Research Article

Cancer Prevention Research

Plasma 25-Hydroxyvitamin D, Vitamin D Binding Protein, and Risk of Colorectal Cancer in the Nurses' Health Study

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Abstract

Total circulating 25-hydroxyvitamin D [25(OH)D)] has been associated with lower risk of colorectal cancer. The physiologic mechanism, however, may be more directly related to the free or bioavailable fraction of 25(OH)D, which is influenced by levels of vitamin D binding protein (VDBP). We assessed the association of prediagnosis total, free, and bioavailable 25(OH)D and VDBP with colorectal cancer risk among predominantly white women in the Nurses' Health Study (NHS) who provided a blood specimen in 1989–1990. We documented 378 cases of colorectal cancer through 2011 and matched them to 689 controls according to age and time of blood draw. We genotyped two common polymorphisms in the gene coding VDBP and calculated free and bioavailable 25(OH)D levels based on total 25(OH)D, VDBP, albumin, and their estimated genotype-specific binding affinities. Total 25(OH)D was associated with lower colorectal cancer risk (P for trend = 0.01). Compared with women in the lowest quintile of total 25(OH)D, those in the highest quintile had a multivariable-adjusted odds ratio (OR) for colorectal cancer of 0.54 [95% confidence interval (CI), 0.33–0.87]. Comparing extreme quintiles, we did not find any significant association with risk of colorectal cancer for VDBP (OR, 0.98; 95% CI, 0.65–1.47), free 25(OH)D (OR, 0.71; 95% CI, 0.46–1.10), or bioavailable 25(OH)D (OR, 0.92; 95% CI, 0.60–1.42). In conclusion, prediagnosis levels of total, but not free or bioavailable 25(OH)D, were associated with lower colorectal cancer risk. Although our findings support an inverse association of vitamin D with colorectal cancer, this association does not appear to be due to the unbound or bioavailable fraction of circulating vitamin D. *Cancer Prev Res; 9(8); 664–72. ©2016 AACR.*

Introduction

Vitamin D plays an important role in regulation of the systemic calcium and bone metabolism. Accumulating evidence suggests that vitamin D also possesses a wide spectrum of anticancer properties, including reduction of inflammation, suppression of cellular proliferation, induction of apoptosis and differentiation, inhibition of invasion and metastasis, and blockade of angiogenesis (1, 2).

Vitamin D is primarily synthesized in the skin upon exposure to ultraviolet B radiation, and can also be consumed from foods and supplements. Once released into circulation, vitamin D undergoes two sequential hydroxylation reactions: first in the liver to 25-hydroxyvitamin [25(OH)D] and then in the kidney and other organs to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. Although 25(OH)D is the primary circulating form of vitamin D and the most widely accepted biomarker for vitamin D status, 1,25$(OH)_2$D is the active form that acts through binding to nuclear vitamin D receptors expressed in various target tissues, including the colorectum. Human studies have documented a lower risk of colorectal cancer associated with a range of surrogates for vitamin D status, including circulating 25(OH)D, dietary and supplement intake (3), and predicted 25(OH)D based on major determinants of vitamin D status (4).

About 85% to 90% of circulating 25(OH)D is bound to vitamin D-binding protein (VDBP), the primary vitamin D carrier protein. The non–VDBP-bound fraction of 25(OH)D is primarily bound to albumin (10% to 25%), leaving less than 1% in a free state (5). Because the binding to albumin is considerably weaker than to VDBP, albumin-bound fraction of 25(OH)D is often grouped with the free fraction and referred to as the "bioavailable" 25(OH)D. In addition, there are common polymorphisms in the gene coding VDBP (group-specific component, *GC*) that produce variant proteins with different affinities for vitamin D (6, 7). Free or

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bioavailable 25(OH)D has been hypothesized to drive the actions of vitamin D in skeletal and extraskeletal sites (8), although supporting evidence is scant. Furthermore, VDBP has bioactivities other than transport function, including macrophage activation, actin scavenging, and chemotaxis, which have been implicated in inhibition of cancer development (9). However, human data on VDBP and colorectal cancer remain sparse. The only two prospective studies so far have not detected an association between circulating VDBP and colorectal cancer risk (10, 11). However, these studies lacked information on both genetic determinants of VDBP binding affinity and levels of albumin, precluding a more complete evaluation of the influence of free or bioavailable 25(OH)D on colorectal cancer risk.

Thus, to extend our knowledge about the role of vitamin D in colorectal cancer, we conducted a comprehensive assessment of plasma total 25(OH)D, VDBP and albumin, and genetic variations of VDBP in a nested case–control study within a large prospective cohort, the Nurses' Health Study (NHS). Earlier examinations of plasma total 25(OH)D in the NHS observed a significant inverse association with colorectal cancer (12–14). In the present study, we offer results that encompass total, free and bioavailable 25(OH)D, and VDBP to provide a more complete understanding about the relationship of vitamin D with colorectal cancer risk. In the secondary analysis, we also assessed whether VDBP and 25(OH)D modify the association of each other with colorectal cancer risk.

