CYP24A1 and CYP27B1 Polymorphisms Modulate Vitamin D Metabolism in Colon Cancer Cells

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Abstract

Vitamin D is a well-studied agent for cancer chemoprevention and treatment. Its chief circulating metabolite, 25-hydroxyvitamin D, is converted into the active hormone 1,25-dihydroxyvitamin D (1,25D) by the cytochrome P450 enzyme CYP27B1 in kidney and other tissues. 1,25D is then deactivated by CYP24A1 and ultimately catabolized. Colorectal carcinoma cells express CYP27B1 and CYP24A1 that locally regulate 1,25D with potential implications for its impact on carcinogenesis. While 1,25D inhibits cancer growth, the effects of polymorphic variations in genes encoding proteins involved in 1,25D homeostasis are poorly understood. Using an RXR-VDR mammalian two-hybrid (M2H) biologic assay system, we measured vitamin D metabolite uptake and activation of the vitamin D receptor (VDR) pathway in colon cancer cells that expressed one of five CYP27B1 single-nucleotide polymorphisms (SNP) or four CYP24A1 SNPs. Compared with the wild-type control, four of five CYP27B1 SNPs reduced enzymatic activity, whereas one (V166L) increased activity. For CYP24A1, all tested SNPs reduced enzyme activity. Quantitative real-time PCR analyses supported the results of M2H experiments. The observed SNP-directed variation in CYP functionality indicated that vitamin D homeostasis is complex and may be influenced by genetic factors. A comprehensive understanding of 1,25D metabolism may allow for a more personalized approach toward treating vitamin D–related disorders and evaluating risk for carcinogenesis.

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Introduction

Vitamin D and two of its metabolites, 25-hydroxyvitamin D (25D) and 1,25-dihydroxyvitamin D (1,25D), have been studied intensively in relation to several disease outcomes, including cancer, using both population-based and basic science approaches. A reduction in risk for colorectal cancer has been consistently associated with higher circulating concentrations of 25D (1), which is the biomarker of vitamin D most commonly used in epidemiologic studies. Nonetheless, it is 1,25D that is considered to be the active molecule, as it binds to the vitamin D receptor (VDR) and exhibits regulatory effects on more than 1,000 genes (2, 3). The activity of 1,25D is particularly relevant with respect to potential chemopreventive or chemotherapeutic actions of vitamin D metabolites (3). Effects of 1,25D on cancer cell lines include inhibition of cell proliferation and promotion of differentiation (4–7).

Colon carcinoma (Caco-2) cells have been shown to display 25-hydroxyvitamin D3 1-alpha hydroxylase (CYP27B1) activity (8). This observation is of interest because CYP27B1 is responsible for the hydroxylation of 25D to form 1,25D, and as such, it indicates that colorectal cells can independently synthesize the active hormone. Increased functional activity of this enzyme in the colon has the potential to elevate 1,25D levels at the local level (3); therefore, a thorough understanding of the cellular-level regulation of 1,25D metabolism through CYP27B1 is crucial to understanding tissue-specific hormone effects.

Another key enzyme influencing circulating and cellular 1,25D concentrations is CYP24A1, which is the primary molecule involved in 1,25D catabolism (2, 3, 9). CYP24A1 hydroxylates 1,25D at the C24 position to form 1,24,25D (9), a metabolite that is less active than 1,25D in activating VDR, and which in turn is catabolized further to excretion products (9). A less active variant of CYP24A1 would likely result in greater concentrations of 1,25D at the tissue level.

Genetic polymorphisms in both CYP27B1 and CYP24A1 have been identified, and some work has shown these to be significantly related to circulating concentrations of vitamin D metabolites (10) or to risk for colorectal cancer (11) in population studies, although these associations are inconsistent (12, 13). Nonetheless, the functional effects of genetic variation in these enzymes is unclear (12) and could result in differences in the amount of 1,25D available in colon cells for antineoplastic activity. Therefore, the objective of the current work was to elucidate the functional effects of selected
single-nucleotide polymorphisms (SNP) in CYP27B1 and CYP24A1 using site-directed mutagenesis in human colon cancer cells.

