Epigenetic Regulation of Vitamin D 24-Hydroxylase/CYP24A1 in Human Prostate Cancer

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Abstract
Calcitriol, a regulator of calcium homeostasis with antitumor properties, is degraded by the product of the CYP24A1 gene, which is downregulated in human prostate cancer by unknown mechanisms. We found that CYP24A1 expression is inversely correlated with promoter DNA methylation in prostate cancer cell lines. Treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (DAC) activates CYP24A1 expression in prostate cancer cells. In vitro methylation of the CYP24A1 promoter represses its promoter activity. Furthermore, inhibition of histone deacetylases by trichostatin A (TSA) enhances the expression of CYP24A1 in prostate cancer cells. Quantitative chromatin immunoprecipitation-PCR (ChIP-qPCR) reveals that specific histone modifications are associated with the CYP24A1 promoter region. Treatment with TSA increases H3K9ac and H3K4me2 and simultaneously decreases H3K9me2 at the CYP24A1 promoter. ChIP-qPCR assay reveals that treatment with DAC and TSA increases the recruitment of vitamin D receptor to the CYP24A1 promoter. Reverse transcriptase-PCR analysis of paired human prostate samples revealed that CYP24A1 expression is downregulated in prostate malignant lesions compared with adjacent histologically benign lesions. Bisulfite pyrosequencing shows that CYP24A1 gene is hypermethylated in malignant lesions compared with matched benign lesions. Our findings indicate that repression of CYP24A1 gene expression in human prostate cancer cells is mediated in part by promoter DNA methylation and repressive histone modifications. Cancer Res; 70(14): 5953-62. ©2010 AACR.

Introduction
1α,25-Dihydroxycholecalciferol (calcitriol), the active form of vitamin D3, plays a major role in regulating calcium homeostasis and bone mineralization (1–3). Calcitriol also modulates cellular proliferation and differentiation in a variety of cell types (4). Calcitriol promotes cell cycle arrest and induces cell apoptosis (5). The cytochrome P450 enzyme 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase), encoded by the CYP24A1 gene, mediates 24-hydroxylation of 1α,25(OH)2D3 to the much less active vitamin D metabolites including 1α,24,25(OH)3D3 and its biliary excretory product calcitriolic acid (6, 7). The important role of CYP24A1 in maintaining vitamin D homeostasis and antitumor effects is supported by several lines of evidence: (a) CYP24A1 overexpression transgenic rats show low vitamin D3 levels (8, 9); (b) the rate of serum calcitriol clearance after calcitriol administration is delayed in CYP24A1-null mouse (10, 11); and (c) pharmacologic inhibition of CYP24A1 in combination with calcitriol enhances antitumor effects in human prostate cancer and other tumor types (12–15).

Similar to CYP27B1, a vitamin D-activating enzyme that is aberrantly expressed in tumors (16), CYP24A1 is dysregulated in a wide range of tumors. Overexpression of CYP24A1 has been reported in several tumors including colon carcinoma (17, 18), ovarian cancer (17, 19), cervical carcinoma (19), lung cancer (17, 20), and cutaneous basal cell and squamous cell carcinoma (21, 22). The overexpression of CYP24A1 is associated with poor prognosis of some human cancers (17). Upregulated CYP24A1 expression may counteract calcitriol antiproliferative activity, presumably by decreasing calcitriol levels. However, microarray expression studies published in ONCOMINE indicate that CYP24A1 is downregulated in prostate cancer (23). The mechanism(s) underlying the dysregulation of CYP24A1 in tumors is not clear. Recently, Novakovic and colleagues showed tissue-specific CYP24A1 promoter methylation in normal human tissues (24). The CYP24A1 gene is methylated in human placenta; no methylation was detected in somatic human tissues (24). A lack of expression of CYP24A1 has also been directly associated with DNA methylation of the upstream promoter and the first exon of the CYP24A1 gene in mouse tumor-derived endothelial cells and in rat osteoblastic ROS17/2.8 cells (12, 25). Differential
methylation of CYP24A1 gene contributed to selective anti-proliferative effects of calcitriol treatment on tumor-derived endothelial cells compared with Matrigel-derived endothelial cells (12).