Materials and Methods

Study population

The NHS is a cohort of 121,700 female U.S. nurses who were enrolled in 1976 at the ages of 30 to 55 years. Details about the cohort have been described elsewhere (15). Briefly, women completed and returned an initial questionnaire on lifestyle and medical factors, and have then been followed biennially using mailed questionnaires designed to update medical, lifestyle, and other health-related information. The average follow-up rate of this cohort has been 95.4%, which is calculated as the number of person-years in the cohort when participants are censored after their last questionnaire response or at death divided by the total number of person-years in the cohort (i.e., participants are censored only upon death), times 100. When participants reported a diagnosis of colorectal cancer on biennial questionnaire, we asked for their permission to acquire medical records and pathologic reports. We identified deaths through the National Death Index and next-of-kin. For all colorectal cancer deaths, we requested permission from next-of-kin to review medical records. A study physician, blinded to exposure information, reviewed records to confirm colorectal cancer diagnosis and record pathologic data (16).

Between 1989 and 1991, 59,923 women in the NHS indicated that they would be willing to send us a blood sample, and among these women, 32,826 (55%) successfully donated blood specimens via overnight courier. The procedures for blood collection, handling, and storage have been previously summarized (17). Among participants who provided plasma samples, we confirmed 378 colorectal cancer cases after blood collection through April 1, 2011. We used risk set sampling to randomly select up to 2 controls for each case matched on age (within 2 years) and month/year of blood donation from eligible participants who were alive and free of cancer (except for nonmelanoma skin cancer) at the time of diagnosis of the colorectal cancer case. A total of 689 controls were included in the analysis. The study protocol was approved by the institutional review board of the Brigham and Women's Hospital.

Laboratory assays

Total 25(OH)D was measured using a radioimmunoassay at the laboratory of Dr. B.W. Hollis (The Medical University of South Carolina, Charleston, SC) and the Heartland Laboratory (Ames, IA; refs. 13, 18, 19). VDBP was measured using a monoclonal enzyme-linked immunosorbent assay (R&D Systems), and albumin was measured by a colorimetric assay (Roche Diagnostics) at the laboratory of Dr. N. Rifai (Children's Hospital, Boston, MA). We used a highly sensitive immunoturbidimetric assay (Denka Seiken Co) to measure C-reactive protein (CRP) and enzyme-linked immunosorbent assays (R&D Systems) to measure interleukin 6 (IL6), soluble tumor necrosis factor receptor 2 (sTNFR-2, also known as TNFRSF1B) and macrophage inhibitory cytokine-1 (MIC-1, also known as growth differentiation factor 15, GDF15), as previously described (18–20). Samples from case patients and their matched control participants were analyzed in the same batch. Quality control samples were randomly interspersed among the case–control samples. Personnel blinded to quality control and case–control status conducted all assays. The mean intra-assay coefficient of variation was 13.5% for total 25(OH)D, 12.7% for VDBP, 3.0% for albumin, 1.6% for CRP, 12.3% for IL6, 8.4% for sTNFR-2, and 9.0% for MIC-1.

Genotyping

Genomic DNA samples from the participants were genotyped for two common single-nucleotide polymorphisms (SNP) in the coding region of the *GC* gene (rs4588 and rs7041; ref. 21). We used the TaqMan Open Array SNP genotyping platform (Biotrove) with 384-well format TaqMan assays. TaqMan primers and probes were designed using Primer Express Oligo Design software v2.0 (ABI PRISM). Primers, probes, and conditions for genotyping assays are available upon request. All genotyping underwent standard quality control, as previously described (22).

Calculation of free and bioavailable 25(OH)D

We calculated free and bioavailable 25(OH)D using the following equations based on the laboratory data on the binding affinity constant of 25(OH)D to albumin (6×10^5) and the genotype-specific binding affinity of 25(OH)D to VDBP (K_{VDBP}) (5, 23). All concentrations are expressed in nmol/L.

$$\text{Free } 25(\text{OH})\text{D} = \frac{\text{Total } 25(\text{OH})\text{D}}{1 + (6 \times 10^5 \times \text{albumin}) + (K_{VDBP} \times \text{VDBP})}$$

$$\text{Bioavailable } 25(\text{OH})\text{D} = \text{Free } 25(\text{OH})\text{D} + 6 \times 10^5 \times \text{Albumin} \times \text{Free } 25(\text{OH})\text{D}$$

Genotype-specific K_{VDBP} values are shown in Table 1. The affinity constants for homozygotes are derived from Arnaud and Constans (6), and the mean affinity for the two homozygotes was used to represent that for heterozygotes. For other *VDBP* phenotypes not listed here, we used the race-specific affinities that accounted for the prevalence and affinity value of each genotype.

Calculated concentrations of free and bioavailable 25(OH)D have been validated previously, and showed a high correlation with direct measurements (r ranged from 0.81 to 0.90; refs. 5, 6). We then calculated the concentration of VDBP-bound 25(OH)D by subtracting bioavailable 25(OH)D from total 25(OH)D.