Materials and Methods

Cell culture

HCT-116 (human colorectal adenocarcinoma) cells were obtained from the American Type Culture Collection (ATCC) biological resource center and grown at 37°C in a humidified atmosphere of 5% CO2. Subconfluent cells were transfected in Costar 24-well polystyrene plates from Corning Inc. using Express-In Transfection Reagent supplied by Thermo Scientific. Cells were plated at a density of 60,000 cells/well approximately 24 hours before transfection in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

The UMR-106 (rat osteosarcoma) cell line was obtained from ATCC and cultured as recommended. Culture media, FBS, and penicillin–streptomycin stocks were obtained from Gibco (Invitrogen Corp.). Crystalline 1,25-dihydroxyvitamin D3 (1,25D) was obtained from Axxora. Cells were cultured at <80% confluence between passages 10 and 30 in 6-well plates (9.4 cm²), seeded at either 175×10⁴ or 200×10⁴/well, and treated with 1,25D for the indicated times, usually 4 or 24 hours.

Site-directed mutagenesis

Synthesis of CYP27B1 and CYP24A1 point mutants within full-length wild-type (WT) pSG5-CYP27B1 and pSG5-CYP24A1 vectors was accomplished using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the protocol of the manufacturer. All mutants were confirmed by DNA sequencing.

Transient transfection and hormone treatment

For the mammalian 2-hybrid (M2H) assays, human RXRα was cloned into pCMV-BD and human VDR was cloned into pCMV-AD (14). The transfection procedure (Fig. 1) was adapted from the manufacturer’s protocol. Briefly, each well received 2 μL Express-In, along with 25 ng pCMV-BD-hRXRα (bait) and 25 ng pCMV-AD-hVDR (prey), plus 200 ng pFR-Luc (reporter gene) and 20 ng pRL-NULL (Renilla reniformis luciferase used to monitor transfection efficiency). All hormone treatments were consistently conducted in 10% FBS-containing medium.

For the experiments to evaluate assay sensitivity and substrate specificity (Fig. 2), cells were additionally transfected with either pSG5-empty or pSG5-CYP27B1 and incubated for approximately 24 hours in minimal DMEM without FBS, penicillin, or streptomycin. The following day, cells were treated with either ethanol or 1,25D for 4 or 24 hours. The following day, cells were treated with either 100 nmol/L 25D or 1 nmol/L 1,25D and incubated for approximately 15 hours before transfection in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

For the experiments to evaluate assay sensitivity and substrate specificity (Fig. 2), cells were additionally transfected with either pSG5-empty or pSG5-CYP27B1 and incubated for approximately 24 hours in minimal DMEM without FBS, penicillin, or streptomycin. The following day, cells were treated with ethanol, 1 nmol/L, 1.25D, 100 nmol/L, 24D, or 10 to 200 nmol/L 25D and incubated for approximately 15 hours before lysis and luciferase assay. For the CYP27B1 SNP analysis experiments (Fig. 3), cells were transfected with either pSG5-empty, human WT pSG5-CYP27B1, or one of the following CYP27B1 SNPs: R107H (rs28934604), A129T (rs85915677), V166L (rs8176344), S356N (rs13377933), or V374A (rs2229103; see Table 1). Following 24-hour incubation, cells were treated with either ethanol or varying concentrations of 25D for approximately 15 hours before lysis and luciferase assay.

For CYP24A1 preliminary experiments (Fig. 4), cells were transfected with either pSG5-empty or pSG5-CYP24A1 and incubated in minimal DMEM as above for approximately 24 hours. The following day, cells were treated with 100 nmol/L 25D or 1 nmol/L 1,25D and incubated for approximately 15 hours. For the CYP24A1 SNP analysis experiments (Fig. 5), cells were transfected with either pSG5-empty, human wild type pSG5-CYP24A1, or one of the following CYP24A1 SNPs: R157Q (rs35051736), T248R (rs16999131), M374T (rs6022990), L409S (rs6068812; see Table 1). Following 24-hour incubation, cells were treated with either ethanol or 1,25D for approximately 15 hours before lysis and luciferase assay.

For the CYP27B1 and CYP24A1 quantitative PCR analyses (Fig. 6), cells were transfected with either an empty pSG5 vector or a pSG5 plasmid containing the cDNA for WT human CYP27B1, WT human CYP24A1, or the CYP27B1 and CYP24A1 SNPs indicated in Table 1. Cells were transfected with 1.0 μg expression vector/well 24 hours postseeding, using XtremaGENE 9 DNA Transfection Reagent (Roche) at a ratio of 3 μL/μg DNA according to manufacturer’s recommendations. Twenty-four hours after transfection, fresh total media were applied, containing either ethanol vehicle, 5 nmol/L 1,25D for 4 hours in the CYP24A1 group, or 100 nmol/L 1,25D for 24 hours in the CYP27B1 group.

