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Vitamin D Related Genes, *CYP24A1* and *CYP27B1,* and Colon Cancer Risk

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Abstract

Genetic association studies investigating the role of vitamin D in colon cancer have primarily focused on the vitamin D receptor(*VDR*), with limited data available for other genes in the vitamin D pathway, including vitamin D activating enzyme 1-alpha hydroxylase(*CYP27B1*) and vitamin D deactivating enzyme 24-alpha hydroxylase(*CYP24A1*). We evaluated whether 12 tagging SNPs in *CYP24A1*, identified by resequencing the gene in 32 Caucasian samples, and 1 SNP in *CYP27B1* were associated with colon cancer risk. In addition, we evaluated whether these two genes modify associations between colon cancer and total vitamin D intake and UV-weighted sun exposure, as well as other variants in *VDR.* Unconditional logistic regression was used to calculate odds ratios(OR) and 95% confidence intervals(95%CI) for the association between polymorphisms and haplotypes in *CYP27B1* and *CYP24A1* in a multi-center population-based case-control study of 1,600 cases and 1,949 controls. *CYP24A1* polymorphism *IVS4-66T>G* showed a statistically significant association with risk of colon cancer overall, particularly for proximal colon cancer. When stratified by anatomic site, we also found statistically significant associations for three *CYP24A1* polymorphisms with risk of distal colon cancer (*IVS4+1653C>T*: OR for CT/TT vs. CC 0.81, 95%CI 0.68–0.96; *IVS9 +198T>C*: OR for CC vs. TT 1.33, 95%CI 1.03–1.73; and within Whites only: *+4125bp 3′ of STPC>G*: OR for GG vs. CC 1.44, 95%CI 1.00–2.05). In addition, a possible interaction between *CYP27B1* and UV-weighted sun exposure with proximal colon cancer was observed. As this is the first study to evaluate these genes in relation to colon cancer, additional studies are needed to confirm these results.

Keywords

vitamin D; colon cancer; genetics; CYP24A1; CYP27B1

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Introduction

Colorectal cancer is the third most commonly diagnosed cancer among men and women in the United States (1). Despite screening and decreasing incidence rates over the past few decades, colorectal cancer is expected to account for 8–9% of all cancer deaths in 2008 (1). Since Garland et al.(2) hypothesized that lower risk of colon cancer was related to higher sunshine exposure and vitamin D, a number of experimental and epidemiologic studies investigating the potential chemopreventive effect of vitamin D have been conducted, most of which are consistent with an inverse association. Conclusions from recent review articles of observational studies strongly support the hypothesis that higher levels of vitamin D (both dietary intake and serum concentrations) reduce risk of colorectal cancer (3,4). Although results from the calcium and vitamin D supplementation trial of the Women's Health Initiative (WHI) (5) failed to demonstrate an effect on colorectal cancer risk, high vitamin D supplementation prior to enrollment and the relatively low vitamin D dose chosen for the intervention (400 IUof vitamin D_3) complicate its interpretation (6). Further, a nested case-control study within the WHI did observe a statistically significant inverse trend between baseline concentrations of serum 25-hydroxyvitamin D and risk of colorectal cancer (5).

Aside from its role in bone metabolism and regulation of blood calcium concentrations, vitamin D is postulated to reduce epithelial-cell proliferation and to promote differentiation in various cell cultures, including colon-derived cells, and animal studies (7–9). In addition, vitamin D has been seen to induce cell-cycle arrest and apoptosis in colorectal tumor cell lines and premalignant adenoma cell lines (10). In the vitamin D pathway, the focus has been primarily on four genetic variants (*Taq1*, *Bsm1*, *Fok1*, and *Apa1*) in the vitamin D receptor (*VDR*) because the cellular effects of vitamin D are mediated primarily through binding, in the biologically active form of 1,25-dihydroxyvitamin D, to VDR, which regulates the transcription of numerous genes (11). However, the discovery that genes encoding for enzymes involved in the activation and inactivation of vitamin D are also expressed in colon cells (12,13), suggests other genes that may contribute towards the association of vitamin D with colorectal cancer risk.

The presence of vitamin D hydroxylase enzymes in colon cells indicates a potential anticarcinogenic effect of 25-hydroxyvitamin D on colon tissue through local production of the biologically active form of vitamin D (1,25-dihydroxyvitamin D) from 25-hydroxyvitamin D (12). One-alpha hydroxylase, encoded by *CYP27B1*, is the enzyme responsible for converting vitamin D into the active VDR-binding form. *CYP24A1* encodes for the catabolic enzyme 24-hydroxylase and is responsible for inactivating vitamin D metabolites. Despite their important role in vitamin D metabolism, genetic variants in *CYP27B1* and *CYP24A1* have not previously been investigated in relation to colon cancer risk.

Detailed information about the genetic variation in *CYP27B1* from resequencing has only recently become available (http://egp.gs.washington.edu/), and such information is missing for *CYP24A1*. The purpose of this study was to take advantage of the recent genetic characterization of *CYP27B1* and to resequence *CYP24A1* for a comprehensive analysis of the relation between genetic variation in these two key vitamin-D-pathway genes and colon cancer risk in a large population-based case-control study of individuals from California, Minnesota, and Utah. We also examined whether the *CYP24A1* and *CYP27B1* variants modified associations between colon cancer and dietary vitamin D and UV-weighted hours of sun exposure, an important factor in endogenous vitamin D production. Due to their close involvement in the local production of vitamin D in colon cells, we also explored the potential gene-gene interaction between variants in *CYP24A1* and *CYP27B1*, as well as with (previously reported) variants in *VDR*. Our study of genetic variants in these key genes sheds light on possible biologic mechanism by which vitamin D may prevent colon cancer, as well as provide

information about whether the chemopreventive effect of vitamin D is modified by genetic variation in *CYP24A1* and *CYP27B1*.