Table 1. Genotype-specific affinity constant used to calculate the concentration of free 25(OH)D

rs7041	rs4588	VDBP phenotype	Affinity constant ($\times 10^9$ per mol/L)
TT	CC	Gc1F homozygote	11.2
GG	CC	Gc1S homozygote	6.0
TT	AA	Gc2 homozygote	3.6
TT	AC	Gc1F/Gc2 heterozygote	7.4
TG	CC	Gc1F/Gc1S heterozygote	8.6
TG	AC	Gc1S/Gc2 heterozygote	4.8

Statistical analysis

We calculated age-adjusted Spearman correlation coefficients between biomarker levels and lifestyle factors among control participants. Plasma markers were categorized into quantiles on the basis of their distributions among control participants. We used conditional logistic regression to estimate the odds ratios (OR) and 95% confidence intervals (CI) of colorectal cancer in association with biomarker levels. Test for trend was performed using the median value for each quantile as a continuous variable in the regression models. Because albumin was only measured in a subset of controls due to limited fund and genotype data were missing in a few participants (see the footnote of Table 2), there were missing data in the calculated free and bioavailable 25(OH)D. Thus, for these two biomarkers, we restricted the analysis to the 300 cases and 337 matched controls with complete data. To control for confounding, we adjusted for several risk factors for colorectal cancer in the multivariable model. More details about covariate assessment and statistical analysis are provided in the Supplementary Materials.

We assessed the association of 25(OH)D and VDBP with risk of cancers according to anatomic subsites (proximal colon, distal colon, and rectum) and calculated the *P* for heterogeneity in the associations by tumor subsite using the contrast test statistic (24). To examine whether 25(OH)D and VDBP modified each other's association with colorectal cancer risk, we performed a stratified analysis and assessed the interaction using a Wald test for the product term between the stratified variable (binary) and main

Table 2. Baseline characteristics of colorectal cancer case and control participants in the Nurses' Health Study (1990)[a]

Variable	Cases ($n = 378$)	Controls ($n = 689$)	*P*
Age at blood draw, year	58.7 (6.8)	58.5 (6.9)	0.95
Colorectal cancer in a parent or sibling, %	23	19	0.15
History of lower gastrointestinal endoscopy, %	35	37	0.60
Postmenopausal, %	87	86	>0.99
Current use of hormones, %[b]	33	41	0.01
Current multivitamin use, %	32	38	0.40
Regular aspirin/NSAID use, %[c]	45	50	0.19
Current smoker, %	14	13	0.77
Pack-years of smoking	14.9 (21.7)	12.7 (18.5)	0.14
BMI, kg/m^2	25.9 (5.0)	25.3 (4.7)	0.03
Physical activity, MET-hours/week	16.1 (18.7)	16.4 (20.5)	0.86
Alcohol consumption, g/d	5.3 (9.6)	5.4 (9.4)	0.41
Folate intake, μg/d	426 (259)	445 (235)	0.03
Calcium intake, mg/d	982 (535)	1,050 (536)	0.003
Total fiber intake, g/d	18.6 (5.7)	18.8 (5.5)	0.54
AHEI diet score	46.2 (9.2)	46.2 (9.6)	0.94
Plasma biomarkers levels, median (IQR)[d]			
Total 25(OH)D, nmol/L	64.5 (48.7–79.9)	68.7 (52.7–84.0)	<0.001
VDBP, nmol/L	4,749 (3,521–6,273)	4,815 (3,656–6,237)	0.37
Free 25(OH)D, pmol/L	18.6 (13.9–27.2)	20.9 (15.2–28.3)	0.15
Bioavailable 25(OH)D, nmol/L	7.8 (5.6–11.4)	8.0 (5.9–11.2)	0.68
VDBP-bound 25(OH)D, nmol/L	54.1 (41.1–70.5)	59.0 (45.6–73.9)	<0.001
Albumin, g/dL	4.60 (4.40–4.70)	4.60 (4.40–4.80)	0.71
CRP, mg/L	1.52 (0.65–3.22)	1.64 (0.73–3.53)	0.29
IL6, pg/mL	1.18 (0.81–1.89)	1.13 (0.76–1.78)	0.59
sTNFR-2, pg/mL	2,658 (2,234–3,162)	2,582 (2,167–3,056)	0.15
MIC-1, ng/L	751 (602–930)	748 (571–964)	0.91
rs7401 genotype, %[e]			0.92
TT	34	33	
GG	45	46	
TG	21	21	
rs4588 genotype, %[f]			0.35
CC	7	9	
AA	37	38	
AC	56	53	

Abbreviations: AHEI, Alternative Healthy Eating Index; IQR, interquartile range; MET, metabolic equivalent; NSAID, non-steroidal anti-inflammatory drug; VDBP, vitamin D binding protein.

[a]Mean (SD) is presented for continuous variable unless otherwise specified. Percentage is presented for categorical variable.

[b]Percentage is among postmenopausal women only.

[c]Regular use is defined as ≥2 standard (325-mg) tablets of aspirin or NSAIDs per week.