Mammalian two-hybrid assay

After transient cell transfection and incubation with ligands, cells were collected and the amount of reporter gene product (luciferase) produced in the cells was measured using the Dual-Luciferase Reporter Assay System according to the manufacturer’s protocol (Promega). To control for transfection efficiency, the relative luciferase units (RLU) produced by the inducible firefly luciferase reporter were divided by the RLUs produced by the constitutively expressed Renilla luciferase. The mean ratio of firefly/Renilla luciferase was determined for each treatment group and the SD was calculated (expressed as error bars). All data are reported as either the average of 3 or more experiments or are representative of 2 or more trials. Each experimental treatment group was replicated in at least 3, and often as many as 6, wells.

Western blotting

Cell lysates were heated at 95°C in SDS sample buffer in the presence of 2-mercaptoethanol and subjected to SDS-PAGE. The separated proteins were transferred by electrophoresis to Amersham Hybond-P polyvinylidene difluoride transfer membranes (GE Healthcare) and then treated with diluted antibodies as follows: CYP24A1 rabbit polyclonal Ab (H-87), CYP24A1 mouse monoclonal Ab (E-7), or CYP27B1 rabbit polyclonal Ab (H-90) (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase–conjugated anti-rabbit or antimouse IgG was used as the secondary antibody (Santa Cruz Biotechnology, Inc.) and signals were detected using SuperSignal (Pierce Biotechnology).
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Functional SNPs in Vitamin D Metabolic Enzymes CYP24A1/CYP27B1

Total RNA isolation and cDNA synthesis

Total RNA was isolated from approximately 2 million cells using an Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 2 μg of RNA using an iScript kit (Bio-Rad) according to the manufacturer’s instructions in 40 μL total volume.

Real-time PCR

Quantitative real-time PCR (qRT-PCR) was carried out with Applied Biosystems SYBR Green 2X PCR Master Mix (Life Technologies) in a System 7500 Fast thermal cycler using 1.5 μL of first-strand DNA and 0.5 μL of 18 μmol/L primer mixture in 10 μL total volume per well in triplicate. For detection of rat FGF23 transcripts, the forward primer was 5′-ACGGAACACCCCCCATCACTATAC-3′ and the reverse primer was 5′-TATCATACGGAGGCCAGCATCC-3′, yielding a 71-nt product, whereas for the reference gene rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the forward primer was 5′-AGGATGAATCCACATCAAGCTGTTC-3′ and the reverse primer was 5′-GATCACCTTTCAAGAAGGAACT-3′, yielding a 94-nt product. Total RNA was isolated from approximately 2 million cells using an Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 2 μg of RNA using an iScript kit (Bio-Rad) according to the manufacturer’s instructions in 40 μL total volume.

Reactions were conducted in 96-well PCR plates and read on an ABI 7500 Fast instrument. Data were analyzed using the comparative cycle threshold (Ct) method as a means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value obtained from vehicle-treated cells) and expressed as 2-ΔΔCt according to the Applied Biosystems manufacturer’s protocol.

Results

Selection of CYP27B1 and CYP24A1 SNPs and development of assays to evaluate colonic intracellular vitamin D metabolism

The 5 CYP27B1 and 4 CYP24A1 human polymorphisms were chosen for analysis based on population diversity, and, using data available in the dbSNP database at the time of selection, including only nonsynonymous SNPs. Each SNP results in a missense mutation where a single amino acid is replaced by another with different physicochemical properties, potentially changing the functionality of the resulting protein. For example, the CYP24A1 R157Q mutant substitutes a polar, uncharged glutamine in place of the original positively charged arginine. These 9 SNPs include few conservatively replaced amino acids and therefore are generally the most common polymorphisms likely to result in a functionally altered protein product.

Figure 1A and B depicts linear schematics of the CYP27B1 and CYP24A1 proteins, respectively. Most notable about the SNP loci selected for analysis is that they are all positioned within the active P450 domain that contains an iron(III) protoporphyrin-IX ring covalently linked to a nearby cysteine ligand (15). This cysteine heme prosthetic group is enzymatically responsible for the oxidative reaction carried out by the proteins; thus, mutations in this domain may have an effect on overall enzyme function.