Methods

Study population

This study was based on a multi-center population based-case-control study with 1,993 colon cancer cases and 2,410 controls recruited from: the Kaiser Permanente Medical Care Program (KPMCP) of Northern California; an eight-county area in Utah; and the metropolitan Twin Cities area of Minnesota. Cases were eligible if they were aged 30–79 years at the time of diagnosis, with first primary colon cancer (ICD-O Ed.2. codes 18.0 and 18.2–18.9) between October 1991 and September 1994; and mental competence to complete the interview. Cases with the following conditions were not eligible: tumors in the rectosigmoid junction or rectum, and cases with pathology report indicating Familial Adenomatous Polyposis, Crohn's disease, or ulcerative colitis. A rapid-reporting system was used to identify all incident cases, with a majority being interviewed within 4 months of diagnosis. Response and participation proportions have been described previously(14). Of the cases that were asked to participate in the study, 76% cooperated. Controls were matched to cases by 5-year age groups and sex.

Controls from KPMCP were randomly selected from membership lists. In Utah, controls who were less than 65 years of age were randomly selected from lists generated using random-digit dialing and driver license lists, and those 65 years and older were randomly selected from Health Care Financing Administration lists. In Minnesota, control participants were identified from driver's license or state identification lists. Of all controls asked to participate, 64% cooperated (14).

Resequencing and tagging single nucleotide polymorphism (tagSNP) selection

To identify relevant and novel polymorphisms in *CYP24A1* (chr20:52,203,395-52,223,931; 20537 bp), we sequenced the promoter region (2 kb upstream), the complete coding sequence (12 exons, including intron/exon boundaries), as well as conserved intronic regions identified by phylogenetic footprinting analysis using the ECR Browser (http://ecrbrowser.dcode.org/) (15). We sequenced 32 of the HapMap CEPH individuals with European ancestry, given that our study population was predominantly Caucasian (95%) and to allow comparability with data from HapMap. Resequencing using standard dideoxy-based sequencing (Applied Biosystems, Foster City, CA) was conducted at the Functional Genomics Lab in the Center for Ecogenetics and Environmental Health at the University of Washington. The subsequent fragments were then aligned and compared with each other and the downloaded GenBank reference sequence using Sequencher (GeneCodes) to determine genetic variation. All alignments were visually inspected to ensure that all variants were comprehensively identified.

Eighty-six variants were detected through our resequencing efforts of which 30 (six with MAF $\geq$ 5%) had not been previously described in dbSNP. These plus six additional variants located in regions that we did not resequence but were genotyped in HapMap (Data release 20, Jan 2006) were included in the tagSNP selection for *CYP24A1*. For *CYP27B1*, we used existing data from NIEHS SNPs (http://egp.gs.washington.edu/). Common SNPs with MAFs $\geq$ 5% were selected using a three-step approach. To include potentially functionally relevant SNPs, we selected all non-synonymous polymorphisms. Thereafter, we identified tagSNPs using the htSNP program developed by Clayton (http://www-gene.cimr.cam.ac.uk/clayton/software/stata) to capture the common variation in the gene (minimum r^2 of 0.80, MAF >5%). Finally, we examined how well the selected tagSNPs were able to identify common haplotypes (>5%) using the software Haploview

leading to the inclusion of additional SNPs. A total of 16 SNPs for *CYP24A1* and 3 SNPs for *CYP27B1* were selected for genotyping.

Genotyping

Of the 4,403 cases and controls with valid study data, 3680 (83% of cases and 85% of controls) provided a blood sample. Genomic DNA was extracted with a success rate >95% for both cases and controls from peripheral blood lymphocytes or immortalized cell lines. Staff was blinded to case/control status. Each plate contained samples from both cases and controls, as well as duplicate quality-control samples interspersed among the plates (147 duplicates of samples from controls). All genotyping was performed by MALDI-TOF mass spectrometry on the Sequenom MassARRAY 7K platform using the iPLEX Gold (low-plex) reaction and conducted at the Translational Genomics Research Institute in March 2007. In total, we received successful genotyping results for 13 out of 19 SNPs (*CYP24A1:* n=12, *CYP27B1*: n=1) for a total of 3,549 subjects (80% of cases and 81% of controls). SNPs were excluded for the following reasons: Two SNPs (*CYP27B1 -1073C>G* and *IVS8+113A>C*) failed at final assay design and could not be replaced as no other tagSNP was available, one SNP (*CYP24A1 EX6+12C>T*) had a call rate <95%, two SNPs (*CYP24A1 EX1+96G>A*, *IVS7+204C>T*) had <98% concordance rate with sequencing data, and one SNP (*CYP24A1 IVS7-1457C>T*) had positive calls in water-negative controls.

The call rate was >95% for all 13 SNPs. Blinded duplicates displayed >99% concordance for any SNP. Using a goodness-of-fit test, the allele frequencies among Caucasian controls did not deviate from Hardy-Weinberg Equilibrium ($p>0.05$) for any SNP. The 12 successfully genotyped tagSNPs covered the genetic variation of all *CYP24A1* SNPs with an r^2 >80% (mean r^2 of 0.89) except for 7 SNPs which were unsuccessfully included in our assays. The tagSNPs had either low or moderate correlation with these 7 SNPs (r^2 range: 0.15 –0.78).