[d]The number of paricipants that had missing measurements: 78 cases and 352 controls for free and bioavailable 25(OH)D, 45 cases and 315 controls for albumin, 41 cases and 81 controls for CRP, 42 cases and 82 controls for IL6, 43 cases and 81 controls for sTNFR-2, 42 cases and 313 controls for MIC-1.

[e]The percentage is calculated after excluding 31 cases and 48 controls that had missing rs7401 genotype.

[f]The percentage is calculated after excluding 30 cases and 53 controls that had missing rs4588 genotype.

Table 3. Age-adjusted Spearman correlation coefficient between plasma biomarkers and lifestyle factors among control participants in the Nurses' Health Study (1990)

	Total 25(OH)D	VDBP	Free 25(OH)D	Bioavailable 25(OH)D	VDBP-bound 25(OH)D	Albumin	CRP	sTNFR-2	IL6	MIC-1	BMI	METs-hours/week	Pack-years of smoking
Total 25(OH)D	1	0.09	0.60[a]	0.58[a]	0.98[a]	−0.02	−0.006	0.10[c]	−0.07	0.04	−0.12[c]	0.16[b]	0.06
VDBP		1	−0.58[a]	−0.57[a]	0.23[a]	0.04	0.03	0.11[c]	0.02	0.06	−0.06	−0.02	0.03
Free 25(OH)D			1	0.99[a]	0.45[a]	−0.02	−0.03	0.03	−0.09	0.02	−0.02	0.12[c]	0.02
Bioavailable 25(OH)D				1	0.44[a]	0.11[c]	−0.05	−0.007	−0.11[c]	0.009	−0.04	0.11[c]	0.03
VDBP-bound 25(OH)D					1	−0.03	0.008	0.11[c]	−0.06	0.04	−0.12[c]	0.14[b]	0.06
Albumin						1	−0.23[a]	−0.19[a]	−0.19[a]	0.003	−0.20[a]	0.04	0.009
CRP							1	0.27[a]	0.48[a]	0.15[b]	0.40[a]	−0.15[b]	0.02
sTNFR-2								1	0.19[a]	0.34[a]	0.28[a]	−0.07	−0.04
IL6									1	0.22[a]	0.26[a]	−0.10	0.10[c]
MIC-1										1	−0.01	−0.08	0.22[a]
BMI											1	−0.16[b]	−0.04
METs-hours/week												1	−0.10
Pack-years of smoking													1

Abbreviation: MET, metabolic equivalent.
[a]$P < 0.001$.
[b]$P < 0.01$.
[c]$P < 0.05$.

exposure (continuous). Using the same strategy, we also examined the potential modification by age and lifestyle factors for the association of 25(OH)D and VDBP with colorectal cancer risk.

We used SAS version 9.3 (SAS Institute, Inc.) for all analyses. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Table 2 shows the baseline characteristics of study participants. Compared with colorectal cancer cases, control participants had a slightly lower body mass index (BMI; 25.3 vs. 25.9 kg/m^2), were more likely to receive postmenopausal hormones (41% vs. 33%), and consumed more folate (445 vs. 426 μg/d) and calcium (1,050 vs. 982 mg/d). The median concentration of total and VDBP-bound 25(OH)D was higher among controls (68.7 and 59.0 nmol/L, respectively) than among cases (64.5 and 54.1 nmol/L, respectively; $P < 0.001$). In contrast, no statistically significant difference was observed between the case and control groups in plasma levels of other biomarkers, including VDBP, free or bioavailable 25(OH)D, or inflammatory markers ($P \geq 0.15$).

Plasma total 25(OH)D was not correlated with VDBP ($r = 0.09$), modestly correlated with free and bioavailable 25(OH)D ($r = 0.60$ and 0.58, respectively, $P < 0.001$), and strongly correlated with VDBP-bound 25(OH)D ($r = 0.98$; Table 3). Total, free, bioavailable, and VDBP-bound 25(OH)D were each correlated with physical activity ($r = 0.16$, 0.12, 0.11, and 0.14, respectively), whereas only total and VDBP-bound, but not free or bioavailable, 25(OH)D was negatively correlated with BMI ($r = -0.12$, -0.12, -0.02, and -0.04, respectively). No statistically significant correlation was detected between vitamin D biomarkers and inflammatory markers, except for a negative correlation between bioavailable 25(OH)D and IL6 ($r = -0.11$), and a positive correlation of total, VDBP-bound 25(OH)D, and VDBP with sTNFR-2 ($r = 0.10$, 0.11, and 0.11, respectively).

Consistent with previous reports in this cohort (12–14), high level of total 25(OH)D was associated with lower risk of colorectal cancer (Table 4). Compared with the lowest quintile, the highest quintile was associated with a multivariable-adjusted OR of 0.54 [95% confidence interval (CI), 0.33–0.87, P for trend = 0.01]. The association remained unchanged after further adjusting for plasma VDBP (OR = 0.54, 95% CI, 0.33–0.88, P for trend = 0.01) or inflammatory markers (OR = 0.49, 95% CI, 0.28–0.83, P for trend = 0.02). Excluding cases diagnosed within the first 2 years after blood draw also did not materially change these results (OR = 0.53, 95% CI, 0.32–0.87, P for trend = 0.02). Similar association was found for VDBP-bound 25(OH)D (comparing extreme quintiles: multivariable-adjusted OR = 0.48, 95% CI, 0.29–0.78, P for trend = 0.006).