Figure 1. Schematic representation of CYP27B1 and CYP24A1 proteins, as well as the novel experimental systems used in this study. These diagrams show the locations of resulting amino acid mutations within the P450 domains of the human cytochrome P450-27B1 protein (A) and the human cytochrome P450-24A1 protein (B), C, a novel experimental assay was used to study the CYP27B1 SNPs. SNP-containing plasmids were introduced into target cells along with an M2H luciferase reporter gene via liposome transfection. This was followed by treatment with inactive 25D. As CYP27B1 catalyzes the addition of a hydroxyl group to 25D, forming 1,25D, the resulting active hormone binds to VDR, causing heterodimerization with DNA-bound RXR and transcription of the luciferase reporter gene. Increased CYP27B1 enzymatic activity is reflected by increased luciferase transcription. D, a similar experimental system was used to study the CYP24A1 SNPs; however, as CYP24A1 causes the inactivation of 1,25D by the addition of a third hydroxyl group, increased CYP24A1 activity is reflected by decreased luciferase transcription.

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To evaluate the relative activity of the SNPs, an assay system was developed to measure intracellular 1,25D levels. As described above, active 1,25D binds to VDR, which heterodimerizes with RXR and then binds to DNA to stimulate transcription. For this system, RXR was cloned into a plasmid containing a heterologous DNA-binding domain (BD), and VDR was cloned into a plasmid containing a transcriptional activation domain (AD). In the presence of 1,25D, AD-VDR (prey) binds the hormonal ligand and dimerizes with BD-RXR (bait). When bait and prey are brought together, the transcriptional activation domain is tethered to the correct DNA sequence by the binding domain, through the RXR–VDR dimer. This assembled complex stimulates transcriptional initiation of the firefly luciferase reporter gene, which can be quantified via luminescence. In this system, a linear relationship exists between the levels of intracellular 1,25D and the amount of luciferase production, allowing for a quantitative assessment of 1,25D levels inside the cell.

Figure 1C and D illustrates the M2H assay system used for the evaluation of both SNPs. In the first system (Fig. 1C), exogenous CYP27B1, RXR-BD, VDR-AD, and inactive 25D are introduced to target cells. As exogenous WT or a CYP27B1 SNP catalyzes the formation of 1,25D, RXR–VDR dimerization occurs, and the firefly luciferase (LUC) gene is transcribed. More active CYP27B1 variants would synthesize more 1,25D, leading to more luciferase production, whereas less active SNPs would produce less luciferase.

The CYP24A1 system (Fig. 1D) used a similar design, except CYP24A1 was introduced and target cells were treated with 1,25D. The 1,25D treatment results in luciferase production, whereas exogenous CYP24A1 expression leads to 1,25D degradation and lowers the overall production of the luciferase protein. The outcome of this system is essentially the reverse of that with CYP27B1, with a more active CYP24A1 enzyme yielding lower firefly luciferase output, due to higher rates of 1,25D inactivation.

Assessment of the 1,25D-luciferase transcription system for evaluating CYP27B1 SNPs

Because of the expression of CYP27B1 in colon carcinoma cells reported previously (8), a luciferase-based transcription system was developed to detect enzymatic activity beyond CYP27B1 endogenous levels. The leftmost column (column one) of Fig. 2A represents target cells transfected with an insertless expression vector, pSG5-empty (indicated by −), and treated with ethanol only. These cells are effectively
untreated, "natural" cells that represent the basal CYP27B1 activity. As indicated by columns one and two, without 25D substrate, synthesis of 1,25D (as measured by the amount of luciferase activity) remains unchanged with or without transfection of exogenous CYP27B1 (indicated by +). A lack of exogenous CYP27B1 and treatment with 25D (Fig. 2A, middle column) showed a slight increase in 1,25D-induced firefly luciferase transcription, indicating modest endogenous CYP27B1-mediated production of 1,25D. Transfection of exogenous CYP27B1, followed by treatment with 25D, yielded a nearly 5-fold increase in 1,25D-induced firefly luciferase transcription (Fig. 2A, column 4), and an increase in CYP27B1 protein as assessed by Western blotting (data not shown). The positive control (far right column), in which transfected cells are treated with 1,25D, indicates a potent induction of luciferase by the active 1,25D–VDR complex. Taken together, these results illustrate that by overexpressing exogenous CYP27B1, fluctuations due to endogenous activity are negligible and the system allows the evaluation of CYP27B1 activity in the various CYP27B1 SNP expression vector constructs.