Data on *VDR* variants (*Bsm1* and *Fok1*) was previously collected and published on this study population and were made available for this analysis (16).

Dietary and lifestyle data

Detailed in-person interviews were used to collect demographic, dietary and lifestyle data from all eligible study participants. Study participants were asked about their lifestyle during the year, 2 years prior to the date of diagnosis or selection. During the in-person interview, information was collected on dietary intake, physical activity, medical history and drug use, demographic factors, smoking, reproductive history (for women) and family history of cancer and colorectal polyps. Quality-control methods were used to monitor the interviews (17). Dietary intake was ascertained using a modified version of the diet-history questionnaire designed and validated for the Coronary Artery Risk Development in Young Adults (CARDIA) study (18). Use of multivitamins, single vitamin and mineral supplements were also ascertained. We defined supplemental vitamin D as 10 μg (400IU)/day if a participant indicated either regular multivitamin or vitamin D supplement use (three times a week for at least one month). Total vitamin D combines dietary and supplemental vitamin D intake. Calcium intake was restricted to dietary intake only because supplemental dosage was not provided and doses in multivitamins are often low and highly variable.

The estimates of UV-weighted hours of sun exposure (UV index-hours/week) were based on the average hours per week spent outdoors in the daylight as reported by subjects during each season (spring, summer, fall, winter) of the referent year, multiplied by the UV index for each season in the geographic area of the study center, and divided by four to average over the four seasons (19).

Statistical analyses

Unconditional logistic regression, adjusted for age, sex, race and study center, was used to estimate odds ratios (OR) and 95% confidence intervals (95% CI) for the association between individual polymorphisms and colon cancer. Trends were tested by including a variable coded 0, 1, and 2 for the number of rare alleles. Statistical significance was defined as a p-value ≤ 0.05. We evaluated associations for the entire study population (all ethnicities), as well as restricted to non-Hispanic whites. Dietary factors included as potential confounders or effect modifiers were energy adjusted using the residual method. Additional adjustment for education, income, BMI, cigarette smoking, physical activity, long-term alcohol use, NSAID use, family history of colorectal cancer, dietary fiber, folate, red meat, fat intake, and multivitamin use did not result in meaningful changes of risk estimates for genotypes and were not included in the reported analyses. Global tests of association were conducted by simultaneously including genotypes (heterozygotes and homozygotes rare allele) of all *CYP24A1* SNPs in a model and comparing it to a model that included none of the genotypes. Adjustment for multiple testing was achieved through this multi-locus global test (degrees of freedom = 2x number of SNPs in a gene) within the likelihood ratio chi-square test (20). When SNPs were highly correlated (r^2>0.80), only one of the SNPs was included in the gene-based model.

Haplotype frequencies were estimated and associations evaluated using HaploStats (version 1.3.1) in R (version 2.4.1), assuming an additive model. If the true underlying model is recessive or dominant, associations for haplotypes may not be apparent due to misspecification. Analyses for haplotypes were restricted to non-Hispanic whites, the largest racial group in our study population, and adjusted for age, sex, and study center. A global score statistic was used to evaluate the overall difference in haplotype frequencies between cases and controls.

Polytomous regression was used to estimate associations between genotypes and risk of proximal colon cancer (cecum, ascending colon, hepatic flexure and transverse colon) and distal colon cancer (splenic flexure, descending colon, and sigmoid colon).

To evaluate whether genetic variants modified the association between measures of vitamin D and colon cancer risk and whether there was evidence of gene-gene interactions among *CYP24A1, CYP27B1,* and *VDR,* we investigated interactions through the inclusion of cross-product terms in the regression models. We performed an omnibus test for multiplicative interaction between each gene and the variable of interest (e.g. total vitamin D) by simultaneously including all cross-product terms for the gene of interest (coded as dummy variables for heterozygotes and variant homozygotes) with the variable of interest in a model (coded as continuous) and comparing that to a model that included only the main effects for the genotypes and variable of interest. For omnibus tests of interaction between two genes, genotypes were reduced to a binary variable combining heterozygotes and variant homozygotes. If the omnibus test for interaction was statistically significant, we further investigated multiplicative interaction between individual SNPs and the variable of interest using the log likelihood ratio test to compare the fit of logistic models with and without interaction variables. Adjustment for multiple testing was also achieved through this multi-locus global test.

Several publicly available web-based tools were used to assess the potential functional significance of variants, including the UCSC Genome Browser to obtain conservation scores (21), SIFT (22), PolyPhen (23), Splice Site Prediction tool by Neural Network (24), ESEfinder (25), RESCUE-ESE (26), and UTRScan (27).

Results

The majority of the study population was non-Hispanic whites (Table 1). Controls had a higher proportion of college graduates, and higher income study participants. Use of multivitamin and vitamin D supplements was higher in controls. Nonsignificant inverse associations for the highest quartiles of total vitamin D (proximal: OR=0.83; 95% CI, 0.66–1.05; distal: OR=0.80; 95% CI, 0.63–1.01) and UV-weighted sun exposure (proximal: OR = 0.71; 95% CI, 0.55–0.92; distal: OR=0.84; 95% CI, 0.65–1.09) was observed. These results have been previously published (28).

