levels of the *CYP1B1* gene were quantified relative to that of G3PDH and expressed as arbitrary units.

***5-Aza-2-deoxycytidine and 2,3,7,8-tetrachlorodigenzo-p-dioxin treatment.*** To investigate whether CYP1B1 was induced by TCDD and if CpG methylation was involved in the inactivation of the *CYP1B1* gene, we analyzed the effect of 5-aza-2-deoxycytidine (5-Aza-dC) on basal and TCDD-inducible target gene mRNA expressions in DU145, DUPro, LNCaP, PC-3, and ND-1 prostate cancer cell lines. All cell lines were treated with 5-Aza-dC (total 1 μmol/L) for 7 days and with TCDD (10 nmol/L) for 1 day, and then were harvested on day 7. Total RNA was isolated and analyzed by One Step RT-PCR using primers specific for human CYP1B1, AhR, and ARNT.

***Methylation-specific PCR.*** Modified DNA was used as a template for methylation-specific PCR (MSP). Sodium bisulfite modification was done using a DNA Modification Kit (Intergen, Oxford, United Kingdom) according to the protocol of the manufacturer. Based on the functional promoter/enhancer sequence of the *CYP1B1* gene (11–13), several sets of primers were designed using MethPrimer (http://itsa.ucsf.edu/~urolab/methprimer; ref. 26) to detect the methylation status of CpG sites in the *CYP1B1* gene. Primer pairs used for amplification of CYP1B1, primer sequences, PCR conditions (annealing temperatures and cycle numbers), and product sizes are described in Table 2.

The enhancer region amplified by the Pan1 sense (S) and Pan2 antisense (AS) primers contained 57 CpG sites including five DREs and two Sp1 sites. The promoter region amplified by Pan5S and Pan5AS primers contained 24 CpG sites including two Sp1 sites, a TATA-like box, and the transcription initiation site. MSP (27) was carried out as a second round of nested PCR (MSP1, MSP2, MSP3, and MSP5) using the universal PCR product amplified by either Pan1S and Pan1AS primers, Pan2S and Pan2AS primers, or Pan5S and Pan5AS as template. The first universal primer pairs used (Pan1S and Pan1AS, Pan2S and Pan2AS, or Pan5S and Pan5AS) have no CpG sites in either forward or reverse primer whereas the MSP1 or unmethylation-specific PCR (USP) 1 primer covers the DRE1 site. Similarly, the MSP2 or USP2 primer covers both the DRE3 and SP-1 sites and the MSP3 or USP3 primer covers the DRE2 site. These three DREs in the enhancer region are particularly important for the expression of CYP1B1 (12, 13). The MSP5 or USP5 primer covers the Sp1 site as the core promoter region. In each assay, absence of DNA template served as negative control. The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The density of each band was calculated by Image J software (http://rsb.info.nih.gov/ij) and the relative methylation level in each sample was determined using the following formula: MSP ratio (%) = MSP × 100 / (MSP + USP). The data were confirmed by visual examination and scanning. A MSP ratio of 5.0% was used as a cutoff value to assess whether samples were MSP positive or negative (28).

***Bisulfite DNA sequencing.*** Bisulfite-modified DNA (1 μL) was amplified using Pan2 primer pairs and Pan5 primer pairs in a total volume of 20 μL. Direct bisulfite DNA sequencing of the PCR products

was done according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA). The Pan2AS primer (Table 2) was used to examine the enhancer region and the Pan5S primer (Table 2) used to examine the core promoter region of CYP1B1.

***Immunohistochemistry of prostate cancer and benign prostatic hyperplasia samples.*** Immunostaining of CYP1B1 protein was done in formalin-fixed, paraffin-embedded materials using a rabbit polyclonal antibody (1:300 dilution) against human CYP1B1 (WB1B1, Gentest Corporation, Woburn, MA). The staining procedure was according to a commercial kit (Santa Cruz Biotechnology, Santa Cruz, CA) and immunoperoxidase activity was developed with 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The sections were counterstained with Harris' hematoxylin. Immunostaining was considered visually positive when CYP1B1 expression was the same or greater than the adjacent smooth muscle tissue as the visual criteria (29, 30). Alternatively, Image J–based analysis was done to quantify the intensity of immunostaining as the quantitative criteria. The average density was measured in randomly selected areas on each slide containing a minimum of 1,000 cells and expressed as arbitrary units.

