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Predicted 25(OH)D score and colorectal cancer risk according to vitamin D receptor expression

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Abstract

Background—Despite accumulating evidence for the preventive effect of vitamin D on colorectal carcinogenesis, its precise mechanisms remain unclear. We hypothesized that vitamin D was associated with a lower risk of colorectal cancer with high-level vitamin D receptor (VDR) expression, but not with risk of tumor with low-level VDR expression.

Methods—Among 140,418 participants followed from 1986 through 2008 in the Nurses' Health Study and the Health Professionals' Follow-up Study, we identified 1,059 incident colorectal cancer cases with tumor molecular data. The predicted 25-hydroxyvitamin D [25(OH)D] score was developed using the known determinants of plasma 25(OH)D. We estimated the hazard ratio (HR) for cancer subtypes using the duplication-method Cox proportional hazards model.

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Results—A higher predicted 25(OH)D score was associated with a lower risk of colorectal cancer irrespective of VDR expression level (*P*, heterogeneity for subtypes = 0.75). Multivariate HRs (95% confidence intervals) comparing the highest to the lowest quintile of predicted 25(OH)D scores were 0.48 (0.30-0.78) for VDR-negative tumor and 0.56 (0.42-0.75) for VDR-positive tumor. Similarly, the significant inverse associations of predicted 25(OH)D score with colorectal cancer risk did not significantly differ by *KRAS*, *BRAF*, or *PIK3CA* status (*P*, heterogeneity for subtypes ≥ 0.22).

Conclusions—A higher predicted vitamin D score was significantly associated with a lower colorectal cancer risk, regardless of VDR status and other molecular features examined.

Impact—The preventive effect of vitamin D on colorectal carcinogenesis may not totally depend on tumor factors. Host factors (such as local and systemic immunity) may need to be considered.

Keywords

vitamin D; nutrition; colorectal neoplasia; carcinoma; vitamin D receptor; *KRAS*; *BRAF*; *PIK3CA*; molecular subtype; molecular pathological epidemiology; epidemiology

Introduction

Vitamin D has long been hypothesized to be associated with a lower risk of colorectal cancer (1-3). A 10ng/mL increment in blood 25-hydroxyvitamin D [25(OH)D] level was associated with 26% lower risk of colorectal cancer (95% confidence interval [CI]; 0.63-0.89) in a meta-analysis of nine prospective studies (4). However, colorectal cancer is a heterogeneous disease in which each tumor evolves through distinct carcinogenic pathways acquiring a unique set of genetic and epigenetic aberrations over time (5). The inhibitory property of vitamin D on colorectal carcinogenesis may differ according to specific tumoral features of the colon (6).

Numerous studies support that vitamin D receptor may mediate the anti-carcinogenic effect of vitamin D (1, 7). The 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$), a hydroxylated form of 25(OH)D, binds to the vitamin D receptor (VDR). Then, activated VDR heterodimerizes with the retinoid X receptor alpha (RXRA) (1, 7). This complex translocates to the cell nucleus and binds to the vitamin D response element to regulate the transcription of a number of genes that control cellular proliferation, differentiation, angiogenesis, inflammation, and apoptosis (1). No previous studies have prospectively investigated the role of vitamin D according to VDR expression level in colorectal tumors. Thus, we hypothesized that a higher vitamin D status was associated with a lower risk of colorectal cancer with high VDR expression level but not with a risk of cancer with low VDR expression level.

To test our hypothesis we prospectively examined the association of a long-term predicted 25(OH)D score (8) with the risk of colorectal cancer according to the level of VDR expression in the Nurses' Health Study (NHS) and Health Professionals' Follow-up Study (HPFS). The predicted 25(OH)D score comprehensively takes into account both endogenous and exogenous sources of plasma 25(OH)D; cumulatively averaged 25(OH)D scores, in particular, could well represent long-term levels of plasma 25(OH)D status (8). In the

secondary analyses, we evaluated the association between the predicted 25(OH)D score and the risk of colorectal cancer as defined by the mutation status of *KRAS*, *BRAF*, and *PIK3CA*, which previously was found to interact with VDR (9-13).