When we investigated the polymorphisms in *CYP24A1* individually, two *CYP24A1* polymorphisms displayed a statistically significant association with risk of colon cancer. A statistically significant trend was observed with increasing number of rare alleles at loci *IVS2 +523C>T* (p_{trend} = 0.05; Table 2) with a statistically non-significant inverse association between this variant and colon cancer risk (all ethnicities: OR CC+TC vs. TT = 0.83; 95% CI, 0.66–1.03; non-Hispanic whites: OR= 0.82; 95% CI, 0.65–1.02). A statistically significant inverse association between colon cancer risk and carrying at least one copy of the rare allele at *IVS4-66T>G* (all ethnicities: OR GG+GT vs. TT = 0.83; 95% CI, 0.72–0.96) was also observed. The global association p-value for SNPs in *CYP24A1* and colon cancer was 0.10 (df=20). A haplotype analysis of *CYP24A1* variants (*IVS7+921C>T, IVS7+1307A>T, IVS9 +198T>C*) did not reveal any statistically significant associations with colon cancer risk (p_{global}=0.27; Table 3).

As the association with colon cancer differs for several risk factors by anatomic site, we examined *CYP24A1* associations for distal and proximal colon cancer. Four *CYP24A1* variants were statistically significantly associated with site-specific colon cancer, although the global tests were not statistically significant (proximal: p_{global}=0.24, df=20; distal: p_{global}=0.13, df=20). The *CYP24A1* variant *IVS4-66T>G*, that demonstrated a statistically significant inverse association with risk of colon cancer, was similarly associated with risk of proximal colon cancer (OR GG+GT vs. TT = 0.82; 95% CI, 0.68–0.99) and was suggestive of an association with distal colon cancer (OR GG+GT vs. TT = 0.85; 95% CI, 0.70–1.02; Table 2). Furthermore, we observed statistically significant associations for three other *CYP24A1* variants with distal colon cancer. Those carrying at least one copy of the rare allele at *IVS4 +1653C>T* had a 19% lower risk (OR = 0.81; 95% CI, 0.68–0.96), which was similar among non-Hispanic whites (OR TT+CT vs. CC = 0.79; 95% CI, 0.66–0.94). In addition, two *CYP24A1* variants had statistically non-significant trends, but genotypes with positive associations with distal colon cancer risk: *IVS9+198T>C* was associated with an increased risk of distal colon cancer (all ethnicities OR CC vs. TT =1.33; 95% CI, 1.03–1.73 and non-Hispanic whites OR =1.46; 95% CI, 1.11–1.91); a borderline association limited to non-Hispanic whites between variant *+4125bp 3' of STP C>G* and distal colon cancer (OR GG vs. CC=1.44; 95% CI, 1.00–2.05) was also observed. Among the four *CYP24A1* variants with a statistically significant association, the variant *IVS4-66T>G* was in strong linkage disequilibrium (D′=1.0) with *IVS4+1653C>T* but in weak linkage disequilibrium (D′ =0.39–0.47) with the other two *CYP24A1* variants. It was only weakly correlated (r^2 <0.1) with the other three variants. The variant *IVS4+1653C>T* was in weak linkage disequilibrium (D′ =0.03–0.37) and weakly correlated (r^2=0.01–0.03) with *IVS9+198T>C* and *+4125bp 3′ of STP C>G*, respectively. The variant *+4125bp 3′ of STP C>G* was in moderate linkage disequilibrium (D′ =0.79) and moderately correlated (r^2 =0.40) with *IVS9+198T>C*. Adjustments for the other *CYP24A1* genotypes did not change any of the observed associations substantially, suggesting that the remaining four SNPs are independently associated with distal colon cancer risk.

Among SNPs in *CYP24A1*, three variants (*IVS7+921C>T, IVS7+1307A>T*, and *IVS9 +198T>C)* were located within a haplotype block spanning intron 7 through intron 9 of

CYP24A1. The haplotype containing the rare allele for *IVS9+198T>C* was possibly associated with risk of distal colon cancer (OR of 1.15; 95% CI, 0.99–1.33; Table 3). Although not statistically significant, this result is consistent with the positive association seen for *IVS9 +198T>C*. The positive association found for the group composed of rare haplotypes is probably due to the majority of these haplotypes carrying the rare allele for I*VS9+198T>C*. However, results are less reliable for rare haplotypes as phase for rare haplotypes may not be estimated correctly (29). We did not observe any noteworthy associations between the *CYP27B1* variant and colon cancer risk either overall or among subtypes (Table 2).

Investigating potential interactions between *CYP24A1*, *CYP27B1* and *VDR* indicated an interaction between SNPs in *CYP24A1* and *CYP27B1* for distal colon cancer risk (p_{global}=0.08; df=10). Further investigation by individual SNPs revealed a statistically significant decreased risk of distal colon cancer among common-allele homozygotes for *CYP27B1 IVS6-29T>C* individuals with at least one copy of the *CYP24A1 IVS2+523C>T* rare allele (OR, 0.51; 95% CI, 0.33–0.81). There was no evidence of an interaction between genotypes in *CYP24A1* or *CYP27B1* and genotypes in *VDR* (*Bsm1* and *Fok1*) either with colon cancer overall or with proximal cancer (data not shown).