In addition to negating the influence of endogenous enzymatic activity, this assay system does not detect CYP27B1...
metabolites other than 1,25D. Cells transfected both with and without exogenous CYP27B1 that have been treated with 24D (Fig. 2B, columns 2 and 4) showed firefly transcription levels only slightly above those of cells treated with ethanol only (Fig. 2A, columns 1 and 2), whereas treatment with 25D (to generate 1,25D) again reveals a vigorous induction of the reporter gene (Fig. 2B, column 3). Increased amounts of transfected CYP27B1, with 25D hormone treatment held constant, lead to elevated detectable 1,25D synthesis (Fig. 2C) and increased CYP27B1 protein expression (data not shown). Similarly, higher concentrations of 25D substrate (Fig. 2D) also resulted in increas-

ing levels of detectable 1,25D synthesis, indicating that the assay system can be used to test dose–response associations.

**CYP27B1 SNPs show both modest and dramatic differences in 1α-hydroxylase activity**

Having designed an assay system that is able to effectively quantify CYP27B1-mediated production of intracellular 1,25D in colonic cells, the next step was to compare the **CYP27B1** SNPs (Table 1) with the WT enzyme. To do this, HCT-116 were transfected with an empty pSG5 vector, pSG5-WT-CYP27B1, or

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| Figure 4. Expression and evaluation of WT human CYP24A1 in target cells. A 1,25D-VDR luciferase transcription system was successfully developed to detect levels of 1,25D inactivation via the CYP24A1 enzyme. In an M2H assay system, HCT-116 cells were transfected with either pSG5-empty (–) or pSG5-CYP24A1 (+) followed by treatment with either 25D or 1,25D. A, cells were transfected as indicated and treated with either 100 nmol/L 25D or 1 nmol/L 1,25D. B, cells were transfected with increasing concentrations of pSG5-CYP24A1 plasmid and treated with 1 nmol/L 1,25D. Data are representative of 3 independent experiments with triplicate samples in each group.
a pSG5-CYP27B1 SNP expression plasmid. Cells were then treated with either a constant concentration of 25D or a range from $10^{-8}$ to $0.5 \times 10^{-8}$ mol/L. All CYP27B1 variants showed statistically significantly ($P < 0.05$) decreased activity with the exception of V166L, a conservative replacement. When CYP27B1-transfected cells were treated with 100 nmol/L 25D (Fig. 3A), R107H (Fig. 3B) displayed nearly undetectable activity at low-to-mid concentrations of hormone, with at most approximately 55% of WT activity ($P < 0.05$) at high concentrations of hormone. A129T (Fig. 3C) also showed little activity at low-to-mid concentrations of hormone, but with higher 25D concentrations, this polymorphism had moderate but statistically significant reductions in activity when compared with WT CYP27B1 ($P < 0.05$). Interestingly, the SNP V166L (Fig. 3D) revealed normal activity at low levels of hormone, with activity higher than WT activity at mid and high concentrations of 25D ($P < 0.05$; similar to Fig. 3A). CYP27B1 S356N (Fig. 3E) was the least active of the SNPs, showing negligible activity at low-to-mid concentrations of hormone and a small increase in activity at higher levels of 25D. However, its overall activity remained markedly lower than the WT enzyme ($P < 0.05$). CYP27B1 V374A (Fig. 3F)
Figure 6. UMR-106 cells transiently cotransfected with cDNA expression vectors containing either WT or SNPs of human CYP27B1 or CYP24A1. A, qRT-PCR shows fold induction of rFGF23 expression in response to treatment with 100 nmol/L 25D for 24 hours. Results are shown as a percentage relative to the average fold induction observed in the hCYP27B1 WT group and did not occur without hCYP27B1 transfection. Actual fold effect of each group was calculated (2∧ΔCt) with the average WT induction of rFGF23 (114-fold) set to 100%. Error bars are SEM of the ratios, with all groups showing statistically significant reductions (P < 0.01; n = 3) compared with WT (first column) using a 2-sided Student t test. B, UMR-106 cells transiently cotransfected with cDNA expression vectors containing either WT or SNPs of human CYP24A1. qRT-PCR shows fold induction of rFGF23 expression in response to treatment with 5 nmol/L 1,25D for 4 hours. 1,25D fold-induction of rFGF23 for each group was calculated (2∧ΔCt) with the average induction of the mock-transfected cells set as the control group (31-fold). Each hCYP24A1-transfected SNP displays an attenuated rFGF23 average fold induction. Two-sided Student t tests showed significant activity of each construct (P < 0.01; n = 3) compared with mock-transfected groups (first column), with R157Q and L409S being significantly different from the WT (P < 0.01; n = 3) and showing reduced attenuation of the 1,25D-induced rFGF23.