Materials and Methods

Study population

The Nurses' Health Study (NHS) enrolled 121,700 US female registered nurses aged 30 to 55 from 11 U.S. States in 1976. The Health Professionals Follow-up Study (HPFS) enrolled 51,529 US male health professionals aged 40 to 75 from all 50 states in 1986. The details for both cohorts have been described previously (14). In brief, NHS and HPFS participants completed questionnaires inquiring about lifestyle factors and chronic disease history at the initiation of each cohort study. This information has been updated via biennial questionnaires thereafter.

In this analysis, the baseline year was 1986, when all the determinants of plasma 25(OH)D were first collected to compute the predicted 25(OH)D score. We excluded participants with a missing predicted 25(OH)D score and those previously diagnosed with cancer (except nonmelanoma skin cancer) or ulcerative colitis. After exclusion, we included 96,239 women from the NHS and 44,197 men from the HPFS. The institutional review board at the Brigham and Women's Hospital (Boston, MA), and the Harvard School of Public Health (Boston, MA) approved the NHS and the HPFS studies, respectively. All participants provided informed consent at enrollment.

Predicted 25(OH)D score

The derivation and validation of the predicted 25(OH)D score in NHS and HPFS have been previously described (8). In brief, significant clinical predictors of circulating plasma 25(OH)D, including race, UV-B flux, dietary vitamin D, supplementary vitamin D, body mass index, physical activity, alcohol intake (NHS only), and postmenopausal hormone use (NHS only), were regressed on a plasma 25(OH)D concentration among the population of NHS (N=2079) and HPFS (N=911) who were disease-free and had their blood measured for plasma 25(OH)D. The predicted 25(OH)D score was computed by summing the value of each significant determinant that was weighted by their individual associations with plasma 25(OH)D.

The validity of the predicted 25(OH)D score was assessed by using the plasma 25(OH)D level, a gold standard indicator of vitamin D status (8) because it reflects vitamin D from both dietary and solar sources (15) with a three-week half-life (16). In the independent subpopulation of NHS and HPFS whose plasma 25(OH)D was not used for the creation of a predicted 25(OH)D score, the correlation coefficients between predicted 25(OH)D score and plasma 25(OH)D, adjusted for laboratory batch, age, and season of blood draw, were 0.33 for NHS and 0.30 for HPFS, respectively. The plasma 25(OH)D concentration increased with the 25(OH)D score; the mean differences of the actual 25(OH)D level between extreme deciles of the predicted 25(OH)D score were 8.7ng/mg for both NHS and HPFS. In addition, the predicted 25(OH)D score per 10ng/mL yielded an odds ratio of 0.78 for colorectal

cancer risk, which is similar to the risk estimate associated with measured plasma 25(OH)D (odds ratio: 0.82 per 10 ng/mL increment) (8, 17).

Ascertainment of colorectal cancer cases

The participants reported newly diagnosed colorectal cancer on each follow-up questionnaire. Among non-respondents, we searched the National Death Index to identify the cause of fatality (18). Through these methods we identified approximately 96-97% of cases (19), and we requested permission to review the medical records and pathology reports. Investigators, blinded to the information of the participants, confirmed the reported cases and extracted information on histologic type, stage, and anatomic location of cancer. Among 2,604 colorectal cancer cases identified during the study follow-up, we collected 1,059 tumor specimens for this study. Among those, we obtained data on tissue microarray immunohistochemistry of VDR expression (N=743) and mutation status of *KRAS* (N=1045), *BRAF* (N=1044), and *PIK3CA* (N=970).

Immunohistochemistry for nuclear VDR expression

For this study we measured the nuclear VDR expression level in colorectal cancer tissue because VDR modulates genomic transcription in cell nuclei (1, 7) and the nuclear VDR level—not the cytoplasmic VDR level—has been a prognostic marker of improved survival (20, 21). We retrieved formalin-fixed paraffin-embedded colorectal cancer tissue blocks from hospitals throughout the United States where colorectal cancer patients had undergone surgical resection. Tissue microarray blocks were constructed as previously described (22). Colorectal cancer cases with VDR expression are limited to those with available tissue microarray for the immunohistochemistry. The pathologists were blinded to patients' information on lifestyle or medical histories. The demographic characteristics of cases with available nuclear VDR expression were similar to those without nuclear VDR expression data (mean age, 56.9 vs. 57.5 years; women, 64 vs. 56%; White, 93% vs. 93%; mean BMI [kg/m^2], 25.9 vs. 25.9; mean physical activity [metabolic equivalent score per week], 14.9 vs.16.3; mean pack years of smoking, 4.8 vs. 4.6; current multivitamin use, 39% vs. 38%; family history of colorectal cancer, 11% vs. 13%; mean red meat intake, 1.2 vs. 1.2 servings/day, mean calcium intake 884.2 vs. 864.8 g/d, mean folate intake 419.0 vs. 421.0 μg/d; $P \geq 0.05$ for all comparisons).