When investigating interaction of *CYP24A1* and *CYP27B1* with vitamin D intake and sun exposure (as a marker of endogenous vitamin D production), we did not observe any statistically significant interactions between genotypes in *CYP24A1* and total vitamin D (p_{global} =0.80; df=20) or UV-weighted sun exposure (p_{global} =0.34; df=20) with overall colon cancer risk or by cancer subsite (data not shown). However, we detected a statistically significant interaction between the *CYP27B1* variant *IVS6-29T>C* and UV-weighted hours of sun exposure for proximal colon cancer ($p_{interaction}$ =0.04; Table 4): the data suggest a lower risk of proximal colon cancer associated with a difference of 75 UV index-hours/week among individuals with one or two copies of the rare allele for *IVS6-29T>C* with an OR of 0.86 (95% CI, 0.74–1.00). 75 UV index-hours/week is equivalent to someone in the Northern California area reporting an average of 10 hours/week in winter (UV index of 1), 19 hours/week in spring (UV index of 5), 15 hours/week in summer (UV index of 9), and 10 hours/week in fall (UV index of 6*)*. We did not observe any statistically significant interactions between *CYP24A1* variants and dietary calcium with overall colon cancer risk (p_{global} = 0.72; df=20) or by cancer subsite (distal p_{global} =0.56; proximal p_{global} =0.82). There were also no statistically significant interactions between *CYP27B1* and dietary calcium with overall colon cancer or by cancer subsite (all $p_{globals}$ >0.38).”

Discussion

We detected a statistically significant inverse association between *CYP24A1* variant *IVS4-66T>G* and colon cancer overall. The association for *IVS4-66T>G* was also seen for proximal colon cancer. The most consistent pattern, however, was with risk of distal colon cancer. Specifically, *CYP24A1* variants *IVS4-66T>G* and *IVS4+1653C>T* were associated with a reduced risk and two *CYP24A1* variants, *IVS9+198T>C* and *+4125bp 3′ of STP C>G*, were associated with increased risk of distal colon cancer. Our findings further suggest evidence of a possible interaction between *CYP24A1* and *CYP27B1* with distal colon cancer, and an interaction between *CYP27B1* and UV-weighted hours of sun exposure with proximal colon cancer.

Based on observations that *CYP27B1* is also expressed in the colon (12), it has been suggested that there may be a paracrine/autocrine role for 1,25-dihydroxyvitamin D, reducing cell proliferation and inducing differentiation locally (30). The involvement of *CYP27B1* in colon carcinogenesis is further supported by the finding that its expression was much lower or even lost in high-grade, poorly differentiated colon cancer cells compared with normal cells (31).

In contrast, the gene encoding for *CYP24A1* has been observed to be more highly expressed in undifferentiated colon cancer cells than in normal colon cells (32), potentially preventing tissue accumulation of 1,25-dihydroxyvitamin D and its anticarcinogenic effects. Taken together, results from experimental studies support the importance of *CYP27B1* and *CYP24A1* in the role of vitamin D on colon cancer.

To our knowledge, this is the first study to examine *CYP24A1* or *CYP27B1* variants and colon cancer using a comprehensive approach. Of the statistically significantly associated *CYP24A1* variants, *IVS9+198T>C*, although not highly conserved (conservation score <0.1, (21)),may be particularly interesting as it could be involved in the regulation of splicing, given that it is located in the intron directly before an alternatively spliced exon. Exon 10 is not present in previously identified human mRNA transcripts listed in GenBank (33), which implies that exon 10 has a weak splicing site, but the functional impact of this transcript is yet to be determined. To investigate whether this intronic variant could be linked with abnormal *CYP24A1* splicing, we conducted a small experimental study evaluating *CYP24A1* expression among EBV-lymphoblastoid cell lines with different genotypes of *IVS9+198T>C* (see supplemental material). We were unable to show, however, that normalized *CYP24A1* expression for all transcripts, or specifically the ratio of exon-10-containing transcripts to other transcripts, differed by genotype. However, a high degree of biologic variability between individual cell lines diminished our ability to determine whether *IVS9+198T>C* is involved in the regulation of *CYP24A1* expression and will require additional experiments to be conducted.

Of the other independently associated *CYP24A1* variants, *+4125bp 3′ of STP C>G* is located in the 3’ UTR, which could influence mRNA stability, but we were not able to detect any regulatory elements in the immediate region surrounding this variant (27). This tagSNP *+4125bp 3′ of STP C>G*, as well as *IVS9+198T>C* are correlated with synonymous SNP *Ex8-33G>A* (r^2 = 0.51 and r^2 = 0.62, respectively) which is highly conserved (conservation score=0.49) (21). Although it does not result in an amino acid change, its residue is located within the K α-helix of the protein, which may be near or within a substrate recognition site (34,35). It has recently been proposed that synonymous mutations are not necessarily silent and could result in a different folding configuration and/or function of the protein (36). Taken altogether, there are two strong candidate SNPs (*IVS9+198T>C* and *Ex8-33G>A*) that may be responsible for the observed associations.

The *CYP24A1* variant *IVS4-66T>G* was associated with a decreased risk of overall and subsite-specific colon cancer. This intronic variant is not considered highly conserved but is correlated (r^2 >0.6) with two potentially interesting variants: a highly conserved synonymous SNP in exon 6 (*Ex6+12G>A*, T248T; conservation score=0.77) and a large insertion/deletion that we detected through resequencing in intron 7 (*IVS7+223*, 99bp long). The functional impact of these two variants is again unclear. We attempted to genotype *Ex6+12G>A* in our study, but excluded results based on a low call rate. Future studies should attempt to include these additional variants.

We observed an inverse trend between a greater number of copies of the rare allele at locus *CYP24A1 IVS2+523C>T* and colon cancer. This variant resides near the starting site of an alternatively transcribed *CYP24A1* protein in which both exons 1 and 2 are missing and a portion of intron 2 is included as an extension of exon 3 (35). The truncated protein is still able to bind vitamin D substrates but is functionally inactive due to its missing mitochondrial targeting domain (35). If *CYP24A1* variant *IVS2+523C>T* was ultimately found to be associated with expression of this splice variant, this could explain our finding.