The above data suggest that epigenetic mechanisms play a key role in regulating CYP24A1 expression in normal human tissues. However, little is known about the mechanism(s) underlying the regulation of CYP24A1 expression in human tumors. Given the importance of CYP24A1 in regulating 1α,25(OH)2D3 levels, we investigated the role of epigenetic regulation of CYP24A1 in human prostate cancer cell lines and primary prostate tumors.

Materials and Methods

Human tissues and cells

Human prostate cancer cell lines DU145, LNCaP, and PC3 were obtained from the American Type Culture Collection and maintained in our laboratory. Human DNA and RNA from 30 paired human prostate benign and primary malignant lesions were obtained from Department of Pathology, Roswell Park Cancer Institute, and approved by the Institutional Review Board.

Drug treatments

LNCaP, PC3, and DU145 cells were seeded at 1 × 10^5 cells in a six-well plate overnight. For dose-response experiments, cells were treated with 5-aza-2′-deoxycytidine (DAC) at 0.1, 0.25, 0.5, 1.0, and 2.0 μmol/L concentrations for 3 days, followed by the addition of calcitriol (100 nmol/L) for an additional 24 hours, and cells were harvested. Cells were treated with tri-chostatin A (TSA) at 50, 100, 200, 300, and 400 nmol/L for 8 hours, followed by the addition of calcitriol (100 nmol/L) for an additional 24 hours, and cells were harvested. For DAC and TSA combination treatments, cells were treated with 1 μmol/L DAC for 3 days, followed by the addition of TSA and calcitriol for an additional 24 hours, and cells were harvested.

Quantitative reverse transcriptase-PCR

Expression of CYP24A1 mRNA was assessed by quantitative reverse transcriptase-PCR (RT-PCR) using the CYP24A1 TaqMan Gene Expression Assay (Hs00167999_m1, Applied Biosystems). The amplification conditions for both CYP24A1 and GAPDH were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds on the 7300 real-time PCR system (Applied Biosystems). Gene expression of CYP24A1 was normalized to the GAPDH and all samples were analyzed in triplicate. Statistical analysis with Wilcoxon signed-rank test was performed to compare the expression levels of CYP24A1 in benign and malignant samples.

Bisulfite DNA sequencing

Genomic DNA was isolated using the genomic DNA isolation kit (Qiagen). DNA (1 μg) was converted with EZ DNA Methyltransferase Kit (Zymo Research Corporation). Primer design was accomplished using Methprimer (26). Bisulfite sequencing primers [region 1 (−500 to +6), 5′-AAAATTTATTTTATTAGTTGGGG-3′ and 5′-TCTCCATATCC-TACCCCCAACAC-3′; region 2 (−17 to +609), 5′-TTTTTTGGGTATAGGAATATGGAGAG-3′ and 5′-CCCCAACATAACACTAAAAAC-3′] were used to amplify the CpG islands in the CYP24A1 promoter region. PCR included an initial incubation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle of 72°C for 10 minutes. The PCR products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen) for sequencing. A total of 10 clones from each sample were sequenced at the Roswell Park Cancer Institute DNA sequencing core facility, and the methylation status for each CpG site was determined by assessing the presence of T (unmethylated) versus C (methylated) at each CpG site.

Bisulfite DNA pyrosequencing

The methylation status of two regions of the CYP24A1 promoter in human prostate tissue samples was analyzed by bisulfite pyrosequencing. For the first region, the primers used were −499F (5′-AAAATTTATTTTATTAGTTGGGG-3′) and −244R (Biotin-5′-AAATACCCCAAAATTATAC-3′) for amplification and primer 5′-TATGTAAGGGGAAGGG-3′ was used for sequencing. For the second region, the primers used for amplification were +66F (5′-GGTGTATTCTTGGAGGAGG-3′) and +301R (Biotin-5′-CAATTTACCTATAAAAACAC-CACTATAATATAT-3′) and the sequencing primer was 5′-TATGTAAGGGGAAGGG-3′. PCR cycling conditions were an initial incubation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C (for region 1) and 51°C (for region 2) for 30 seconds, and 72°C for 30 seconds. Pyrosequencing was accomplished in duplicate using the PSQ 96 Pyrosequencing System (Biotage) and was performed two times.