Assessment of a 1,25D-luciferase transcription system for CYP24A1

A similar M2H system as used for the CYP27B1 experiments was used for evaluating the CYP24A1 SNPs (Fig. 1). Over-expression of exogenous transfected CYP24A1 in target cells (Fig. 4A, 3 far-right columns) reduced levels of intracellular 1,25D, as shown by the reductions in basal (column 1), 25D-induced (column 2), or 1,25D-induced (column 3) luciferase transcription. Analogous to the CYP27B1 system, elevated induction of exogenous WT and SNPs, followed by treatment with 1,25D, resulted in increased expression of the CYP24A1 protein and in reduction of luciferase activity, indicating increased 1,25D catabolism.

CYP24A1 SNPs all exhibit reduced 24-hydroxylase activity

Western blot analysis reveals the induction of endogenous CYP24A1 in colon carcinoma cells by the active 1,25D metabolite (top inset, Fig. 5A–D). As expected, further transfection of CYP24A1 vectors results in additional and pronounced expression of exogenous WT or SNP CYP24A1 protein, indicating that a significant proportion of CYP24A1 expression was derived from cellular transfection. For all CYP24A1 SNP experiments, endogenous CYP24A1 activity in colon cells transfected with pSG5-empty was set to 100% (Fig. 5A–D, second column, black bar). Transfection of exogenous CYP24A1 WT and SNPs, followed by treatment with 1,25D, resulted in increased expression of the CYP24A1 protein and in reduction of luciferase activity, indicating increased 1,25D catabolism.

RT-PCR data results

To evaluate the effects of the human CYP27B1 and CYP24A1 SNPs in conjunction with an endogenous 1,25D-VDR target gene, we selected the rat osteosarcoma bone cell line UMR-106 and the FGF23 gene as the reporter of 1,25D activity. Prior studies have shown robust 1,25D-induced FGF23 expression that is both dose- and time-dependent in this cell line, with maximum mRNA levels observed at 24 hours when evaluated by qRT-PCR (16). However, FGF23 levels gradually decline in UMR-106 cells with advanced (>30) passages. We avoided this issue and observed internal consistency in FGF23 expression.
by evaluating multiple SNPs in parallel experiments in UMR-106 cells grown between passages 15 and 30 only. The induction of rFGF23 depicted in Fig 6A represents the relative amount of 1,25D-mediated induction via production of intracellular 1,25D from the 25D prohormone by the transfected CYP27B1, following treatment of cells with 100 nmol/L 25D for 24 hours. The WT CYP27B1–directed expression of FGF23 observed under these conditions is 114-fold (set to 100% in Fig. 6A, column 1). This level of FGF23 induction is almost entirely abrogated under similar conditions for the R107H and S356N groups (Fig. 6A, columns 2 and 5; P < 0.05), although when the dose was increased to 200 nmol/L 25D, we did observe a modest but significant increase in activity (data not shown). Interestingly the V166L showed a rare statistically significant hyperactivity (P < 0.05), whereas the A129T and V374A SNPs induced about 50% FGF23 compared with WT (P < 0.05).