***Statistical analysis.*** Statistical analysis was done using the Mann-Whitney *U* test for expression of CYP1B1 mRNA and protein. The correlation between clinicopathologic findings and the methylation status of CYP1B1 was assessed using the $\chi^2$ test and logistic regression analysis. $P < 0.05$ was regarded as statistically significant.

## Results

***CYP1B1 protein expression in prostate cancer and benign prostatic hyperplasia.*** Figure 1A shows typical immunostaining of CYP1B1 in BPH and prostate cancer samples. Representative sections of the epithelium show that CYP1B1 protein expression was localized to the cytoplasm of prostate cancer cells. CYP1B1 expression was also observed in smooth muscle cells of both BPH and prostate cancer samples. As visual criteria, we considered immunostaining to be positive when expression of CYP1B1 was the same or greater than the surrounding smooth muscle. Using visual criteria, positive CYP1B1 immunostaining was observed in 78.8% (26 of 33) of the prostate cancer samples. We also analyzed the intensity of immunostaining using Image J software as quantitative criteria. Using quantitative criteria, the intensity of CYP1B1 immunostaining was significantly higher in prostate cancer samples than in BPH samples [BPH versus prostate cancer: 44.4 ± 17.5 versus 78.6 ± 23.9 ($P < 0.001$)].

***Reverse transcription-PCR of CYP1B1 mRNA expression in clinical prostate samples.*** The expression of CYP1B1 mRNA was investigated by RT-PCR in 152 cancer regions and 164 normal regions as the control for prostate tissues (Fig. 1B). The mRNA transcript expression levels were higher in prostate cancer samples than in control prostate samples. There was a significant increase in the expression of CYP1B1 mRNA in prostate cancer [control versus cancer: 1.1 ± 0.28 versus 1.5 ± 0.52 ($P < 0.001$)]. There was no significant correlation between CYP1B1 mRNA levels and Gleason grade or pathologic stage in prostate cancer (data not shown).

***Reverse transcription-PCR of CYP1B1, AhR, and ARNT mRNA expression in prostate cancer cell lines.*** Prostate cancer cell lines were treated with 5-Aza-dC (1 μmol/L) and/or TCDD (10 nmol/L) and then analyzed for expression of human CYP1B1, AhR, and ARNT mRNA by RT-PCR. DUPro and LNCaP cells did not have detectable basal or TCDD-inducible CYP1B1 mRNA in

