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A novel SNP in a vitamin D response element of the CYP24A1 promoter reduces protein binding, transactivation, and gene expression

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ABSTRACT

The active form of vitamin D ($1\alpha,25(OH)_2D_3$) is known to have antiproliferative effects and has been implicated in cancers of the colon, breast, and prostate. These cancers occur more frequently among African Americans than Caucasians, and individuals with African ancestry are known to have approximately twofold lower levels of serum vitamin D (25(OH)D) compared with individuals of European ancestry. However, epidemiological studies of the vitamin D receptor (VDR) have shown inconsistent associations with cancer risk, suggesting that differences in other genes in the pathway may be important. We sought to identify functionally significant polymorphic variants in CYP24A1, a gene that is highly inducible by $1\alpha,25(OH)_2D_3$ and that encodes the primary catabolic enzyme in the pathway. Here we report the identification of six novel SNPs in the human CYP24A1 promoter, including one at nucleotide −279 occurring within the distal vitamin D response element (VDRE2). Our experiments demonstrate that the VDRE2 variant results in decreased protein binding and transactivation *in vitro*, and reduced expression of CYP24A1 in cultured primary human lymphocytes provides evidence for an effect *in vivo*. This variant was only observed in our African American population, and represents a first step toward understanding differences in disease risk among racial/ethnic groups.

1. Introduction

The biologically active form of vitamin D ($1\alpha,25(OH)_2D_3$) has multiple roles in the body, including regulation of intestinal calcium absorption, maintenance of bone mineral density, and modulation of cell growth and apoptosis [1]. Genomic actions of $1\alpha,25(OH)_2D_3$ are mediated through ligand-binding to the vitamin D receptor (VDR), which forms a heterodimer with retinoid x receptor alpha (RXRα) and subsequently binds to vitamin D response elements (VDRE) to either enhance or repress transcription of various genes. These response elements are typically comprised of two conserved hexameric half-sites separated by a three nucleotide spacer, referred to as a DR3 type element. Although it is known that the sequence of a VDRE can have a strong influence on the degree of protein binding, particularly at the fifth position in the half-site [2], previous studies have focused on synthetic variations of response elements and not naturally occurring sequences [3].

It has been known for some time that there are significant racial/ethnic differences in serum vitamin D status, with individuals of African ancestry having approximately twofold lower levels than those with European ancestry [4–6]. Low levels of serum vitamin D (25(OH)D) have been associated with cancers of the colon, breast, and prostate, as well as an increased risk of cardiovascular disease [7–10]. Compared with whites, African Americans are more frequently diagnosed with these cancers and are at a greater risk for cardiovascular disease [11–13], indicating the possibility that polymorphic variants in the vitamin D pathway could be influencing these disparities. There are several well-known variants in the VDR, but epidemiological studies have shown inconsistent associations with disease outcomes, particularly among different racial/ethnic groups [14–19]. Taken together, this suggests that other variants or combinations of variants within the pathway may underlie the differences in serum vitamin D and disease risk.

The CYP24A1 gene has very low basal expression, but is strongly upregulated by $1\alpha,25(OH)_2D_3$ through two VDRE in the proximal promoter region [20,21]. The resulting 24-hydroxylase enzyme catalyzes the first step in the catabolic pathway, converting $1\alpha,25(OH)_2D_3$ into the less active intermediate $1,24,25(OH)_3D_3$ [22]. Through activation of this and other negative feedback loops, $1\alpha,25(OH)_2D_3$ can regulate its own metabolism. We hypothesized that there were unidentified SNPs in the promoter of the

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CYP24A1 gene that could alter expression of the 24-hydroxylase enzyme and impact the rate at which an individual can metabolize 1α,25(OH)$_2$D$_3$. Population studies to date have sequenced only a small number of individuals, particularly individuals of African ancestry, so it is possible that significant polymorphic variants in this region have yet to be identified.

In the work presented here, we report the identification of six novel SNPs in the human CYP24A1 promoter, including one that occurs in the fifth position of the distal vitamin D response element (VDRE2). We demonstrated that this VDRE2 polymorphism results in impaired receptor protein binding and decreased transactivation *in vitro*, and more importantly, we provide evidence that the variant can lead to decreased expression of the CYP24A1 gene in a heterozygous polymorphic individual.

2. Materials and methods

2.1. Subjects

Healthy individuals who classified themselves as at least 50% Caucasian or at least 50% African American were recruited at Pennsylvania State University General Clinical Research Center during the winter and spring months. The 100 total participants were matched on age (within 2 years), sex, and opposite self-reported race. Each participant donated a venous blood sample that was used to prepare dried blood spot cards and for Ficoll separation with Ficoll-Paque PLUS (GE Healthcare) to obtain lymphocytes.