The assay to assess CYP24A1 SNP activity applied the same cell line and reporter gene as above, although the experimental scheme is modified for the CYP24A1 catabolic enzyme. In this experiment, the cultures were transfected with CYP24A1 WT or SNP variants and then treated with 1,25D instead of the 25D prohormone. In this assay, the attenuation of 1,25D-mediated FGF23 induction is monitored at a shorter time point of 4 hours. The pSG5-empty group (∼) treated with 5 nmol/L 1,25D reveals an average 31-fold induction of FGF23 (Fig. 6B, column 1). This level of FGF23 induction is almost identical to that observed with the pSG5-empty control (P < 0.01). The other CYP24A1 SNPs analyzed, namely, R157Q, T248R, and M374T, each displayed a statistically significant (P < 0.01) reduction in activity compared with WT CYP24A1, as evidenced by a blunting of the attenuation of FGF23 induction (Fig. 6B, 21, 20, and 16-fold, respectively, vs. 13-fold for WT). Importantly, these qPCR data with an endogenous 1,25D target gene reporter effectively mimic the results obtained previously with CYP27B1 (Fig. 3) and CYP24A1 (Fig. 5) using the M2H assay.

Discussion

The objective of the present work was to examine whether genetic variations in CYP27B1 and CYP24A1 might affect enzymatic function in the 1,25D metabolic pathway in colon cancer cell lines and therefore potentially alter tissue-level exposure to the hormone. In these experiments, we observed differential activity of CYP27B1 and CYP24A1 in the presence of SNPs that result in missense mutations with both an M2H system and qRT-PCR analysis of an endogenous 1,25D target gene. These findings have potentially important implications for cancer prevention and control given the significance of these enzymes in regulating the metabolism of 1,25D, which has been shown to possess potent anticarcinogenic effects in the colon (6, 7, 17).

CYP27B1 and CYP24A1 are both of critical importance in governing 1,25D concentrations (2, 3, 9), and it has been proposed that these 2 enzymes work in concert to regulate the concentrations of this hormone at the tissue level (9). The hydroxylation that converts 25D to 1,25D is conducted by CYP27B1 (3), whereas CYP24A1 begins the catabolic processing of 1,25D by incorporating a hydroxyl group at the C24 position to form 1,24,25D and continues to catalyze the further hydroxylation of this metabolite until degradation (9). CYP24A1 may also hydroxylate 25(OH)D to begin catabolism of this molecule, a process that has the potential to limit the availability of active prohormone (9).

Several factors may influence the degree to which these enzymes can alter cellular exposure to the anticarcinogenic effects of 1,25D. First, compared with normal cells, the colonic expression of CYP27B1 is increased in early-stage colon tumors but is reduced or abrogated as colon cells become increasingly undifferentiated (18). Therefore, the production of 1,25D may be stimulated in response to carcinogenic initiation but repressed in progression to undifferentiated tumors, thus excluding late-stage tumors from the antiproliferative effects of this hormone allowing for further uncontrolled growth (17). In contrast, expression of CYP24A1 remains high during tumorigenesis, allowing for sustained catabolism of 1,25D, and therefore again reducing tumor exposure to this hormone (17). Genetic variation in CYP24A1 and CYP27B1 is another factor that may contribute to availability of 1,25D during carcinogenesis.

Polymorphisms in CYP27B1 and CYP24A1 have been studied in relation to both vitamin D metabolite concentrations and risk of cancer. Genetic variation in both CYP27B1 and CYP24A1 has been linked to colorectal cancer risk in a human population study; however, the mechanism of action for this association is unknown (11). It may be the result of alterations in circulating or tissue-level availability of vitamin D metabolites. Significant associations between CYP27B1 and circulating 25D concentrations have been observed in some studies (10, 19), although a large genome-wide association study (GWAS) did not identify a significant relationship (20). However, the same GWAS investigation showed that variation in CYP24A1 was significantly related to blood 25D levels (20). While no CYP27B1 or CYP24A1 polymorphisms are known to affect concentrations of 1,25D in circulation, both enzymes are expressed in the colon (8, 21). The activity of these enzymes is likely critically important in tissue-level exposure to 1,25D (9) and as such could have major implications for any inhibition of the carcinogenic pathway by this hormone.