For VDR immunostaining, deparaffinized tissue sections were heated using a pressure cooker in a microwave for 15 minutes in Antigen Retrieval Citra Solution, pH 6 (BioGenex Laboratories, San Ramon, CA). Tissue sections were incubated with Dual Endogenous Enzyme Block (DAKO, Carpinteria, CA), then Serum Free Protein Block (DAKO), each for 15 minutes. Slides were incubated at 4°C overnight with a primary antibody against VDR (1:500, rabbit polyclonal; Novus Biologicals, NBP1-19478, Littleton, CO), diluted in Da Vinci Green Diluent (Biocare Medical, Concord, CA). Envision™ anti-rabbit HRP-labeled polymer (DAKO) was applied to the sections for 30 minutes followed by visualization using the chromogen 3,3-diaminobenzidine (DAKO), and hematoxylin counterstain; VDR (N204) detects endogenous levels of VDR protein and synthetic peptide corresponding to the residues surrounding Asparagine 204 of human VDR.

The level of nuclear VDR expression in the tumor tissue was assessed using a semi-quantitative immunoreactivity scoring (SIS) system (23). The staining intensity was scored as 1 (no immunostaining) to 4 (strong). The percentage of immunoreactive nuclear cells was rated from 0-100%. The SIS score was calculated by multiplying the scores for expression intensities with the percentage of positive cells resulting in the score variation from 0 to 400. We defined VDR expression as positive (SIS score≥ 180) and negative (SIS score<180); the cutoff for dichotomization of nuclear VDR expression level was chosen at the nuclear VDR level where survival length among colorectal cancer survivors significantly differs. In addition, we classified colorectal tumor into three subgroups using the tertile of VDR expression level in secondary analyses.

Sequencing of *BRAF*, *KRAS*, and *PIK3CA*

DNA was extracted from paraffin-embedded tissues. PCR and pyrosequencing were performed for *KRAS* (codons 12, 13, 61 and 146) (24, 25), *BRAF* (codon 600) (26), and *PIK3CA* (exons 9 and 20) (27), as described previously

Assessment of covariates

Lifestyle and other information (body mass index, physical activity, smoking status, aspirin use, multivitamin use, family history of colorectal cancer in first-degree relatives, and history of endoscopy) was self-reported on biennial questionnaires. Dietary information was assessed via validated semi-quantitative food frequency questionnaires with ∼130 food items every 4 years in the NHS (28) and the HPFS (29). Daily nutrient intake was calculated by multiplying the frequency response of each specified food item by the nutrient content of the specified portion sizes then summing these products for all food items.

Statistical analyses

We categorized predicted 25(OH)D into quintiles separately within each cohort. To investigate long term 25(OH)D exposure and minimize the influence of measurement error, we used the cumulatively averaged predicted 25(OH)D score as our exposure. Sensitivity analyses using a simple updated and baseline predicted 25(OH)D were conducted.

We calculated hazard ratios (HRs) and 95% confidence intervals (95% CIs) separately for incidence of overall and subtypes of colorectal cancer using duplication-method Cox proportional cause-specific hazards regression for competing risk data (30). Cases without information on relevant molecular subtypes were censored at the time of colorectal cancer diagnosis. Person-years of follow-up were calculated from the date of baseline questionnaire return to the date of diagnosis of colorectal cancer, date of death, loss to follow-up, or the end of follow-up (2008 for NHS; 2008 for HPFS), whichever came first. We included age (in months) and the year of questionnaire cycle as stratification variables. In multivariate analyses we included potential confounding variables. We updated covariates in each questionnaire cycle to take into account potential changes over time. A missing indicator for missing responses of each covariate was created, if applicable. We tested for trend using the Wald test of the continuous variable set to the median values of quintiles of 25 (OH)D score. We pooled participants from the NHS and the HPFS and included a cohort indicator as stratification variable in the model. Before pooling the cohorts we tested between-studies

heterogeneities using Q statistic (31, 32) and found no significant heterogeneities (*P*, heterogeneity between studies > 0.19 for all analyses). To test the significance of differential association by the tumor molecular characteristic, we conducted the likelihood ratio test comparing the model fit that allows separate associations by different molecular subtypes (i.e., VDR-positive vs. VDR-negative cancers) to the model fit that assumed a common effect. With 80% power, the ratio of RR for detecting significant heterogeneity by tumor subtypes were 1.30 for the analysis of VDR expression, 1.15 for *KRAS* mutation, 1.65 for *BRAF* mutation, and 1.59 for *PIK3CA* mutation.