CYP24A1 activity assay

Cells were treated with DAC for 72 hours and/or TSA for 8 hours, followed by 100 nmol/L calcitriol for 48 hours, and were washed with 1% bovine serum albumin (BSA)-PBS twice. Then, cells (1 × 10^6) were incubated with 25(OH)D_3 (1 μmol/L) in 2 mL of 1% BSA medium for 12 hours. 25(OH)D_3 metabolites from cells and medium were extracted by liquid-liquid partition with 8 mL of chloroform/methanol (3:1). Before the extraction, 20 μL of EB1089 (10 μg/mL) were added as an internal standard. Dried sample extracts were dissolved in 100 μL of high-performance liquid chromatography (HPLC) mobile phase, hexane/isopropanol/methanol in 90:5:5 (v/v/v). Vitamin D_3 metabolites and internal standard in 50 μL of the extract were separated by HPLC on a 4.6 × 250-mm Zorbax SIL column (Agilent Technologies, Inc.) using HPLC mobile phase at a flow rate of 2 mL/min. Metabolites were monitored at λ_228 nm and identified based on retention time and Diode array collected vitamin D_3 chromophore spectral characteristics (UV_max = λ_265, UV_min = λ_228; UV_max/UV_min = 1.75). CYP24A1 activity was calculated as the area ratio of 24,25-(OH)_2D_3 recovered 25-(OH)D_3 + 24,25-(OH)_2D_3/12 h/10^6 cells.
In vitro CYP24A1 promoter methylation assay

A 587-bp region of the human CYP24A1 promoter gene that was shown (27, 28) to confer vitamin D responsiveness was amplified using primers Xho-CYP24−556F (5′-GCTAGCCGCAGAAAGCCAATTCCTC-3′) and NheICYP24+40R (5′-GCAAGCTCGAGAGATGCTGGCCTGCGT-3′) with PfuUltra high-fidelity DNA polymerase (Stratagene). The Xho- and NheI-digested amplicon was cloned into the promoterless luciferase expression vector pGL4.21 (Promega) to produce the plasmid pGL4.21/547/+40. Methylation of the CYP24A1 promoter region was done as described (29). Briefly, the fragments were methylated in vitro with SssI, HpaII, and HhaI methylases (New England Biolabs), or no enzyme (mock), according to the manufacturer’s instructions. Methylation efficiency was confirmed using HhaI, HpaII, and McrBC digestions (New England Biolabs). Methylated or mock-methylated fragments were ligated back into pGL4.21 vector (Promega). PC3 cells were transfected with 100 ng of the differentially methylated construct along with 20 ng of a Renilla luciferase control construct (Promega). All transfections were carried out in triplicate wells of 96-well plates. Twenty-four hours after transfection, cells were treated with calcitriol (100 nmol/L) for an additional 24 hours and harvested, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Statistical analysis with Student’s t test on luciferase values was performed to compare the CYP24A1 promoter activities in unmethylated and methylated CYP24A1 promoter constructs. The experiment was repeated twice to confirm the reproducibility of results.