In this study, *CYP24A1* variant *IVS4+1653C>T* was associated with a decreased risk of distal colon cancer. As it resides within an intronic area which is not conserved and investigation of

its possible function did not reveal any interesting leads, it is unlikely to be responsible for the observed association. However, this variant is not in strong linkage disequilibrium (r^2>0.5) with other identified *CYP24A1* variants. Thus, we could not determine what variant or factor, if any, is driving the observed association with *IVS4+1653C>T*.

As *CYP24A1* and *CYP27B1* are involved in the regulation of vitamin D, it is highly plausible that these two genes may modify an association between measures of vitamin D and colon cancer risk. Although there was no evidence of an interaction between *CYP24A1* and either vitamin D measure, an inverse association between UV-weighted sun exposure and proximal colon cancer risk may depend on *CYP27B1 IVS6-29T>C* status: among those carrying the rare allele, increasing sun exposure was associated with lower cancer risk. This variant is located within a poorly conserved area and is in strong linkage disequilibrium with one other *CYP27B1* variant, *−1073C>G*, located in the 5' promoter region of the gene. The functional significance of both *−1073C>G* and *IVS6-29T>C* is unknown, but variants located in the 5' region of a gene may have an impact on transcriptional or translational control (37). Although we detected a statistically significant interaction with *CYP27B1*, our global test was based on only one SNP for *CYP27B1* (df=2), and may be due to chance.

In this study, we observed an association between *CYP24A1* variants and colon cancer, which appeared to be most consistent for distal colon cancer. There is extensive support from epidemiologic and experimental studies that risk factors have a different association with the development of colon tumors by anatomic site. The proximal and distal colon subsites have been characterized with both morphologic and genetic differences that would signify a potential difference in their etiology (38,39). Of the epidemiologic studies that evaluated vitamin D by subsite, several suggested a stronger inverse association with distal tumors (40–42), whereas other studies did not find a meaningful difference by subsite (28,43–46) or found an association with proximal only (47). However, the majority of studies that evaluated serum levels of 25-hydroxyvitamin D by subsite suggested a stronger inverse association with distal adenomas or cancer (48–52). An explanation for why the associations with vitamin D and *CYP24A1* may be specific to distal colon cancer is unclear. Further studies assessing the role of *CYP24A1* variants are required to verify whether this association is truly specific to the distal colon.

The selection of tagSNPs based on resequencing data allowed us to conduct a more comprehensive analysis of common genetic variation in *CYP24A1* than a candidate SNP approach. As *CYP24A1* is a large gene (20 kb), we may have missed some genetic variation by not resequencing the entire *CYP24A1* intronic region (77% sequenced). However, our resequencing strategy focused on regions of the gene most likely to be functionally relevant. We were not able to genotype all preselected tagSNPs due to incompatibility of some SNPs with the multiplexing assay; however the average coverage for *CYP24A1* was high (mean r^2 =0.89). TagSNP selection for *CYP27B1* was also based on resequencing data (98% sequenced); however, there was very little genetic variation (nucleotide diversity among multiethnic panel =2.04 x10^{-4}), perhaps due to its important role in maintaining calcium homeostasis. This study included a large number of cases and collected detailed data on dietary and environmental factors, thus allowing us to control for known confounders. As this is a case-control study, dietary data were collected retrospectively and may be subject to recall bias; however, data were ascertained rapidly after enrollment and collected with established and validated procedures (18). A limitation is that vitamin D status was assessed by vitamin D intake and sun exposure. Serum 25-hydroxyvitamin D in prospectively collected samples provides a more direct measurement. Although some *CYP24A1* results were nominally statistically significant, they should be interpreted with caution given that the global test for *CYP24A1* was not statistically significant and there remains a potential role of chance in these findings. None of these SNPs reached genome-wide significance. Nonetheless, the probable functional

significance of some SNPs provides us with the rationale to present results for individual SNPs and will make replication a worthwhile exercise.

The primary focus of our study was to evaluate the impact of common genetic variants in key genes in the vitamin D pathway other than *VDR*. Our results suggest that several variants in *CYP24A1* are associated with colon cancer. Etiologic studies and vitamin D prevention strategies should take the genetic variation of *CYP24A1* and the potential interaction between UV-weighted sun exposure and *CYP27B1* into account.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of cases and controls

	Cases (n=1600)	Controls (n=1949)
Age (years)*	64.9 ± 9.8	65.0 ± 10.1
Sex		
Male	895 (55.9)	1036 (53.2)
Female	705 (44.1)	913 (46.8)
Race		
White, not Hispanic	1461 (91.4)	1814 (93.1)
Hispanic	62 (3.9)	78 (4.0)
African American	72 (4.5)	53 (2.7)
Other	4 (0.2)	3 (0.2)
Study center		
Kaiser	769 (48.1)	804 (41.3)
Utah	578 (36.1)	796 (40.8)
Minnesota	253 (15.8)	349 (17.9)
Education		
Less than 12 years	262 (16.4)	249 (12.8)
High school graduate	451 (28.2)	545 (28.0)
Some college or post-high school	530 (33.1)	634 (32.6)
College graduate or higher	357 (22.3)	520 (26.7)
Income		
<$20,000	401 (27.0)	455 (24.8)
$20,000–40,000	533 (35.9)	627 (34.2)
$40,000–$60,000	348 (23.4)	438 (23.9)
$60,000 +	203 (13.7)	313 (17.1)
Mean UV-weighted hours of sun exposure (UV index hours/ week)	85.8 ± 70.9	87.4 ± 68.7
Mean total vitamin D (μg/day)†	10.6 ± 6.0	11.1 ± 6.2
Multivitamin supplement use‡	520 (32.5)	654 (33.6)
Vitamin D supplement use‡	25 (1.6)	53 (2.7)
Tumor site		
Distal	790 (49.4)	-
Proximal	771 (48.2)	-
Unknown	39 (2.4)	-

Continuous variables are displayed as mean values ± standard deviation and frequencies are displayed as counts (percentage).