In addition, to evaluate the nonlinearity of the association of predicted 25(OH)D score with the risk of overall and molecular subtypes of colorectal cancer, we compared the model fit between the model with the linear term and cubic spline terms and the model without spline terms (33-35); the log-likelihood ratio test comparing those two model was statistically significant suggesting a nonlinear association. Therefore, we considered the predicted 25(OH)D score as a categorical variable in our analyses.

We used the SAS software (SAS Institute, Inc., Version 9, Cary, NC). All tests were two-sided and a $P<0.05$ was considered statistically significant.

Results

Among 140,418 participants followed up from 1986 through 2008, we documented 1,059 incident colorectal cancer cases (41% of all colorectal cancer cases) with tumor molecular data on the level of VDR expression (N=743) and mutation status for *KRAS* (N=1,045), *BRAF* (N=1,044), and *PIK3CA* (N=970). Table 1 summarizes the age-standardized characteristics of the study population at the median follow-up time according to the level of predicted 25(OH)D score. As expected, participants with higher predicted 25(OH)D scores were more likely to live in the Southern states, have a lower body mass index, report higher physical activity, have higher multivitamin intake, and consume more vitamin D, calcium, and folate. The differences in the median across the extreme quintiles of predicted 25(OH)D score were 7.8 ng/ml in men and 10.4 ng/ml in women, which is equivalent to 22-29 μg/d increase in vitamin D intake (36) (Table 1).

Since the results from men and women did not significantly differ (Supplementary Tables 1 and 2), here we present the results from the pooled analyses. We first examined the association of predicted 25(OH)D score with the risk of overall colorectal cancer with available molecular data in our cohorts (Table 2). The predicted 25(OH)D score was inversely associated with the risk of colorectal cancer. In the pooled cohort, the multivariate HR (95% CI) was 0.52 (0.42-0.64) comparing the highest to the lowest quintile of the predicted 25(OH)D score (*P*, trend <.001). Because some individual components of predicted 25(OH)D score are associated with colorectal cancer, we evaluated whether the observed association between predicted 25(OH)D score and colorectal cancer incidence was confounded by those individual components (8). We found that the inclusion of body mass index, physical activity, and alcohol consumption into the model yielded similar results to our original main model (Table 2).

We further evaluated the association of predicted 25(OH)D score with the risk of colorectal cancer according to VDR expression level (Table 2). We found that the predicted 25(OH)D score had a statistically significant inverse association with the risk of both VDR-positive and VDR-negative colorectal tumors. In the pooled cohort, multivariate HRs (95% CI) were 0.48 (0.30-0.78) for VDR-negative and 0.56 (0.42-0.75) for VDR-positive colorectal cancer comparing the highest to the lowest quintile of predicted 25(OH)D score. The difference in the associations for VDR-positive vs. VDR-negative tumors was not statistically significant (*P*, heterogeneity for subtypes = 0.75). Additionally, we grouped the colorectal tumors using the tertile of VDR expression level to evaluate whether the inverse association of the predicted 25(OH)D score with colorectal cancer risk differed by VDR expression level with a linear trend; we found no evidence of linear trend for heterogeneity. Pooled multivariate HRs (95% CI) of predicted 25(OH)D comparing extreme quintiles were 0.47 (0.29-0.74), 0.63 (0.43-0.93) and 0.51 (0.33-0.78) for colorectal tumors with low, medium, and high VDR levels, respectively (*P*, heterogeneity for subtypes = 0.67).