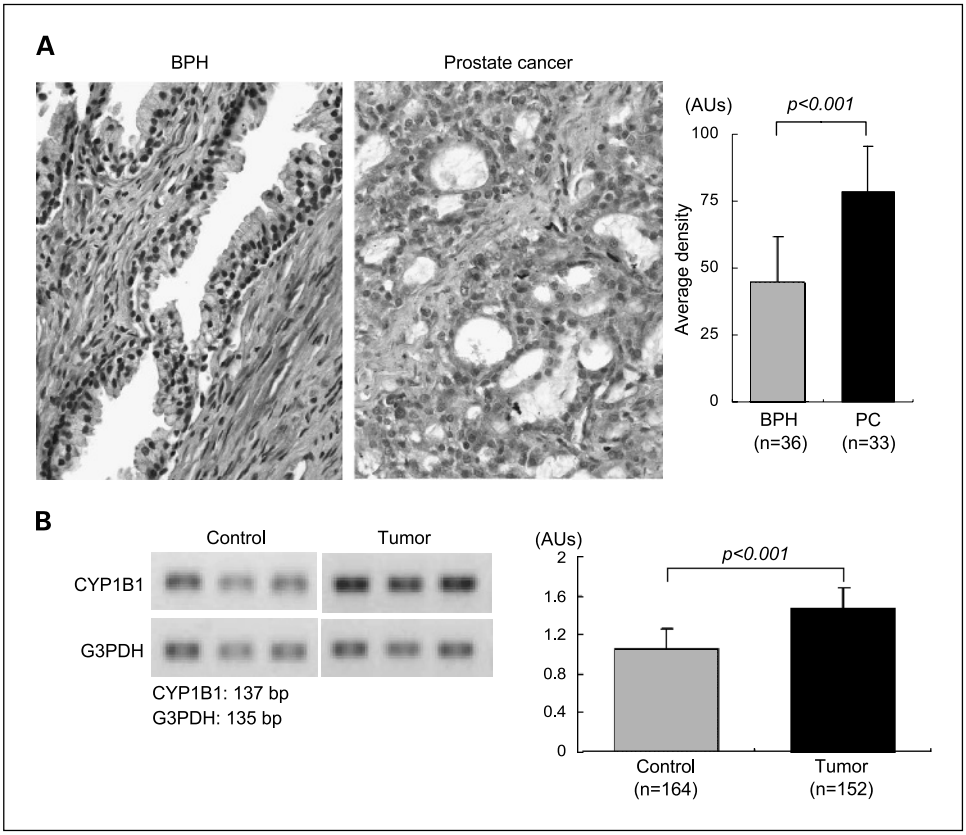



**Fig. 1.** *A,* typical immunohistochemical staining of human CYP1B1 in prostate cancer and BPH samples. CYP1B1 was expressed in the glandular epithelium and immnoreactivity localized to the cytoplasm of prostate cancer. The average intensity of CYP1B1 immunostaining is significantly increased in prostate cancer samples compared with BPH samples ($P < 0.001$). *B*, RT-PCR of CYP1B1 mRNA in clinical prostate samples. Expression of CYP1B1 mRNA transcripts was increased in prostate cancer samples and was significantly higher as compared with control samples ($P < 0.001$).

control or 5-Aza-dC treated cells. DU145 and ND-1 cells expressed CYP1B1 mRNA constitutively, but there was no induction by TCDD. Treatment of these cell lines with 5-Aza-dC had little effect on basal CYP1B1 expression (data not shown). In contrast, as shown in Fig. 2, levels of CYP1B1 mRNA in PC-3 cells were induced by TCDD. In addition, treatment of PC-3 cells with 5-Aza-dC resulted in a moderate increase in basal and TCDD-inducible CYP1B1 mRNA expressions. On the other hand, TCDD and 5-Aza-dC had little effect on the constitutive levels of AhR and ARNT mRNAs in all cell lines (data not shown) including PC-3 cells (Fig. 2). Taken together, these results suggest that in PC-3 cells, decreased promoter methylation is important for the increased expression of the *CYP1B1* gene rather than increased expression of AhR and/or ARNT.

***Methylation status of the CYP1B1 enhancer in prostate tissues.*** The methylation status of the *CYP1B1* gene was analyzed in 175 prostate cancer samples and 96 BPH samples. We designed primers to discriminate between methylated and unmethylated alleles following bisulfite treatment (Table 2; Fig. 3A). Typical examples of the MSP and USP bands are shown in Fig. 3B. In the 175 prostate cancer samples, a positive MSP1 band was detected in 11 samples (6.3%), MSP2 in 10 samples (5.7%), MSP3 in 25 samples (14.2%), and MSP5 in 30 samples (17.1%). On the other hand, in 96 BPH samples, a positive MSP1 band was detected in 35 samples (36.5%), MSP2 in 66 samples (68.8%), MSP3 in 76 samples (79.2%), and MSP5 in 59 samples (61.5%). The prevalence of methylation in three enhancer regions and the promoter region was significantly higher in BPH samples than in prostate cancer samples (Figs. 3B and 4). To confirm the methylation status of the enhancer and promoter regions of CYP1B1, bisulfite-modified DNA was amplified and sequenced. Typical bisulfite-modified DNA sequencing around the promoter and enhancer regions in prostate cancer and BPH samples is shown in Fig. 3C. In prostate cancer samples, CpG sites were not methylated whereas in BPH samples, most CpG sites were methylated in the promoter region including Sp1 sites and all CpG sites were methylated in the enhancer region including DRE2 and DRE3 (Fig. 3C). In addition, the DRE1 region in BPH samples was methylated whereas it was not methylated in prostate cancer samples (data not shown). As shown in Table 3, logistic regression analysis revealed that MSP-positive patients had a significantly lower risk for prostate cancer as compared with MSP-negative patients [MSP1: odds ratio, 7.3; MSP2: odds ratio, 40.5; MSP3: odds ratio, 25.8; MSP5: odds ratio, 8.7, respectively (Table 3)]. However, no significant correlation of CYP1B1 methylation with Gleason grade and pathologic category was found in this study (Fig. 4).