Quantitative chromatin immunoprecipitation-PCR

Quantitative chromatin immunoprecipitation-PCR (ChiP-qPCR) was performed essentially as described by Väisänen and colleagues (30). Briefly, cells were treated with DAC (1 μmol/L), TSA (300 nmol/L), or both agents. One hour after adding calcitriol, cells were fixed to cross-link nuclear protein to DNA by adding formaldehyde as described previously (30). Chromatin was sheared to an average length of 500 bp. The chromatin was precipitated with the indicated antibodies. Antibodies against histone H3 dimethylated lysine 9 (H3K9me2), dimethylated lysine 4 (H3K4me2), and acetylated lysine 9 (H3K9ac) were purchased from Upstate Biotechnologies; an antibody against vitamin D receptor (VDR; sc-1008x) was obtained from Santa Cruz Biotechnologies. ChiP-qPCR primers for the CYP24A1 promoter region 1 and region 2 were −292F (5′-AGCACACCCGGT-GAACTC-3′) and −152R: (5′-TGGAAGGAGGATGGAGTCAG-3′); +130F (5′-TTCAAGAGGTCCCCAGACAC-3′) and +333R.
(5′-AGTCGGGGCTTAACGATTCT-3′). ChIP-qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with the following cycling parameters: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Copy number was determined using a standard curve containing serial dilutions (10⁻⁷–10⁰ copies) of the CYP24A1 DNA amplicon. Samples were run in triplicate and data were normalized to 5% input DNA amplifications after subtraction of signals obtained from antibody isotype control. ChIP was repeated twice to confirm the reproducibility of results.

Results

Correlation of CYP24A1 expression in human prostate cell lines with promoter DNA methylation status

To investigate the mechanism(s) regulating CYP24A1 mRNA expression in prostate cancer, we analyzed LNCaP, PC3, and DU145 cells, which express variable levels of CYP24A1. Quantitative RT-PCR showed that LNCaP and PC3 cells express CYP24A1 weakly in the presence of calcitriol, whereas DU145 cells express CYP24A1 at high baseline and show further induction following calcitriol treatment (Fig. 1A). We further tested whether CpG island methylation could account for differential CYP24A1 expression in the prostate cell lines. Analysis of the human CYP24A1 gene by Webgene indicated that it contains two CpG islands (580 and 1,232 bp, respectively) that span the transcriptional start site (Fig. 1B; ref. 31). The 5′ CpG island of the CYP24A1 gene encompasses the promoter region and contains two known VDR response elements (VDRE): the proximal element VDRE (VDREp; −172/−143) and the distal element VDRE (VDRED; −293/−273; Fig. 1B; ref. 27). To examine whether CYP24A1 is differentially methylated in these cell lines, we conducted bisulfite sequencing on 506-bp region 1 and 627-bp region 2 of the CYP24A1 5′-CpG island promoter (Fig. 1B). This assay revealed that the overall methylation of CYP24A1 promoter regions in LNCaP and PC3 cells is 24% and 50%, respectively. The hypermethylation is particularly evident in region 2 of the CYP24A1 gene. In contrast, the CYP24A1 5′-CpG island was completely unmethylated in DU145 cells, which is consistent with constitutive CYP24A1 expression in this cell line (Fig. 1C). These data suggest that promoter DNA methylation status may regulate differential CYP24A1 expression in prostate cancer cells.

Activation of CYP24A1 gene expression by epigenetic modulatory drugs in prostate cancer cells

To determine the role CYP24A1 gene methylation in CYP24A1 expression, we treated LNCaP and PC3 cells with DAC, a DNA methyltransferase inhibitor. Quantitative RT-PCR revealed that DAC elicited a dose-dependent induction of CYP24A1 expression in LNCaP and PC3 cells that was dependent on calcitriol treatment (Fig. 2A). To confirm the demethylation effect of DAC, bisulfite sequencing was done on treated cell lines. Overall methylation of the CYP24A1 promoter in LNCaP and PC3 cells was reduced from 24% to 5% and from 50% to 13%, respectively (Figs. 1C and 2B).
To determine whether histone deacetylation is also involved in repression of \textit{CYP24A1} expression, we treated LNCaP and PC3 cells with TSA, a histone deacetylase inhibitor. Quantitative RT-PCR revealed that TSA elicited a dose-dependent induction of \textit{CYP24A1} expression in LNCaP and PC3 cells that was dependent on calcitriol treatment (Fig. 3A). Combination treatment with DAC and TSA induced a significant increase in \textit{CYP24A1} mRNA and protein expression in LNCaP and PC3 cells (Fig. 3B, top and middle; Supplementary Fig. S1). In contrast, DU145, which has an unmethylated promoter, showed no increase in \textit{CYP24A1} expression with DAC treatment but a robust effect with TSA treatment (Fig. 3B, bottom). The slightly less \textit{CYP24A1} expression with combination treatment of DAC and TSA compared with TSA alone could be due to the additive toxicity of this approach in DU145. To test whether the increased \textit{CYP24A1} expression by epigenetic modulators is associated with increased \textit{CYP24A1} activity, we performed enzyme activity assay by HPLC. Increased \textit{CYP24A1} enzyme activity was observed in LNCaP and PC3 cells treated with TSA (Fig. 3C). In addition, there was no significant increase in enzyme activity in DU145 cells treated with DAC, which has hypomethylated \textit{CYP24A1} promoter (Fig. 3C). In all cell lines, combination treatment of DAC and TSA causes a significant increase in \textit{CYP24A1} activity (Fig. 3C). However, it is not clear why the differences in the enzyme activity between DU145 cells and the other two cell lines are less than the relative differences at the mRNA expression level. We speculate that the enzyme activity may be affected by other endogenous factors and that the mRNA and protein expression of \textit{CYP24A1} may closely, but not exactly, parallel.