In contrast, VDBP, or free or bioavailable 25(OH)D was not associated with colorectal cancer risk. The multivariable ORs (95% CIs) comparing the extreme quintiles were 0.98 (0.65–1.47) for VDBP; 0.71 (0.46–1.10) for free 25(OH)D; and 0.92 (0.60–1.42) for bioavailable 25(OH)D. To test for potential nonlinearity, we performed smoothing spline analysis and did not find strong statistical evidence for any of the biomarkers [including total 25(OH)D] to have a nonlinear relationship with colorectal cancer risk (data not shown).

The two SNPs in the *GC* gene were not associated with colorectal cancer risk. The adjusted OR was 1.01 (95% CI, 0.84–1.22, P for trend = 0.93) per one T-allele increment for rs7041 and 1.11 (95% CI, 0.90–1.37, P for trend = 0.34) per one C-allele increment for rs4588. Given the concern that the monoclonal VDBP assay gives much lower values for individuals with the Gc1F variant (25), we excluded the 38 participants carrying Gc1F homozygote and the results were essentially unchanged (comparing extreme quintiles of VDBP: OR = 1.02, 95% CI, 0.66–1.58, P for trend = 0.91). Similar results were also obtained after excluding 30 non-white participants (OR = 1.06, 95% CI, 0.69–1.62, P for trend = 0.89).

In the secondary analysis, we stratified by VDBP or total 25(OH)D according to their median levels in controls (Table 5). The inverse association between total 25(OH)D and colorectal cancer risk was stronger among individuals with low VDBP (194 cases, 344 controls; comparing the highest to the lowest quartile: OR = 0.57, 95% CI, 0.32–1.02, P for trend = 0.01) than among those

Table 4. Risk of colorectal cancer according to vitamin D-related biomarkers in the Nurses' Health Study (1990–2011)

Biomarker	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	Per 1-standard deviation increment[a]	*P* for trend
Total 25(OH)D							
Median, nmol/L[b]	38.0	55.8	68.7	79.8	101.5		
No. of cases/controls (n = 378/689)	94/138	87/138	69/138	76/138	52/137		
Age-adjusted OR (95% CI)[c]	1 (referent)	0.85 (0.58–1.25)	0.68 (0.46–1.02)	0.73 (0.49–1.09)	0.51 (0.33–0.79)	0.76 (0.64–0.91)	0.002
Multivariable-adjusted OR (95% CI)[d]	1 (referent)	0.88 (0.59–1.32)	0.76 (0.50–1.16)	0.77 (0.50–1.18)	0.54 (0.33–0.87)	0.78 (0.65–0.95)	0.01
VDBP							
Median, nmol/L[b]	2,528	3,994	4,815	5,904	7,749		
No. of cases/controls (n = 378/689)	83/137	82/138	70/139	64/138	79/137		
Age-adjusted OR (95% CI)[c]	1 (referent)	1.01 (0.68–1.51)	0.87 (0.57–1.30)	0.80 (0.54–1.20)	0.96 (0.65–1.43)	0.96 (0.84–1.11)	0.59
Multivariable-adjusted OR (95% CI)[d]	1 (referent)	1.01 (0.67–1.53)	0.88 (0.58–1.34)	0.82 (0.54–1.24)	0.98 (0.65–1.47)	0.97 (0.84–1.12)	0.68
Free 25(OH)D							
Median, pmol/L[b]	10.8	16.3	20.8	26.3	39.6		
No. of cases/controls (n = 378/689)	94/138	95/137	42/138	82/139	65/137		
Age-adjusted OR (95% CI)[c]	1 (referent)	0.97 (0.66–1.42)	0.43 (0.28–0.67)	0.81 (0.55–1.19)	0.65 (0.43–0.99)	0.85 (0.73–1.00)	0.05
Multivariable-adjusted OR (95% CI)[d]	1 (referent)	1.01 (0.68–1.49)	0.45 (0.28–0.71)	0.83 (0.55–1.25)	0.71 (0.46–1.10)	0.88 (0.74–1.04)	0.13
Bioavailable 25(OH)D							
Median, nmol/L[b]	4.1	6.3	8.0	10.4	15.4		
No. of cases/controls (n = 378/689)	88/137	84/139	49/137	75/139	82/137		
Age-adjusted OR (95% CI)[c]	1 (referent)	0.90 (0.61–1.34)	0.53 (0.35–0.82)	0.82 (0.55–1.21)	0.84 (0.56–1.26)	0.97 (0.83–1.13)	0.71
Multivariable-adjusted OR (95% CI)[d]	1 (referent)	0.92 (0.61–1.39)	0.54 (0.34–0.84)	0.84 (0.55–1.29)	0.92 (0.60–1.42)	1.01 (0.86–1.19)	0.92
VDBP-bound 25(OH)D							
Median, nmol/L[b]	33.1	48.0	59.0	70.4	90.4		
No. of cases/controls (n = 378/689)	96/138	90/138	60/137	83/138	49/138		
Age-adjusted OR (95% CI)[c]	1 (referent)	0.89 (0.61–1.31)	0.58 (0.38–0.87)	0.82 (0.55–1.21)	0.45 (0.29–0.71)	0.75 (0.63–0.89)	0.001
Multivariable-adjusted OR (95% CI)[d]	1 (referent)	0.93 (0.62–1.38)	0.63 (0.41–0.97)	0.86 (0.57–1.32)	0.48 (0.29–0.78)	0.77 (0.63–0.93)	0.006