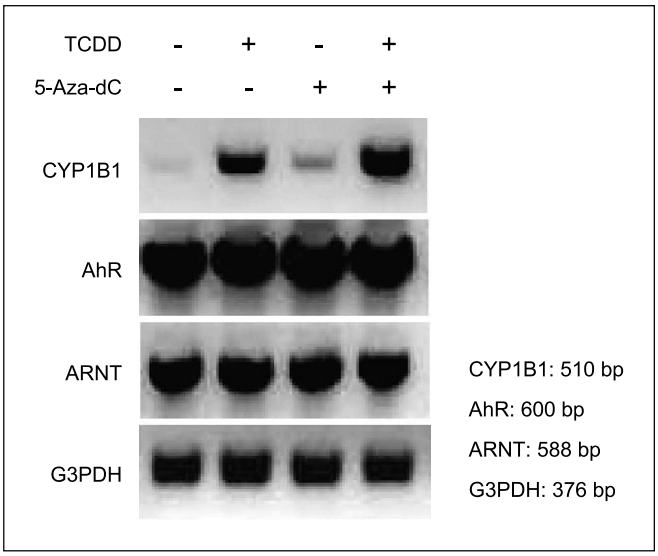
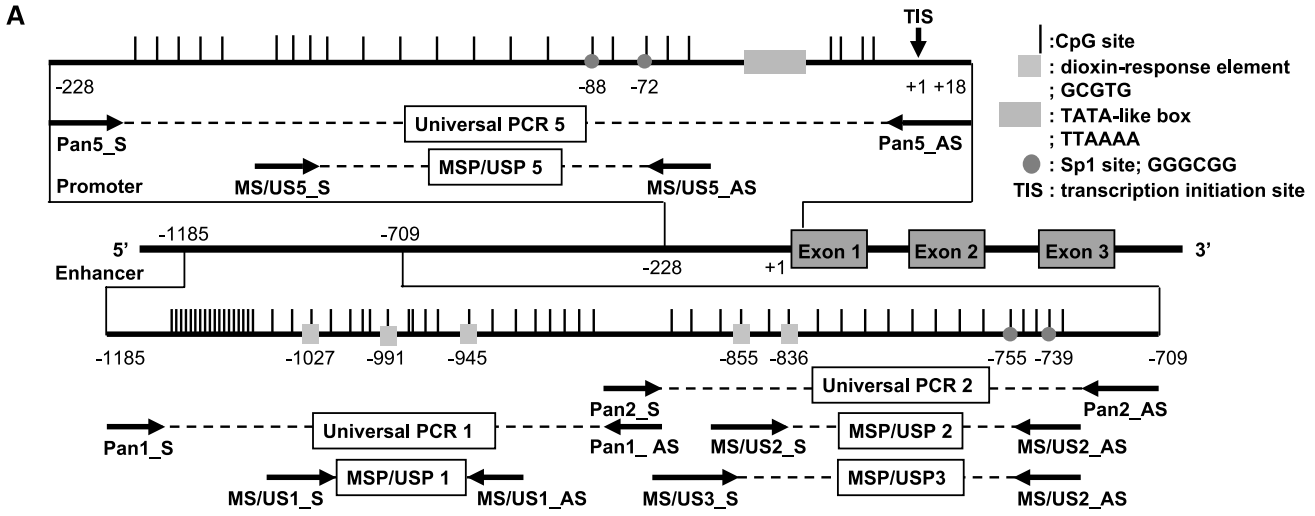



**Fig. 2.** Treatment of PC-3 cancer cells with 5-Aza-dC (1 μmol/L) and/or TCDD (10 nmol/L). Expression levels of mRNA were analyzed by RT-PCR with primer pairs specific for human CYP1B1, AhR, ARNT, and G3PDH. Basal expression of CYP1B1 mRNA in PC-3 cells was observed, which was induced by TCDD. Treatment of PC-3 cells with 5-Aza-dC resulted in a moderate increase in basal and TCDD-inducible CYP1B1 expression. In contrast, expressions of AhR and ARNT mRNAs were not affected by TCDD and 5-Aza-dC in PC-3 cells.