We also examined whether the association of predicted 25(OH)D score with the risk of colorectal cancer differed according to the mutation status of *KRAS*, *BRAF*, or *PIK3CA* (Table 3). A higher predicted 25(OH)D score was inversely associated with colorectal cancer risk, regardless of *KRAS*, *BRAF*, or *PIK3CA* status (*P*, heterogeneity for subtypes ≥ 0.22). Pooled multivariate HRs (95% CI) were 0.46 (0.35-0.60) for *KRAS* wild-type versus 0.70 (0.50-0.98) for *KRAS*-mutant, 0.52 (0.41-0.65) for *BRAF* wild-type versus 0.58 (0.35-0.94) for *BRAF*-mutant, and 0.51 (0.40-0.65) for *PIK3CA* wild-type versus 0.54 (0.32-0.90) for *PIK3CA*-mutant (*P*, trend ≤ 0.01). Given the interrelationship of key regulatory molecules that may obscure an association, we further evaluated the association of the predicted 25(OH)D score with colorectal cancer risk according to the combination of VDR with *KRAS* or *PIK3CA* mutation status (9, 10, 12, 13). However, we did not observe substantial or statistically significant differences in risk estimates according to any of these categories of tumor subtypes (data not shown).

We conducted sensitivity analyses excluding the participants who were diagnosed within the first 4 years of follow-up to minimize the influence of alterations in lifestyle due to prediagnostic symptoms or occult cancer. These results were not materially different from our original findings (data not shown).

Discussion

In this large prospective study, the inverse association between the predicted 25(OH)D score and colorectal cancer incidence did not significantly differ according to VDR expression level. In addition, we did not observe significant differences in the association by mutation status of *KRAS*, *BRAF*, and *PIK3CA*. To the best of our knowledge, our study is the first to examine the association of vitamin D with the risk of colorectal cancer according to these tumor molecular features. Our results suggest that the apparent benefit of vitamin D on the risk of colorectal cancer may be uniform regardless of the heterogeneous carcinogenic pathways analyzed in this study.

In the current literature, several lines of mechanistic evidence have suggested that vitamin D may have a stronger inverse association with the risk of colorectal tumor with high level of VDR expression. Colon epithelial and cancer cells express both VDR (37) and 1-α-hydroxylase (CYP27B1) (38), which converts 25(OH)D into the $1,25(OH)_2D$ that binds to VDR. The recent genome-wide association study identified numerous VDR binding sites in the colon (39), thus suggesting that the colon may be more likely to be influenced by VDR signaling than are other organs. Furthermore, experimental studies observed that the growth arrest induced by vitamin D disappeared in VDR knock-out cell lines (40, 41).

However, we found no evidence of differential association of the predicted 25(OH)D score with the risk of colorectal cancer according to VDR expression level. Further, we also did not observe significantly varied association of predicted 25(OH)D score with colorectal tumor defined by mutation status of *KRAS*, *BRAF*, and *PIK3CA* that are downstream of EGFR signaling (42). Previously, some studies suggested the anti-cancer effect of vitamin D in the oncogenic pathways involving VDR and RAS-mitogen-activated protein kinase (MAPK) (9, 12, 13) or phosphatidylinositol-4,5-bisphosphonate 3-kinase (PI3K)-AKT pathways (9). Despite these experimental data suggesting the potential variation of association between vitamin D and colorectal cancer according to tumor features, there was no empirical evidence from prospective population-based studies.

The consistent and significant inverse association of the predicted 25(OH)D score with the risk of all subtypes of colorectal cancer we observed suggests that vitamin D may not act through a single mechanism to inhibit carcinogenesis. Supporting this speculation, experimental studies have demonstrated that vitamin D reduces proliferation, inflammation, and angiogenesis, stimulates differentiation and apoptosis, and enhances the immune system (1, 2). Recent observations that suggest the interaction of vitamin D with IGF1 (43) and the influence of vitamin D on the regulation of microRNAs (44) and chromatin epigenetic alteration (44, 45) add to the mechanistic complexity of the action of vitamin D. Confirmation from a future large study is warranted given that our study is the first epidemiologic study that used VDR expression level in colorectal tumor tissue. Nonetheless, the consistent inverse associations we observed may reflect the pleiotropic anti-tumoral biological function of vitamin D.

Our result of the inverse association between the predicted 25(OH)D score and the risk of colorectal cancer is aligned with current literature. Previous results have consistently supported the benefit of vitamin D in preventing colorectal cancer (46). The largest nested case-control study that measured 25(OH)D level among 1,248 cases and 1,248 controls across 10 Western European countries reported the relative risk (RR) for colorectal cancer of 0.60 (95% CI, 0.46-0.80) comparing the highest to the lowest quintile of plasma 25(OH)D (47). The meta-analyses of 9 studies (7 cohort studies and 2 nested case-control studies) with 2,767 cases and 3,948 controls reported that the summary RR was 0.67 (95% CI, 0.54-0.80) comparing the highest versus lowest categories of blood 25(OH)D levels (4).