[a]The standard deviation among controls was 26.5 nmol/L for total 25(OH)D, 1,955 nmol/L for VDBP, 12.0 pmol/L for free 25(OH)D, 4.7 nmol/L for bioavailable 25(OH)D, and 24.0 nmol/L for VDBP-bound 25(OH)D.

[b]Median was calculated among control participants.

[c]Adjusted for matching factors (age and time of blood draw).

[d]Additionally adjusted for family history of colorectal cancer, multivitamin use, pack-years of smoking (0, 0–9.9, 10–24.9, 25–49.9, ≥50), alcohol consumption (0, 0–5.0, 5.1–15.0, >15 g/d), BMI (continuous), physical activity (≤5, 5.1–10, 10.1–20, >20 METs), regular aspirin/NSAID use, postmenopausal status and hormone use (premenopausal, never use of postmenopausal hormone, ever use of postmenopausal hormone), calcium intake (in quartiles), and Alternative Healthy Eating Index (in quartiles).

with high VDBP (184 cases, 345 controls; OR = 0.84, 95% CI, 0.49–1.46, P for trend = 0.19). However, this difference did not reach statistical significance (P for interaction = 0.34). Similar results were observed for VDBP-bound 25(OH)D (P for interaction = 0.33). VDBP did not appear to modify the association of free or bioavailable 25(OH)D with colorectal cancer risk. We did not find any association between VDBP and colorectal cancer risk among high or low strata of total 25(OH)D.

We further examined the biomarker–colorectal cancer association by tumor subsite and did not detect any statistically significant heterogeneity between cancers in the proximal colon, distal colon, and rectum [P = 0.70 for total 25(OH)D, 0.49 for VDBP, 0.99 for free 25(OH)D, and 0.78 for bioavailable 25(OH)D; Supplementary Table S1].

Lastly, we assessed whether the association of total 25(OH)D and VDBP with colorectal cancer risk was modified by age and lifestyle factors (Supplementary Table S2). Total 25(OH)D was more strongly associated with lower colorectal cancer risk among women who were obese (comparing extreme quartiles: OR = 0.14, 95% CI, 0.03–0.76, P for trend = 0.002, P for interaction = 0.03) or regularly took aspirin/NSAIDs (OR = 0.39, 95% CI, 0.21–0.70, P for trend = 0.006, P for interaction = 0.03). However, given the small number of cases in each stratum derived in the context of multiple testing, these findings need to be interpreted with caution. There was no statistically significant interaction for 25(OH)D with age, physical activity, smoking, or alcohol consumption (P for interaction ≥ 0.07). The association of VDBP with colorectal cancer did not differ by age or lifestyle factors either (P for interaction ≥ 0.41).

Discussion

We prospectively assessed the association of prediagnosis vitamin D with colorectal cancer risk accounting for the various forms of circulating 25(OH)D according to levels of albumin, VDBP, and genetic variability in the binding affinity of VDBP. Consistent with our previous findings (12–14), plasma concentration of total 25(OH)D was associated with lower risk of colorectal cancer. This association appears to be driven by VDBP-bound component of 25(OH)D. In contrast, we did not find any association between circulating VDBP or calculated free or bioavailable 25(OH)D and colorectal cancer risk. VDBP did not appear to modify the association between 25(OH)D and colorectal cancer risk. These findings extend our knowledge about the inverse association between vitamin D and colorectal cancer risk, and have implications for the underlying biological mechanisms.

Substantial data support the antineoplastic properties of vitamin D (2, 26). A recent meta-analysis of 9 prospective studies showed that a 10 ng/mL (25 nmol/L) increment in blood 25(OH)D level was associated with 26% lower risk of colorectal cancer (3). Despite these compelling epidemiologic data and biological plausibility, randomized controlled trials (RCT) provide little support for the effect of vitamin D supplements on lowering colorectal cancer incidence (27–31). A recent RCT reported that

Table 5. Association between plasma 25(OH)D stratified by VDBP, and between VDBP stratified by 25(OH)D, and risk of colorectal cancer in the Nurses' Health Study (1990–2011)