## Discussion

This study shows that the expressions of CYP1B1 protein and mRNA were significantly higher in prostate cancer compared with benign prostate. CYP1B1 is overexpressed in a variety of human tumor cells such as the lung, breast, liver, gastrointestinal tract, and ovarian cancer (7–9). Using immunohistochemical analysis, Carnell et al. (10) reported that CYP1B1 protein is frequently overexpressed in the cytoplasm of prostate cancer cells compared with premalignant and hyperplastic tissues. The CYP1B1 is expressed during premalignancy and is up-regulated at an early stage of carcinogenesis in prostate cancer. Thus, CYP1B1 seems to have a potentially crucial role in tumor development and progression. However, the mechanisms controlling the tissue-specific and cancer-specific expression of CYP1B1 are not known (4). In the present study, we found that CYP1B1 protein was overexpressed in the cytoplasm of prostate cancer cells with a high frequency whereas it was largely absent in BPH samples. This data was confirmed by RT-PCR in radical prostatectomy samples that clearly showed that CYP1B1 mRNA expression was significantly higher in prostate cancer than in nonmalignant prostate tissue. Our results are consistent with previous studies (7–9) showing that CYP1B1 protein is specifically localized to tumor cells.

It is well documented that the *CYP1B1* gene is induced by the AhR signaling pathway to which the polycyclic aromatic hydrocarbons bind with high affinity. After ligand binding, the AhR complex is heterodimerized with ARNT and translocated to the nucleus. The AhR/ARNT heterodimer complex activates gene transcription by binding to core recognition motifs contained within the DREs of CYP1B1 (14, 31). In the present study, CYP1B1 mRNA was induced by TCDD and expression of CYP1B1 mRNA was increased in PC-3 cells after treatment with 5-Aza-dC, a DNA methyltransferase inhibitor. In contrast, levels of AhR and ARNT mRNAs were not induced by either TCDD or 5-Aza-dC compared with basal levels. Thus, increased levels of CYP1B1 in these studies were not related to increased expressions of AhR and ARNT. These results suggested that CpG methylation of the *CYP1B1* gene promoter might be important in regulating its expression. On the basis of these findings, we hypothesized that CYP1B1 in human prostate cancer tissue might be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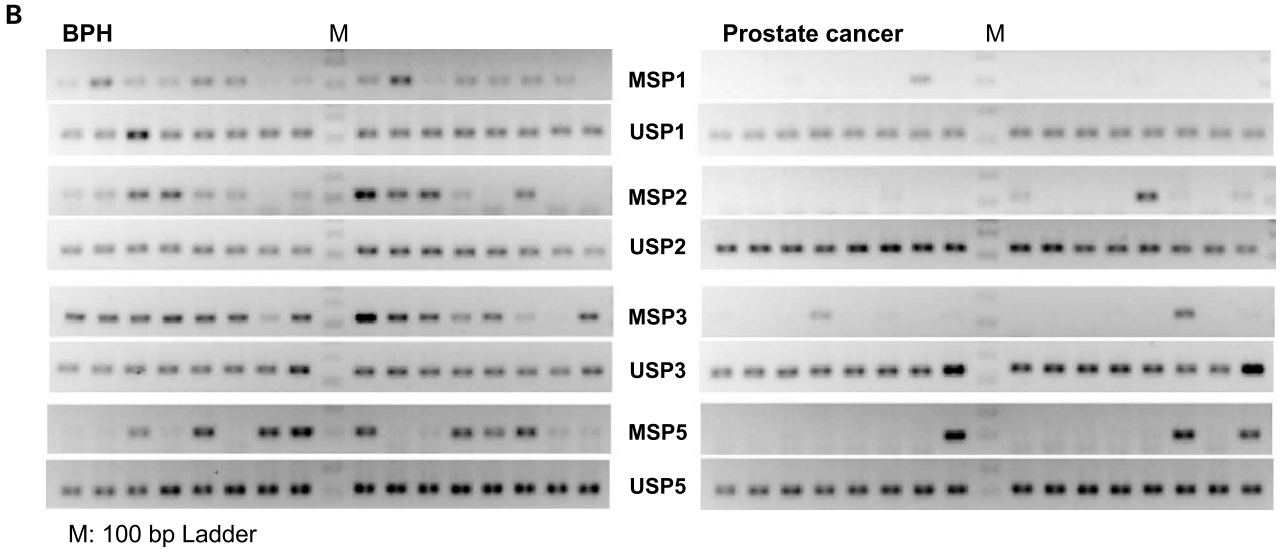
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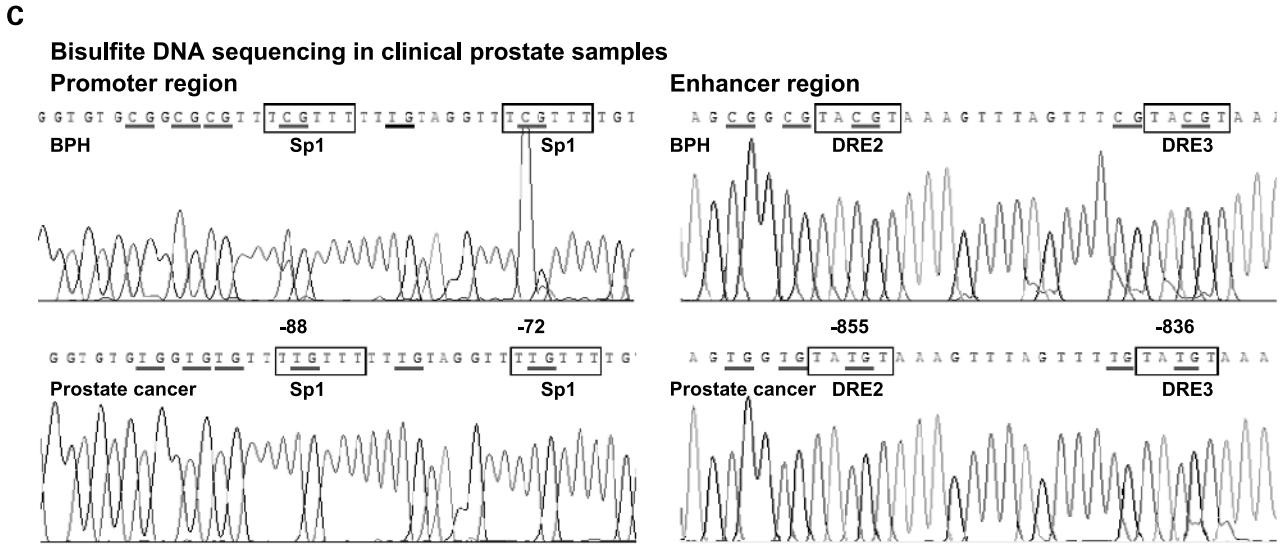
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DRE3