Repression of human \textit{CYP24A1} gene promoter activity by DNA methylation

To examine whether DNA methylation directly represses \textit{CYP24A1} promoter activity, we cloned a 587-bp fragment of the 5' end of \textit{CYP24A1}, which contains 46 CpG sites, into a luciferase reporter construct. We methylated the cloned insert (29) using \textit{Sss}I methylase (methylation of 46 CpGs), \textit{Hpa}II methylase (methylation of 14 CpGs), and \textit{Hha}I methylase (methylation of 4 CpGs). \textit{Sss}I methylase methylates all 5'-CpG-3' sites, \textit{Hpa}II methylase methylates only the CpG within the sequence 5'-CCGG-3', and \textit{Hha}I methylase methylates only the CpG within the sequence 5'-GCGC-3'. Proper methylation of inserts was confirmed by digesting with the restriction enzymes \textit{Mcr}BC, \textit{Hpa}II, and \textit{Hha}I (Fig. 4A).
CYP24A1 promoter activity was tested by transfection of the luciferase construct containing methylated CYP24A1 promoter. Prior methylation with SssI, HpaII, or HhaI methylases in PC3 cells repressed CYP24A1 promoter activity (Fig. 4B). Repression was methylation dose dependent in that SssI, which methylates all CpG sites, showed the greatest repression, and HhaI, which methylates four CpG sites, showed the least repression on CYP24A1 promoter activity (Fig. 4B).

Histone modifications associated with the CYP24A1 promoter region

The association of CYP24A1 promoter DNA methylation and gene silencing in relation to histone modifications has not been investigated previously. To clarify the role of histone modifications in the regulation of the CYP24A1 gene, we examined the active histone marks H3K4me2 and H3K9ac and the repressive histone mark H3K9me2 in the chromatin associated with the CYP24A1 promoter region using ChIP-qPCR. The results showed similar histone acetylation patterns and H3 methylation levels in LNCaP and PC3 cells with relative high H3K9me2 compared with DU145 cells (Fig. 5A). In contrast, the active histone markers (H3K4me2 and H3K9ac) were highest in DU145 cells, as expected (Fig. 5A). We also examined whether treatment with DAC and/or TSA changed the profile of histone modifications in the CYP24A1 promoter. The ChIP-qPCR assay revealed that TSA treatment induced increases of H3K9ac and H3K4me2 and a decrease of H3K9me2 in PC3 cells, giving rise to an active pattern of histone modifications following treatment (Fig. 5B). Similar histone modification changes were also observed in LNCaP cells after treatment (data not shown).

Increased recruitment of VDR to the CYP24A1 promoter in PC3 cells treated with epigenetic modulatory drugs

We next examined whether these epigenetic changes affect the binding of the VDR to the CYP24A1 promoter region. ChIP-qPCR analysis using a VDR-specific antibody revealed that there was significantly increased binding of VDR to the CYP24A1 promoter in PC3 cells on treatment with DAC and TSA, in addition to calcitriol (Fig. 5C). These data provide functional evidence that decreased VDR binding to the CYP24A1 promoter in PC3 cells, due to hypermethylation and a repressive histone modifications, contributes to the suppression of CYP24A1 expression in prostate cancer cells.