*Defined as age at diagnosis for cases and age at recruitment for controls.

†Energy adjusted (residual method) dietary intake.

‡Supplement use defined as regular use (at least three times a week for at least one month) over the referent period.

Table 2

ORs and 95% CIs for the association between polymorphisms in *CYP24A1* and *CYP27B1* and colon cancer risk

Position/Genotype	MAF (%)†	Controls N	All colon cancer Cases N	OR*	95%CI	P for trend	Proximal colon cancer Cases N	OR*	95%CI	P for trend	Distal colon cancer Cases N	OR*	95%CI	P for trend
CYP24A1														
IVS1-105G>C (rs2248137)	0.401													
GG		687	546	1.00	-		269	1.00	-		260	1.00	-	
CG		862	709	1.01	0.87–1.18		347	1.01	0.84–1.23		347	1.04	0.86–1.25	
CC		332	286	1.01	0.83–1.23	0.91	127	0.93	0.72–1.20	0.66	153	1.11	0.87–1.42	0.41
CC + CG vs. GG				1.01	0.88–1.17			0.99	0.83–1.19			1.06	0.88–1.26	
IVS2+523C>T (rs2762942)	0.062													
TT		1712	1430	1.00	-		691	1.00	-		702	1.00	-	
TC		214	149	0.85	0.68–1.06		70	0.82	0.62–1.10		77	0.90	0.68–1.19	
CC		9	2	0.26	0.06–1.19	**0.05**	0	-	--	0.06	2	0.52	0.11–2.41	0.31
CC + TC vs. TT				0.83	0.66–1.03			0.79	0.59–1.05			0.88	0.67–1.16	
IVS2-105A>G (rs2259735)	0.416													
AA		662	510	1.00	-		249	1.00	-		245	1.00	-	
AG		895	756	1.08	0.93–1.25		376	1.11	0.91–1.34		363	1.07	0.88–1.30	
GG		363	308	1.03	0.84–1.25	0.67	135	0.94	0.73–1.21	0.88	167	1.13	0.89–1.44	0.30
GG + AG vs. AA				1.06	0.92–1.23			1.06	0.89–1.27			1.09	0.91–1.30	
IVS3+103T>C (rs6022999)	0.236													
TT		1112	933	1.00	-		444	1.00	-		463	1.00	-	
TC		692	538	0.90	0.78–1.04		273	0.97	0.81–1.16		254	0.85	0.71–1.02	
CC		128	120	1.00	0.76–1.32	0.37	50	0.89	0.62–1.28	0.55	68	1.11	0.79–1.55	0.51
CC + TC vs. TT				0.92	0.80–1.05			0.96	0.81–1.14			0.89	0.75–1.05	
IVS4+1653C>T (rs2181874)	0.243													
CC		1105	921	1.00	-		412	1.00	-		485	1.00	-	
CT		713	576	0.95	0.83–1.10		311	1.15	0.97–1.37		255	**0.80**	**0.67–0.95**	
TT		123	98	0.95	0.71–1.26	0.49	46	1.00	0.70–1.43	0.32	47	0.86	0.60–1.23	**0.03**
TT + CT vs. CC				0.95	0.83–1.09			1.13	0.95–1.34			**0.81**	**0.68–0.96**	
IVS4-66T>G (rs4809958)	0.174													
TT		1314	1144	1.00			555	1.00			562	1.00		