In the current work, the majority of the CYP27B1 variants resulted in attenuation of enzymatic activity to different degrees; these SNPs included R107H, A129T, S356N, and V374A. Although the R107H variant appears relatively rarely in human populations, it is perhaps the most well-studied of these 2 enzymes due to adverse outcomes when it is present (22, 23). Kitanaka and colleagues conducted DNA sequencing of CYP27B1 among 4 patients with pseudovitamin D deficiency rickets (PDDR) and discovered 4 genes with missense mutations, including R107H, which abrogated CYP27B1 activity (22). Sawada and colleagues (23) confirmed these findings by expressing cDNAs from normal and patients with PDDR in Escherichia coli JM109 cells. Among the cells expressing the
R107H mutant CYP27B1, activity was reported as less than 1% than that of WT (23). Our results support and extend this observation, as R107H was 1 of the 2 SNPs that resulted in striking reductions in CYP27B1 activity in both the M2H and qRT-PCR experiments. The results also revealed another SNP that exhibited such drastic attenuation of enzymatic activity, namely, S356N; however, to date there are no allele frequency or functional data available for this polymorphism. It is possible that its potent effects on CYP27B1 function render it a rare variant in human populations.

Two other SNPs, A129T and V374A, caused a reduction in activity compared with WT CYP27B1. Very little information is currently available for A129T, which has a minor allele frequency (MAF) in human populations of approximately 0.013% (24). Regarding V374A, a SNP for which the MAF is 0.02 (24), it was 1 of 791 SNPs in human cytochrome P450 enzymes recently evaluated by Wang and colleagues using sorting intolerant from tolerant calculations and polymorphism phenotyping (25). Both methods predicted that V374A would be a deleterious SNP (25), which is supported by our results. It is unknown whether there are differences in MAF for these 2 SNPs by race/ethnicity and whether the impact of such SNPs varies between populations with differing genetic backgrounds, although these are strong possibilities given prior findings for genetic variation in genes within the vitamin D pathway (19, 26). In fact, variation in MAF by race/ethnicity has been reported for V166L, the only SNP that exhibited increased enzymatic activity over CYP27B1 WT at high doses of 25D (24). The overall MAF frequency for V166L has been reported as 0.03, whereas in a small sample of those reporting Pacific Rim heritage, the MAF is 0.13 (24). Very little is known about circulating concentrations of vitamin D metabolites associated with this SNP. However, the functional differences in the presence of V166L and the variation in MAF between racial and ethnic groups, further study is warranted to evaluate whether this SNP may affect the availability of 1,25D at the cellular level, as this may affect susceptibility to cancer (27). A more efficient enzyme for conversion of prohormone 25D to 1,25D would be expected to produce higher concentrations of the hormone at the cellular level and possibly confer protective or chemopreventive effects. Also of interest was the finding that the activity of V166L remained at normal levels when exposed to low levels of hormone but increased to higher than WT at mid and high concentrations of 25D. These results suggest the possibility that higher concentrations of circulating 25D may overcome genetic background to induce rapid production of 1,25D at the target cell. It is therefore possible that the intake of vitamin D needed to achieve and maintain optimal circulating concentrations of vitamin D metabolites may vary by genotype.

As observed with CYP27B1, CYP24A1 SNPs exhibited a broad range of effects on activity; however, in this case, each of the 4 tested polymorphisms resulted in lower activity than in WT enzyme. Little is known about the effects of these SNPs in human populations, as they tend to be rare variants. However, one SNP examined in the current work, R157Q, is located 2 amino acids from R159Q, a SNP that has been associated with idiopathic infantile hypercalcemia (28). There is also marked variation in MAF between different racial/ethnic groups for some CYP24A1 polymorphisms. The MAF for M374T, for example, appears to be absent in those reporting white race but is 0.15 among African-Americans (24). Because the reduced catabolism of 1,25D at the cellular level associated with this SNP would likely result in higher concentrations of this metabolite, variation such as this may partially account for the observation that while African-Americans tend to have lower concentrations of the prohormone 25D, this population has also been reported to have higher circulating levels of 1,25D than whites (27, 29, 30).

Vitamin D metabolism, predominantly controlled by CYP27B1 and CYP24A1, is a vital mediator of mineral homeostasis (31). Moreover, CYP expression in a variety of cancer cells likely affects tissue level 1,25D bioavailability and thus could potentially limit vitamin D concentrations in the tumor microenvironment (32). In summary, the present study shows that CYP27B1 and CYP24A1 polymorphisms result in marked changes in enzymatic activity in colon cancer cells. These alterations would likely result in differential 1,25D exposure in colonic cells, which could render individuals more susceptible to the development of colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.T. Jacobs, C. Van Pelt, R.E. Forster, M.A. Galligan, P.W. Jurutka

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