Expression and methylation of CYP24A1 in primary normal and cancerous human prostate tissues

We analyzed CYP24A1 expression in human normal prostate, benign prostatic hyperplasia, prostate carcinoma, and metastatic prostate cancer using microarray expression studies published in ONCOMINE (23). CYP24A1 expression was significantly lower in prostate cancer compared with normal prostate in three of four (P < 0.01) microarray expression studies (Fig. 6A). However, VDR expression was not downregulated in studies published in ONCOMINE (data not shown). We obtained 30 matched human prostate benign and malignant prostate samples. Gleason scores were 3 + 3 (n = 6), 3 + 4 (n = 15), 4 + 3 (n = 7), 4 + 5 (n = 1), and 5 + 4 (n = 1). Quantitative RT-PCR revealed that CYP24A1 expression was significantly downregulated in prostate malignant lesions compared with benign lesions (P = 0.0336; Fig. 6B and C). Bisulfite pyrosequencing assays for the CYP24A1 gene were designed to span VRDEd and VRDEp (21) and a putative regulatory sequence located in exon 1 within the associated CpG island. Pyrosequencing revealed that the CYP24A1 gene was significantly hypermethylated in malignant lesions as compared with matched benign lesions (Fig. 6D). There was no correlation of the level of CYP24A1 expression and methylation with Gleason score. To determine whether malignant samples with decreased CYP24A1 expression correlated with CYP24A1 hypermethylation, we built a 2 × 2 contingency table by dividing the 30 samples based on the
CYP24A1 expression change (≥1.5-fold down versus other) and methylation change (≥2.0-fold up versus other). Ten of the 15 samples with CYP24A1 downregulation in malignant lesions have increased CYP24A1 DNA methylation, whereas only 4 of the other 15 samples have increased CYP24A1 DNA methylation. Fisher’s exact test shows that decreased CYP24A1 expression is significantly associated with increased CYP24A1 DNA methylation (P < 0.05).

Discussion

In this study, we showed that both DNA hypermethylation and repressive histone modifications at the CYP24A1 promoter were shown to repress CYP24A1 gene expression. Epigenetic repression also impairs the recruitment of VDR to the CYP24A1 regulatory regions, providing a plausible mechanism for how the repressive epigenetic markers exert their effects on CYP24A1 expression.

This study indicates a direct molecular relationship between 5’CpG island methylation and downregulation of CYP24A1 expression in human prostate cancer. This conclusion is supported by the following observations. First, the presence of a classic 5’CpG island in the CYP24A1 promoter suggests a role for DNA methylation–based regulation. Second, the CYP24A1 promoter is hypermethylated in human prostate cell lines that weakly express CYP24A1 (LNCaP and PC3), whereas it is completely unmethylated in a human prostate cell line with high CYP24A1 expression (DU145). Third, treatment of low CYP24A1 expression prostate cell lines with DNA methyltransferase inhibitor induces CYP24A1 expression in a dose-dependent manner, which coincides with DNA hypomethylation of the CYP24A1 promoter. However, no increased induction of CYP24A1 expression was observed with DAC treatment in DU145, which has hypomethylated CYP24A1. Furthermore, in vitro methylation of the CYP24A1 promoter directly represses its promoter activity. Finally, ChIP-qPCR shows that treatment of PC3 cells with DAC and/or TSA increases the recruitment of VDR to the CYP24A1 promoter. Data obtained from prostate cell lines were further supported by the analysis of human matched prostate benign and malignant lesion samples. The expression of CYP24A1 was significantly downregulated in malignant prostate tissues comparing with benign prostate tissues. This finding was consistent with the data obtained from ONCOMINE (23). We further show that the decreased expression of CYP24A1 in malignant tissues was significantly associated with hypermethylation of the CYP24A1 promoter, showing a relationship between 5’-CpG island methylation and reduced CYP24A1 expression.
in prostate tumor specimens. These observations suggest that DNA methylation may serve as a mechanism for controlling CYP24A1 expression in human cancers.