Analysis	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* for trend	*P* for interaction[a]
Total 25(OH)D						
Low VDBP[b]						
No. of cases/controls	65/92	62/91	39/88	28/73		
Multivariable OR (95% CI)[c]	1 (referent)	0.96 (0.59–1.55)	0.62 (0.37–1.05)	0.57 (0.32–1.02)	0.01	0.34
High VDBP[b]						
No. of cases/controls	50/80	43/81	47/83	44/101		
Multivariable OR (95% CI)[c]	1 (referent)	0.84 (0.49–1.46)	0.84 (0.49–1.46)	0.84 (0.49–1.46)	0.19	
Free 25(OH)D						
Low VDBP[b]						
No. of cases/controls	35/41	39/63	48/97	72/144		
Multivariable OR (95% CI)[c]	1 (referent)	0.70 (0.38–1.29)	0.49 (0.27–0.91)	0.57 (0.32–1.00)	0.15	0.79
High VDBP[b]						
No. of cases/controls	83/131	53/110	32/74	16/29		
Multivariable OR (95% CI)[c]	1 (referent)	0.74 (0.47–1.17)	0.71 (0.43–1.20)	0.88 (0.43–1.77)	0.2	
Bioavailable 25(OH)D						
Low VDBP[b]						
No. of cases/controls	29/48	39/57	45/94	81/146		
Multivariable OR (95% CI)[c]	1 (referent)	1.21 (0.64–2.30)	0.74 (0.39–1.38)	0.97 (0.55–1.72)	0.87	0.73
High VDBP[b]						
No. of cases/controls	76/124	51/116	38/78	19/26		
Multivariable OR (95% CI)[c]	1 (referent)	0.67 (0.41–1.09)	0.85 (0.50–1.43)	1.27 (0.63–2.54)	0.77	
VDBP-bound 25(OH)D						
Low VDBP[b]						
No. of cases/controls	76/106	59/91	40/81	19/67		
Multivariable OR (95% CI)[c]	1 (referent)	0.88 (0.55–1.39)	0.69 (0.42–1.13)	0.41 (0.22–0.76)	0.007	0.33
High VDBP[b]						
No. of cases/controls	46/67	38/80	53/92	47/105		
Multivariable OR (95% CI)[c]	1 (referent)	0.67 (0.38–1.19)	0.82 (0.48–1.42)	0.59 (0.33–1.06)	0.14	
VDBP						
Low total 25(OH)D[b]						
No. of cases/controls	71/99	56/84	47/90	46/71		
Multivariable OR (95% CI)[c]	1 (referent)	1.00 (0.62–1.62)	0.83 (0.51–1.35)	0.95 (0.57–1.57)	0.46	0.79
High total 25(OH)D[b]						
No. of cases/controls	32/73	35/88	41/82	50/102		
Multivariable OR (95% CI)[c]	1 (referent)	0.95 (0.53–1.72)	1.12 (0.63–2.00)	1.12 (0.64–1.96)	0.50	

[a]Wald test was used for the product term between stratified variable (binary) and main exposure (continuous).

[b]Low level was defined as ≤median concentration among control participants [279 mg/mL for VDBP, 27.5 ng/mL for total 25(OH)D], and high level otherwise.

[c]Conditional logistic regression adjusted for matching factors (age and time of blood draw), family history of colorectal cancer, multivitamin use, pack-years of smoking (0, 0–9.9, 10–24.9, 25–49.9, ≥50), alcohol consumption (0, 0–5.0, 5.1–15.0, >15 g/d), BMI (continuous), physical activity (≤5, 5.1–10, 10.1–20, >20 METs), regular aspirin/NSAID use, postmenopausal status and hormone use (premenopausal, never use of postmenopausal hormone, ever use of postmenopausal hormone), calcium intake (in quartiles), and Alternative Healthy Eating Index (in quartiles).

daily supplement of 1,000-IU vitamin D_3 did not lower risk of adenoma recurrence among patients with a history of adenoma, a precursor lesion for colorectal cancer (27). Similarly, in the Women's Health Initiative, women who received 400 IU of vitamin D_3 with 1,000 mg of calcium daily had similar colorectal cancer incidence as compared with those receiving placebos (28). However, as discussed previously (32–34), these RCT data are far from conclusive given their limitations, including the relatively low dose used, suboptimal adherence, and "drop-in" of individuals who self-supplemented into the placebo group. In addition, for the RCTs of cancer as an endpoint, the relatively short duration of treatment and follow-up may have obscured any association. In this context, our current observation of a dose-dependent reduction of long-term colorectal cancer risk associated with total 25(OH)D, which is robust to adjustment for various confounding factors, including inflammatory markers, provides additional evidence for the potential benefit of vitamin D for colorectal cancer prevention.