Position/Genotype	MAF (%)†	Controls N	All colon cancer Cases N	OR*	95%CI	P for trend	Proximal colon cancer Cases N	OR*	95%CI	P for trend	Distal colon cancer Cases N	OR*	95%CI	P for trend
GT		553	397	**0.82**	**0.71–0.96**		196	0.84	0.69–1.01		193	**0.82**	**0.67–0.99**	
GG		49	39	0.92	0.60–1.42	**0.03**	12	0.59	0.31-1.12	**0.02**	25	1.19	0.73–1.96	0.21
GG + GT vs. TT				**0.83**	**0.72–0.96**			**0.82**	**0.68–0.99**			0.85	0.70–1.02	
IVS5-162T>C (rs6013905)	0.169													
TT		1296	1120	1.00			543	1.00			550	1.00		
CT		514	378	**0.85**	**0.72–0.99**		185	0.86	0.70–1.04		186	0.85	0.70–1.03	
CC		52	41	0.91	0.60–1.38	0.06	13	0.60	0.33–1.12	**0.04**	26	1.17	0.72–1.90	0.35
CC + CT vs. TT				**0.85**	**0.73–0.99**			0.83	0.69–1.01			0.88	0.73–1.06	
IVS5-149C>G (rs2762939)	0.252													
CC		1055	876	1.00	-		404	1.00	-		448	1.00	-	
GC		740	586	0.94	0.82–1.08		300	1.06	0.89–1.26		273	0.85	0.71–1.02	
GG		130	118	1.04	0.79–1.36	0.74	59	1.14	0.82–1.59	0.38	57	0.97	0.69–1.37	0.23
GG + GC vs. CC				0.96	0.83–1.09			1.07	0.90–1.27			0.87	0.73–1.03	
IVS7+921C>T (rs2762938)	0.410													
TT		654	564	1.00	-		261	1.00	-		289	1.00	-	
TC		950	771	0.95	0.82–1.10		384	1.02	0.85–1.23		369	0.89	0.74–1.07	
CC		325	254	0.92	0.75–1.12	0.37	121	0.94	0.73–1.21	0.71	126	0.90	0.70–1.15	0.28
CC + TC vs. TT				0.94	0.82–1.08			1.00	0.84–1.19			0.89	0.75–1.06	
IVS7+1307A>T (rs2762936)	0.199													
AA		1211	1014	1.00	-		484	1.00	-		503	1.00	-	
TA		633	487	0.90	0.78–1.04		242	0.95	0.79–1.14		234	0.87	0.72–1.04	
TT		80	79	1.13	0.81–1.56	0.58	37	1.12	0.74–1.68	0.91	41	1.17	0.78–1.74	0.53
TT + TA vs. AA				0.93	0.81–1.07			0.97	0.81–1.15			0.90	0.75–1.07	
IVS9+198T>C (rs1570669)	0.340													
TT		830	651	1.00	-		321	1.00	-		313	1.00	-	
CT		880	714	1.02	0.88–1.18		349	1.01	0.85–1.21		349	1.03	0.86–1.24	
CC		225	225	1.21	0.97–1.50	0.15	97	1.08	0.82–1.42	0.66	122	**1.33**	**1.03–1.73**	0.07
CC + CT vs. TT				1.06	0.92–1.21			1.02	0.86–1.22			1.09	0.92–1.29	
+4125bp 3' of STP C>G (rs927648)	0.242													

Position/Genotype	MAF (%)†	All colon cancer					Proximal colon cancer				Distal colon cancer			
		Controls N	Cases N	OR*	95%CI	P for trend	Cases N	OR*	95%CI	P for trend	Cases N	OR*	95%CI	P for trend
CC		1082	855	1.00	-		410	1.00	-		423	1.00	-	
GC		733	595	1.01	0.88–1.16		291	1.04	0.87–1.24		293	1.00	0.84–1.19	
GG		115	125	1.29	0.98–1.70	0.21	55	1.18	0.84–1.68	0.4	64	1.32	0.95–1.85	0.29
GG + GC vs. CC				1.05	0.91–1.20			1.06	0.89–1.25			1.04	0.88–1.23	
Global P						0.10				0.24				0.13
CYP27B1														
IVS6-29T>C (rs4646536)	0.314													
TT		910	729	1.00	-		346	1.00	-		367	1.00	-	
CT		796	663	1.05	0.91–1.21		324	1.08	0.90–1.29		318	1.00	0.84–1.19	
CC		199	170	1.08	0.86–1.35	0.42	80	1.06	0.80–1.42	0.47	88	1.11	0.84–1.47	0.60
CC + CT vs. TT				1.06	0.92–1.21			1.08	0.91–1.28			1.02	0.86–1.21	

*Adjusted for age, race, sex and study center.

†MAF (minor allelic frequency) calculated from results from genotyping restricted to non-Hispanic White controls.

Table 3

Association between *CYP24A1* haplotypes and Colon Cancer among non-Hispanic whites

			All colon cancer		Proximal colon cancer			Distal colon cancer		
Haplotype	**Controls %**	**Cases %**	**OR* (95%CI)**	**p**	**Cases %**	**OR* (95%CI)**	**p**	**Cases %**	**OR* (95%CI)**	**p**
Block 1 (*IVS7+921C>T, IVS7+1307A>T, IVS9+198T>C*)†										
C-A-T	39.4	38.0	1.00		39.2	1.00		37.1	1.00	
T-A-C	31.1	32.3	1.07 (0.95–1.20)	0.29	30.6	0.98 (0.84–1.15)	0.83	34.2	1.15 (0.99–1.33)	0.07
T-T-T	17.4	15.8	0.94 (0.81–1.08)	0.39	16.5	0.95 (0.79–1.14)	0.56	15.2	0.93 (0.77–1.12)	0.42
T-A-T	8.6	9.4	1.14 (0.95–1.38)	0.16	9.5	1.14 (0.90–1.43)	0.28	9.0	1.11 (0.88–1.41)	0.37
rare			1.32 (1.01–1.73)	**0.05**		1.23 (0.87–1.72)	0.24		1.40 (1.01–1.95)	**0.05**
Global P				0.27			0.78			0.16

*Among non-Hispanic whites adjusted for age, sex and study center.

†Loci of SNPs included in Block 1 are in the following order: rs2762938, rs2762936, rs1570669.

Table 4

Association between UV-weighted hours of Sun Exposure and Proximal Colon Cancer Risk stratified by *CYP27B1* Genotype

Position/Genotype	UV-weighted Sun Exposure* Δ75 UV index-hours/week			
	Cases/Controls	OR†	95% CI	
***IVS6-29T>C* (rs4646536)**				
TT	342/897	1.02	0.89–1.18	
CT	325/783	**0.84**	**0.70–1.00**	
CC	80/198	0.94	0.69–1.28	
$P_{interaction}$				**0.03**
CC + CT		**0.86**	**0.74–1.00**	

*UV-weighted sun exposure based on the average hours per week of daylight reported, weighted by the UV index for each season in the geographic area of the study center.

†Adjusted for age, race, sex, and study center.