In the human CYP24A1 promoter, VDREs are located within the hypermethylated regions. Therefore, hypermethylation of these regions identified in this study could lead to a chromatin state in which VDR is prevented from binding to the VDREs, which results in transcription silencing of CYP24A1. This model is supported by our observations that recruited VDR on the CYP24A1 promoter is increased in PC3 cells treated with DAC or TSA. H3K9me2, a repressive histone modification, was high in LNCaP and PC3 cells, which have low CYP24A1 expression. In contrast, low H3K9me2 and high H3K9ac and H3K4me2 in DU145 cells, which have the highest CYP24A1 expression, suggest an association of an open chromatin structure with active gene expression (32, 33). Notably, we found that treatment of CYP24A1 hypermethylated prostate cells with histone deacetylase inhibitor and DNA methyltransferase inhibitor induces CYP24A1 expression in a dose-dependent manner, accompanied by an active pattern of histone modifications. These results are consistent with the hypothesis that aberrant DNA methylation and repressive histone modifications function in combination to silence important tumor suppressor genes in human cancers (34, 35). Studies have shown that hypermethylated promoters often involve recruitment of methyl binding domain proteins and histone-modifying enzymes such as histone deacetylase (36–39). Binding of these proteins leads to a chromatin closed state and prevents binding of transcription factors like VDR to target promoters. Although evidence in the current study and previously reports (12, 24, 25, 40) supports promoter DNA hypermethylation–mediated CYP24A1 suppression, quantitative RT-PCR analysis of human prostate tissues reveals that the level of CYP24A1 methylation does not entirely account for strong inverse correlation with the level of CYP24A1 expression in human prostate tissues (Fig. 6C and D). Our results suggest that histone code modifications could provide an alternative regulatory mechanism that affects CYP24A1 gene expression in vitro and in vivo (41–43).

Analysis of the CYP24A1 gene exon 1 region by NUBIScan (44) identified a putative VDRE. We found that treatment with DAC or TSA increases the recruitment of VDR to this putative VDRE region, which is methylated in LNCaP and PC3 cells (Figs. 1C and 5C). These results suggest that the
region of downstream of the transcriptional start site may be involved in the recruitment of CYP24A1 regulators. Further studies are in progress to investigate this possibility.

Taken together, these data define promoter DNA methylation and altered histone codes as a key mechanism controlling CYP24A1 expression in human prostate cancers. Information obtained from this study has implications for developing strategies to optimize the vitamin D antitumor activity. Based on the apparent role of CYP24A1 as a key enzyme to degrade the activity of vitamin D, one could envision that direct inhibition of CYP24A1 function could enhance the antitumor activity of vitamin D (14, 45, 46). CYP24A1 expression is heterogeneous in prostate cancer (Fig. 6B). Prostate cancer patients with high expression of CYP24A1 may not be as responsive to vitamin D analog therapy, presumably due to the ability of CYP24A1 in prostate tumors to decrease 1,25(OH)2D3 levels. Decreased expression of CYP24A1 in some malignant prostate tissue is caused, in part, by methylation and histone modification associated with the CYP24A1 promoter. Furthermore, Chung and colleagues reported that induction of CYP24a1 by hypomethylating agents may attenuate responses to vitamin D in mouse tumor-derived endothelial cells that do not express CYP24 due to epigenetic silencing (12). Therefore, the use of DNA methyltransferase inhibitors and histone deacetylase inhibitors in vitamin D-based therapy for prostate cancer should be avoided due to the reactivation of CYP24A1 expression, potentially rendering the tumor less sensitive to vitamin D. In addition, decreased activity of extrarenal 1α-hydroxylase in prostate cancer may be also responsible for the increased proliferation of the cancer cells (47). In conclusion, the data obtained from this study contribute to our understanding of the mechanisms regulating CYP24A1 gene expression in malignant and normal human tissues and facilitate the optimization of antitumor therapy of vitamin D in combination with repression of CYP24A1 in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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