Due to the high binding affinity of 25(OH)D to VDBP and the much higher plasma concentration of VDBP (μmol/L) than vitamin D (nmol/L), the vast majority of circulating 25(OH)D is present in a complex with VDBP (9). As a reservoir for 25(OH)D, VDBP may prolong its half-life and augment its beneficial effects. In addition to the binding capacity, VDBP functions as an actin scavenger, macrophage activating factor, and cochemotactic factor (9). Actin is released into circulation following tissue injury and cell death, and can result in vascular obstruction and organ dysfunction. The ability of VDBP to rapidly sequester actin protects against these complications and may account for its great molar excess compared with 25(OH)D (35). VDBP can also be deglycosylated to VDBP-macrophage activating factor (36), which has antiproliferation and antiangiogenic properties in tumor (37, 38). In addition, VDBP has a chemotactic effect by attracting neutrophils to the site of inflammation (39, 40). Although these data together suggest that VDBP may have an independent effect on cancer development, in the current study, we did not find any evidence that VDBP was associated with colorectal cancer risk. Rather, we noted that VDBP may modify the association between total 25(OH)D and lower colorectal cancer risk, which was restricted to the 538 individuals with relatively low VDBP. However, the confidence intervals were fairly wide and the interaction test did not achieve statistical significance. Two previous studies

have assessed circulating VDBP in relation to colorectal cancer incidence (10, 11). In line with our results, neither of the studies found an association between VDBP and colorectal cancer risk. Interestingly, one study of male smokers found that total 25(OH)D was associated with higher risk of colorectal cancer among individuals with high VDBP, with no association among those with low VDBP (11).

According to the "free hormone theory," if vitamin D directly inhibits colorectal carcinogenesis, the unbound free 25(OH)D should have a stronger anticancer effect because it is able to enter target cells and influence transcriptional and downstream biological events. Although our finding that neither free nor bioavailable 25(OH)D was associated with colorectal cancer risk appears inconsistent with this hypothesis, it is in line with two previous studies that did not find an association between the molar ratio of total 25(OH)D to VDBP, the surrogate for free 25(OH)D, and lower colorectal cancer risk (10, 11). Our results are more consistent with a critical role for the 25(OH)D–VDBP complex in synthesis of bioactive $1,25(OH)_2D$ (41). It has been shown that the 25(OH)D–VDBP complex, rather than free 25(OH)D, is taken up by the endocytic receptor megalin into the proximal tubules of the kidney or the epithelial cells of other target organs (including colon; refs. 42, 43). Endocytosed VDBP is degraded in lysosomes, while the 1α-hydroxylase enzyme, CYP27B1, converts 25(OH)D into $1,25(OH)_2D$, which exerts potential anticancer effects by activating vitamin D receptor in the colonic nucleus and regulating gene expression (26). Megalin knockout mice are unable to retrieve 25(OH)D from glomerular filtrate and develop severe vitamin D deficiency and bone disease (42). Therefore, the abundant 25(OH)D component of the endocytosed complex represents the major precursor for production of $1,25(OH)_2D$, making it likely that the trace-concentration free 25(OH)D is unrelated to colorectal cancer risk.

A major advantage of our study is the comprehensive assessment of total 25(OH)D, VDBP, genetic variation of *GC*, and albumin, which allowed us to evaluate the association of various forms of circulating vitamin D with colorectal cancer risk. Other strengths of our study include prospective blood collection, high follow-up rate of participants over 20 years, and detailed information on covariates and inflammatory marker levels, which permitted us to adjust for potential confounding and evaluate possible effect modification.

A limitation of the current study is lack of direct measurement for free or bioavailable 25(OH)D. Although the calculated levels based on VDBP and its estimated genotype-specific affinities have been shown to be well correlated with the measured concentrations (5, 6), the VDBP assay used for this study has not been standardized for diagnostic use. In addition to the different binding affinities for 25(OH)D, the variants of VDBP have distinct patterns of glycosylation, which could influence the performance of the assay (44, 45). Nevertheless, our findings are unlikely to be explained by the VDBP assay used in this study because samples from cases and their matched controls were measured in the same batch and under the same condition. In addition, the assay issue is more of a concern for ethnically mixed populations (25, 46, 47). Our study population is largely white (97%) and the results are robust to exclusion of the participants with Gc1F homozygote in VDBP. Another limitation of the study is potential measurement error in 25(OH)D due to the relatively high coefficient of variation of the assay, which may have diluted the association with colorectal cancer risk. However, this coefficient of variation for the 25(OH)D assay is comparable to previous studies (11, 48). In addition, we measured plasma markers at only one point in time that may not represent their long-term status. However, VDBP concentrations appear to be stable over much of the life course of an individual (49), and prior data indicate that 25(OH)D measurements in samples collected up to 14 years apart are well correlated (correlations of 0.50–0.70; ref. 50–52). Moreover, only a subset of randomly selected controls was measured for albumin, and therefore statistical power for free and bioavailable 25(OH)D analysis was reduced. Finally, we cannot exclude residual confounding due to the observational study design.

In conclusion, prediagnosis levels of total, but not free or bioavailable, 25(OH)D were associated with lower risk of colorectal cancer. We did not find any evidence that VDBP was associated with colorectal cancer risk. Although our findings support a protective effect of vitamin D against colorectal cancer, this effect does not appear to be due to the unbound or bioavailable fraction of circulating vitamin D.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The authors assume full responsibility for analyses and interpretation of these data. The funders had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript; and the decision to submit the manuscript for publication.

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