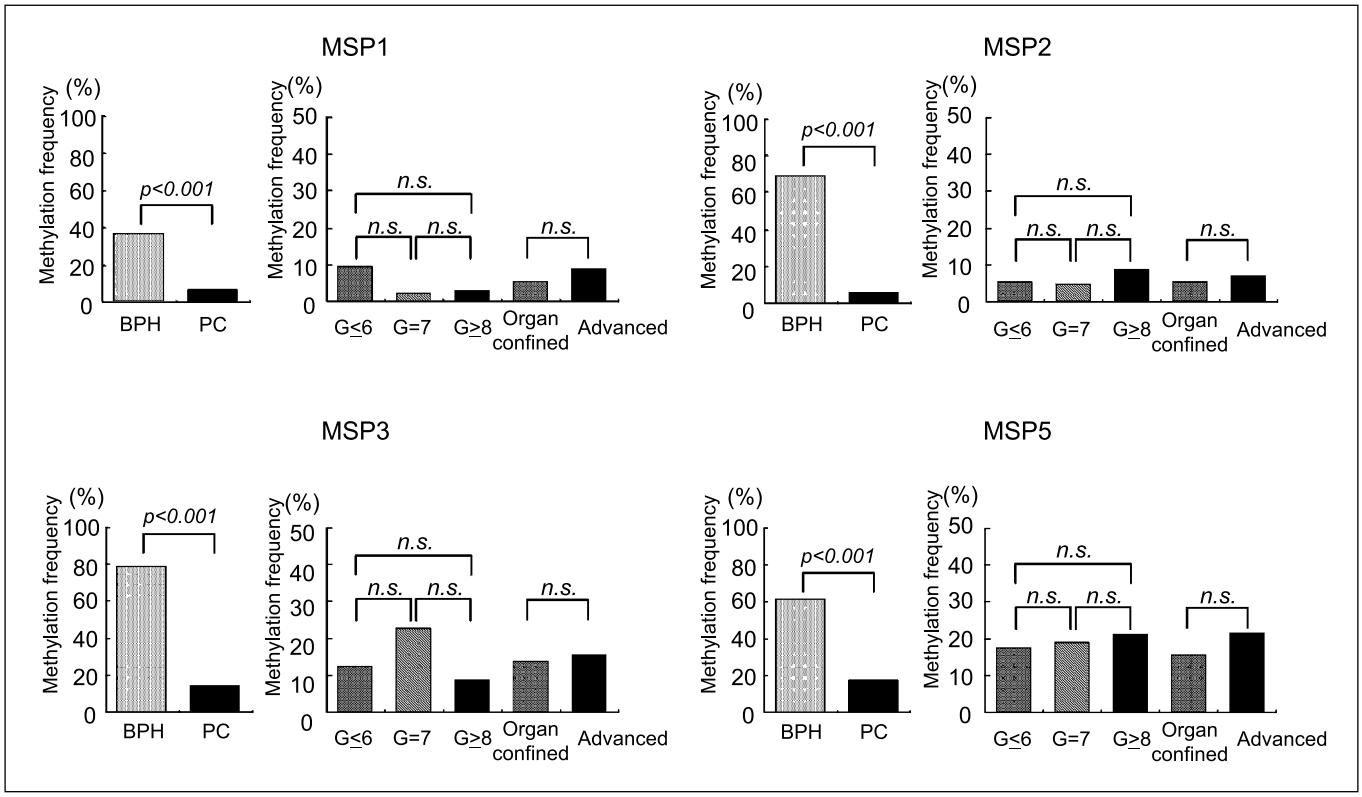


**Fig. 4.** Methylation frequency of the CYP1B1 promoter/enhancer region. Methylation frequency (MSP1, MSP2, MSP3, and MSP5) was significantly higher in BPH samples as compared with prostate cancer samples ($P < 0.001$). In prostate cancer samples, there was no significant difference in methylation associated with Gleason grade and pathologic stage in all enhancer/promoter regions.

regulated by aberrant CpG methylation of the CYP1B1 promoter/enhancer.

The upstream regulatory region of the human *CYP1B1* gene has both basal regulatory sequences and DREs. The core promoter region contains a TATA-like box and two Sp1 binding sites. Full CYP1B1 promoter activity requires both Sp1 motifs located within the core promoter region (12). In addition, important enhancer elements of the human CYP1B1 promoter are located between nt −1,022 and −835 (11, 12), a region that contains several DRE sites [DRE1 (nt −945), DRE2 (nt −855), and DRE3 (nt −836)] that are necessary for TCDD-mediated induction of CYP1B1. Other investigators have reported that DRE2 and DRE3 are particularly important in the TCDD-mediated response (13). Because the CpG dinucleotide exists within the Sp1 binding sites and the core motif of DREs, we investigated the methylation status of the upstream regulatory region in clinical prostate samples. As shown in Fig. 3, the frequency of promoter/enhancer methylation at these four sites was significantly higher in BPH samples compared with prostate cancer samples. Thus, the CYP1B1 promoter/enhancer regions were relatively hypomethylated in prostate cancer tissue compared with BPH tissue. Taken together, these results indicate that increased CYP1B1 expression in human prostate cancer is related to CpG hypomethylation of its promoter/enhancer region.

Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. DNA methylation affects histone modifications and chromatin structure, which, in turn, can alter gene expression. DNA methylation can directly interfere with the binding of specific transcription factors to their recognition sites in their respective promoters such as activator protein 2, c-Myc, and nuclear factor κB (17, 32). In addition, Takahashi et al. (33) have shown that the AhR/ARNT complex was not able to bind to methylated DREs. Our results show that several binding sites containing CpG dinucleotides are methylated in BPH samples. Methylation of