2.2. DNA isolation and sequencing to screen for CYP24A1 promoter polymorphisms

Genomic DNA for SNP discovery was isolated from dried blood spot cards from 20 randomly selected African American participants in our study using the QIAamp DNA Micro Kit (QIAGEN). A 677-bp region of the proximal CYP24A1 promoter (−618 to +59) including two known VDRE was PCR-amplified using Phusion DNA polymerase (New England Biolabs) and the following primers: 5′-GTGTCAAGGAGGGTAGATGAGATG-3′ (forward) and 5′-TTGCTCAAGTTAAGAAAGTCTCCTC-3′ (reverse). The desired PCR products were gel-isolated using the QIAquick Gel Extraction Kit (QIAGEN), run on agarose gels to verify DNA recovery, and sequenced using the forward PCR primer at the University of Pennsylvania School of Medicine DNA Sequencing Facility by automated cycle sequencing. Polymorphisms were identified by BLAST alignment with the wild-type sequence for human chromosome 20 (GenBank accession number NT_011362) and by visual inspection of printed chromatograms. Some SNPs were also confirmed by restriction fragment length polymorphism analysis. Resulting sequence variants were compared to data from NCBI (build 36, dbSNP b126) and the HapMap project (www.hapmap.org, release 22).

2.3. DNA isolation and SNP genotyping for the VDRE2 polymorphism

Genomic DNA was isolated from frozen lymphocytes of all 100 participants with the QIAamp DNA Mini Kit (QIAGEN). A custom TaqMan SNP Genotyping assay (Applied Biosystems) was designed for the newly identified VDRE2 polymorphism and subsequently tested on samples of known genotype to conduct an internal validation of the assay. Quantitative real-time PCR and allelic discrimination were conducted at the Functional Genomics Core Facility at the Penn State College of Medicine using 10 ng genomic DNA per assay. Genotypes were successfully determined for 99% of the samples with a reliability rate of 100% when a random 10% sample was again genotyped in a separate experiment. Samples were also genotyped for the presence or absence of the M1T polymorphism in VDR (rs10735810) using a predesigned TaqMan assay (Applied Biosystems, assay ID# C_12060045_20) with similar success.

2.4. Plasmid construction and site-directed mutagenesis

The hCYP24p-Luc construct was generated by first PCR-amplifying and gel-isolating a 677-bp region of the proximal CYP24A1 promoter (−618 to +59) as described above. The purified product was then subjected to a second round of amplification using two nested primers designed to introduce XhoI and HindIII restriction sites for subcloning: 5′-TGCTCgAGTTAAGAAAGTCTCC-TCTTC-3′ (forward) and 5′-GGACCAaGCtTTTATGGAGACAGA-3′ (reverse). The new PCR product of 603 bp (−617 to −15) was digested with the above restriction endonucleases to expose the cohesive ends and was gel-purified as before. The pGL3-Promoter vector (Promega) was similarly digested and gel-purified, then ligated overnight with the CYP24A1 promoter fragment and transformed into chemically competent *Escherichia coli*. Sequencing was done to confirm the orientation and integrity of the newly created hCYP24p-Luc construct, which contains the proximal CYP24A1 promoter (−611 to −25) driving expression of firefly luciferase under the control of two VDRE: GAGTCAgcgAGGTGAgcgAGGGCG at −169 to −145 (VDRE1) and GAGTTCaccGGGTGT at −289 to −274 (VDRE2).

To create the hCYP24pV2SNP-Luc construct, we used the hCYP24p-Luc plasmid as template for site-directed mutagenesis with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer. Briefly, the primers 5′-CGAAGCA-CACCCGGTGGACTCCGGGCTT-3′ (sense) and 5′-AAGCCCGGAGTCC-ACCGGGTGTGCTTCG-3′ (antisense) were annealed to the hCYP24p-Luc plasmid to allow synthesis of the mutated promoter with *Pfu* DNA polymerase, followed by digestion of the parental plasmid by DpnI, transformation into XL1-Blue chemically competent cells, and screening of the resulting colonies by restriction endonuclease digestion. Positive clones were verified by sequencing, and the new construct was identical to the wild-type promoter with the exception of a single base pair change in VDRE2 (GAGTTCaccGGGTGT was mutated to GAGT**C**CaccGGGTGT) to reflect the polymorphism identified at −279 during sequencing of our human subjects.

The r24OHase-Luc construct, a generous gift from Dr. H. DeLuca at the University of Wisconsin-Madison, contains the proximal 942 bp of rat 24-hydroxylase (CYP24A1) promoter with two VDRE cloned into pMAMneo-Luc vector upstream of firefly luciferase. This was used as a positive control for induction by 1α,25(OH)$_2$D$_3$ in transfection experiments.

Expression constructs for VDR and RXRA were generated from an existing human cDNA pool made by reverse-transcription of human liver RNA, a gift from Dr. Philip Lazarus at Penn State University. Coding sequences for both VDR and RXRA were specifically amplified with the following primers: 5′-GGTCTGAAGTGTCTGTGA-GACCTC-3′ (VDR-forward), 5′-ACAAACAGCAACTCCTCATGGCTG-3′ (VDR-reverse), 5′-GGGCATGAGTTAGTCGCAGA-3′ (RXRA-forward), and 5′-AAACAGGCCAGGCAGAGAAG-3′ (RXRA-reverse). Amplified cDNAs were gel-isolated and sequenced to verify that they were wild-type, then TA-cloned separately into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) and transformed into chemically competent *E. coli*. Multiple transformed colonies were screened for the presence and orientation of the cDNA inserts, and positives were confirmed by bidirectional DNA sequencing. The resulting constructs (pcDNA3.1-VDR and pcDNA3.1-RXRA) contain the wild-type coding sequences with native stop codons, and expression is driven by the CMV promoter.