**Fig. 3.** *A*, schematic representation of the promoter/enhancer region of the human *CYP1B1* gene and location of primers. The regions Universal PCR1 and Universal PCR2 cover the functional enhancer of CYP1B1 and were amplified using two different sets of universal primers (Pan1S and Pan1AS; Pan2S and Pan2AS). The universal primers contained no CpG sites within their sequence. Using the universal PCR1 and PCR2 products as templates, a second round of nested PCR (MSP1, MSP2, and MSP3 and corresponding USP1, USP2, and USP3, respectively) was done. The reverse primer of MS1_AS and US1_AS covers the DRE1 (−945) site. The forward primer of MS2_S and US2_S covers DRE3 (−836). The forward primer of MS3_S and US3_S covers DRE2 (−855) and the reverse primer of MS2_AS and US2_AS covers the consensus motif of the SP1 binding site (GGGCGG). On the other hand, Universal PCR5 covers the core promoter region of CYP1B1 and was amplified using different sets of universal primers (Pan5S and Pan5AS). These universal primers also contained no CpG sites within their sequence. Using the universal PCR5 product as templates, a second round of nested PCR (MSP5 and USP5) was done. The reverse primer of MS5_AS and US5_AS covers the SP1 binding site. Primer sequences and expected PCR product sizes are shown Table 2. *B,* typical PCRs (MSP and USP) in BPH and prostate cancer samples are shown. MSP bands are detected in 11 (MSP1), 12 (MSP2), 15 (MSP3), and 8 (MSP5) of the BPH samples, and in only 1 (MSP1), 2 (MSP2), 2 (MSP3), and 3 (MSP5) of the prostate cancer samples. *C*, typical bisulfite-modified DNA sequencing at the enhancer and promoter regions in prostate cancer and BPH samples. Bars under the sequence highlight the CpG sites. In prostate cancer samples, CpG sites of the core promoter region including SP1 binding sites and the enhancer region including DRE2 and DRE3 were not completely methylated whereas in BPH samples, most CpG sites were methylated.

**Table 3.** Logistic regression analysis of CYP1B1 methylation in BPH and prostate cancer

| | Odds ratio* | 95% confidence interval | *P* value† |
|---|---|---|---|
| MSP1 (−) | 7.26 | 3.01-15.92 | <0.001 |
| MSP2 (−) | 40.45 | 16.96-96.46 | <0.001 |
| MSP3 (−) | 25.79 | 12.45-53.44 | <0.001 |
| MSP5 (−) | 8.70 | 4.63-16.39 | <0.001 |

NOTE: Logistic regression analysis showed that MSP-positive patients had a significantly lower risk for prostate cancer as compared with MSP-negative patients.
*Age adjusted.
† BPH versus prostate cancer.

these specific sites may be one of the mechanisms that prevent transcription factor binding in nonmalignant prostate tissue. Additionally, Guo et al. (18) have shown that both increased DNA methyltransferase activity and reduced demethylase activity contribute to the methylation and silencing of gene expression during breast cancer tumor progression. Although further experiments are required to determine the exact role of these types of epigenetic changes, our data suggest that demethylation of the CYP1B1 enhancer/promoter region is an epigenetic event that occurs during the tumor initiation process in prostate cancer.

Metabolic activation of carcinogens by hydroxylation is an important function of CYP1B1 and has been postulated to be a key factor in prostate carcinogenesis (5, 6) because activated carcinogens induce DNA single-strand breaks (34) and mutation (35). For instance, activation of estrogens is capable of inducing ovarian adenocarcinoma in mice (36). Some epidemiologic studies suggest a positive association between exposure to polycyclic aromatic hydrocarbons and increased risk of prostate cancer (2, 37, 38). These findings support a direct role for activated metabolites in prostate carcinogenesis and indicate that CYP1B1 is a key player in the process. In the present study, mRNA expression and the methylation status of CYP1B1 were not associated with prostate cancer clinicopathologic findings. However, expressions of CYP1B1 mRNA and protein, as well as the methylation status of its promoter/enhancer regions, were significantly different when comparing nonmalignant prostate and prostate cancer tissues. Logistic regression analysis showed that MSP-positive patients had a significantly lower risk for prostate cancer as compared with MSP-negative patients. Although expression levels of CYP1B1 mRNA are low, it is detected in normal regions. Therefore, normal tissue may contain CYP1B1, which is capable of metabolically activating compounds suspected of being prostate carcinogens (10). Thus, hypomethylation of the *CYP1B1* gene may play an important role in the initiation of prostate tumors rather than in their progression. Because expression of CYP1B1 is regulated by the methylation of its promoter/enhancer, this region may be useful as a target for anticancer drugs and in preventive medicine.

In conclusion, this is the first study to demonstrate that increased CYP1B1 expression in clinical prostate cancer tissues is caused by promoter/enhancer hypomethylation of the *CYP1B1* gene.

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