Our study has several strengths. Prospectively collected data eliminated recall bias. Bias that could have occurred in the selection of controls in a case-control design was avoided in our prospective study design. The follow-up rate of the participant in NHS and HPFS is high

over 90%. With a long-term follow-up of 24 years, we were able to examine several potential latency periods relating vitamin D to cancer risk. We were able to evaluate several tumor markers concurrently through comprehensive assessment of tumor pathological and molecular characteristics. Our integrative molecular pathological epidemiology approach aimed to examine potential links between exposures and molecular signatures of disease, and obtain mechanistic insights on biological influences of exposures on molecular pathways to the disease (48-50). Tumor molecular analyses have become increasingly common in research and clinical practice (51-54). By cumulatively updating the predicted 25(OH)D score, we could examine average long-term vitamin D status compared to using a single measurement of actual 25(OH)D (half-life 3 weeks) (16). The predicted 25(OH)D score in our analyses accounts for both diet and sun exposure, capturing the variation of circulating plasma 25(OH)D comprehensively (8). In addition, detailed information on lifestyle, diet, and other risk factors allowed us to finely adjust for potential confounding factors for colorectal cancer.

Our study has several limitations. Regarding the use of the predicted 25(OH)D score, the predicted 25(OH)D level may be confounded by its predictors; however, our results did not change materially when we added individual components of the score into the main model. Although the predicted 25(OH)D score is a validated predictor of the plasma 25(OH)D level in the NHS and HPFS, the misclassification of the actual level of 25(OH)D is still possible due to factors that were not considered at the derivation of the predicted score. For example, a recently large-pooled GWAS study identified polymorphisms that significantly predict circulating 25(OH)D levels (55). The measurement error might be non-differential attenuating the associations observed.

In addition, we could not retrieve the tumor marker information for all of our colorectal cancer cases. However, the risk estimates for incident colorectal cancer using cancer cases with tumor marker information were similar to those for incident cancer in the entire population (56). Due to limited cases, our study had limited power to detect differential associations of colorectal cancer risk by molecular subtypes (5, 48). Another limitation is that our measurement of VDR expression level may not reflect changes in VDR levels as a tumor progresses (37). There is currently no standardized method to assess VDR expression level in colorectal tumors or a consensus on cut-off to classify VDR over-expression. However, results did not change when we alternatively divided colorectal cancer into two groups using the median value of tumoral VDR expression level (data not shown), and we did not observe any trend for the association of the predicted 25(OH)D score with the risk of colorectal cancer when we further classified the colorectal tumor by tertile categories of tumoral VDR expression level. In addition, we cannot rule out whether our result is driven by residual confounding. For example, VDR signaling needs not only vitamin D as a ligand but also the retinoid X receptor (57) and protein complexes (58) to be activated and to unwind the chromosomal constraint (57). However, the distribution of those multiple components may not be differential with respect to the level of either vitamin D or VDR expression in a colorectal tumor.

In conclusion, we observed that prediction of a high predicted 25(OH)D score was significantly associated with the lower risk of colorectal cancer regardless of VDR

expression level or molecular features of colorectal tumors. Our observation supports a broad influence of vitamin D on colorectal carcinogenesis that may involve multiple biological pathways including host immunity. As vitamin D status is determined by many modifiable lifestyle factors, our results may further indicate that diet and lifestyle associated with high level of vitamin D level can be recommended to prevent colorectal cancer. Future studies with large numbers of cases are warranted to replicate our observations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nature reviews Cancer. 2007; 7:684–700.
2. Krishnan AV, Feldman D. Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. Annual review of pharmacology and toxicology. 2011; 51:311–36.
3. Giovannucci E. Epidemiology of vitamin D and colorectal cancer: casual or causal link? The Journal of steroid biochemistry and molecular biology. 2010; 121:349–54. [PubMed: 20398758]
4. Ma YL, Zhang P, Wang F, Yang JJ, Liu ZH, Qin HL. Association Between Vitamin D and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies. J Clin Oncol. 2011; 29:3775–82. [PubMed: 21876081]
5. Ogino S, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. Gut. 2011; 60:397–411. [PubMed: 21036793]
6. Colussi D, Brandi G, Bazzoli F, Ricciardiello L. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. International journal of molecular sciences. 2013; 14:16365–85. [PubMed: 23965959]
7. Lamprecht SA, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nature reviews Cancer. 2003; 3:601–14.
8. Bertrand KA, Giovannucci E, Liu Y, Malspeis S, Eliassen AH, Wu K, et al. Determinants of plasma 25-hydroxyvitamin D and development of prediction models in three US cohorts. The British journal of nutrition. 2012; 108:1889–96. [PubMed: 22264926]
9. Kure S, Nosho K, Baba Y, Irahara N, Shima K, Ng K, et al. Vitamin D receptor expression is associated with PIK3CA and KRAS mutations in colorectal cancer. Cancer epidemiology,

biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2009; 18:2765–72.

10. Hughes PJ, Lee JS, Reiner NE, Brown G. The vitamin D receptor-mediated activation of phosphatidylinositol 3-kinase (PI3Kalpha) plays a role in the 1alpha,25-dihydroxyvitamin D3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cell lines. Journal of cellular biochemistry. 2008; 103:1551–72. [PubMed: 17879954]
11. Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget. 2011; 2:135–64. [PubMed: 21411864]
12. Buitrago CG, Pardo VG, de Boland AR, Boland R. Activation of RAF-1 through Ras and protein kinase Calpha mediates 1alpha,25(OH)2-vitamin D3 regulation of the mitogen-activated protein kinase pathway in muscle cells. The Journal of biological chemistry. 2003; 278:2199–205. [PubMed: 12417593]
13. Narayanan R, Sepulveda VA, Falzon M, Weigel NL. The functional consequences of cross-talk between the vitamin D receptor and ERK signaling pathways are cell-specific. The Journal of biological chemistry. 2004; 279:47298–310. [PubMed: 15331595]
14. Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. The New England journal of medicine. 2012; 367:1596–606. [PubMed: 23094721]
15. Wei MY, Giovannucci EL. Vitamin D and multiple health outcomes in the Harvard cohorts. Molecular nutrition & food research. 2010; 54:1114–26. [PubMed: 20486209]
16. Wootton AM. Improving the measurement of 25-hydroxyvitamin D. The Clinical biochemist Reviews / Australian Association of Clinical Biochemists. 2005; 26:33–6. [PubMed: 16278775]
17. Wu K, Feskanich D, Fuchs CS, Willett WC, Hollis BW, Giovannucci EL. A nested case control study of plasma 25-hydroxyvitamin D concentrations and risk of colorectal cancer. Journal of the National Cancer Institute. 2007; 99:1120–9. [PubMed: 17623801]
18. Stampfer MJ, Willett WC, Speizer FE, Dysert DC, Lipnick R, Rosner B, et al. Test of the National Death Index. American journal of epidemiology. 1984; 119:837–9. [PubMed: 6720679]
19. Giovannucci E, Liu Y, Rimm EB, Hollis BW, Fuchs CS, Stampfer MJ, et al. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. Journal of the National Cancer Institute. 2006; 98:451–9. [PubMed: 16595781]
20. Srinivasan M, Parwani AV, Hershberger PA, Lenzner DE, Weissfeld JL. Nuclear vitamin D receptor expression is associated with improved survival in non-small cell lung cancer. The Journal of steroid biochemistry and molecular biology. 2011; 123:30–6. [PubMed: 20955794]
21. Kim SH, Chen G, King AN, Jeon CK, Christensen PJ, Zhao L, et al. Characterization of vitamin D receptor (VDR) in lung adenocarcinoma. Lung cancer (Amsterdam, Netherlands). 2012; 77:265–71.
22. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. The New England journal of medicine. 2007; 356:2131–42. [PubMed: 17522398]
23. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Der Pathologe. 1987; 8:138–40. [PubMed: 3303008]
24. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, et al. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. The Journal of molecular diagnostics: JMD. 2005; 7:413–21. [PubMed: 16049314]
25. Imamura Y, Lochhead P, Yamauchi M, Kuchiba A, Qian ZR, Liao X, et al. Analyses of Clinicopathological, Molecular, and Prognostic Associations of KRAS Codon 61 and Codon 146 Mutations in Colorectal Cancer: Cohort Study and Literature Review. Mol Cancer. 2014 in press.
26. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. The Journal of molecular diagnostics: JMD. 2006; 8:582–8. [PubMed: 17065427]
27. Liao X, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. Clinical cancer

research: an official journal of the American Association for Cancer Research. 2012; 18:2257–68. [PubMed: 22357840]

28. Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, et al. Reproducibility and validity of a semiquantitative food frequency questionnaire. American journal of epidemiology. 1985; 122:51–65. [PubMed: 4014201]
29. Rimm EB, Giovannucci EL, Stampfer MJ, Colditz GA, Litin LB, Willett WC. Reproducibility and validity of an expanded self-administered semiquantitative food frequency questionnaire among male health professionals. American journal of epidemiology. 1992; 135:1114–26. discussion 27-36. [PubMed: 1632423]
30. Lunn M, McNeil D. Applying Cox regression to competing risks. Biometrics. 1995; 51:524–32. [PubMed: 7662841]
31. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials. 1986; 7:177–88. [PubMed: 3802833]
32. Cochran WG. The combination of estimates from different experiments. Biometrics. 1954; 10:101–29.
33. Durrleman S, Simon R. Flexible regression models with cubic splines. Stat Med. 1989; 8:551–61. [PubMed: 2657958]
34. Smith PL. Splines As a Useful and Convenient Statistical Tool. The American Statistician. 1979; 33:57–62.
35. Govindarajulu U, Spiegelman D, Thurston S, Eisen E. Comparing smoothing techniques for modeling exposure-response curves in Cox models. Stat Med. 2007; 26:3735–52. [PubMed: 17538974]
36. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. The American journal of clinical nutrition. 2003; 77:204–10. [PubMed: 12499343]
37. Lointier P, Meggouh F, Dechelotte P, Pezet D, Ferrier C, Chipponi J, et al. 1,25-Dihydroxyvitamin D3 receptors and human colon adenocarcinoma. The British journal of surgery. 1991; 78:435–9. [PubMed: 1851650]
38. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, et al. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. The Journal of clinical endocrinology and metabolism. 2001; 86:888–94. [PubMed: 11158062]
39. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome research. 2010; 20:1352–60. [PubMed: 20736230]
40. Wu W, Zhang X, Zanello LP. 1alpha,25-Dihydroxyvitamin D(3) antiproliferative actions involve vitamin D receptor-mediated activation of MAPK pathways and AP-1/p21(waf1) upregulation in human osteosarcoma. Cancer letters. 2007; 254:75–86. [PubMed: 17412493]
41. Eelen G, Verlinden L, van Camp M, van Hummelen P, Marchal K, de Moor B, et al. The effects of 1alpha,25-dihydroxyvitamin D3 on the expression of DNA replication genes. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. 2004; 19:133–46.
42. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. The New England journal of medicine. 2009; 361:2449–60. [PubMed: 20018966]
43. Wu K, Feskanich D, Fuchs CS, Chan AT, Willett WC, Hollis BW, et al. Interactions between Plasma Levels of 25-Hydroxyvitamin D, Insulin-Like Growth Factor (IGF)-1 and C-Peptide with Risk of Colorectal Cancer. Plos One. 2011; 6:e28520. [PubMed: 22216097]
44. Pereira F, Larriba MJ, Munoz A. Vitamin D and colon cancer. Endocrine-related cancer. 2012; 19:R51–71. [PubMed: 22383428]
45. Pereira F, Barbachano A, Singh PK, Campbell MJ, Munoz A, Larriba MJ. Vitamin D has wide regulatory effects on histone demethylase genes. Cell cycle (Georgetown, Tex). 2012; 11:1081–9.
46. Zhang X, Giovannucci E. Calcium, vitamin D and colorectal cancer chemoprevention. Best practice & research Clinical gastroenterology. 2011; 25:485–94. [PubMed: 22122765]

47. Jenab M, Bueno-De-Mesquita HB, Ferrari P, van Duijnhoven FJB, Norat T, Pischon T, et al. Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations: a nested case-control study. Brit Med J. 2010; 340
48. Ogino S, Stampfer M. Lifestyle factors and microsatellite instability in colorectal cancer: the evolving field of molecular pathological epidemiology. Journal of the National Cancer Institute. 2010; 102:365–7. [PubMed: 20208016]
49. Ogino S, Lochhead P, Chan AT, Nishihara R, Cho E, Wolpin BM, et al. Molecular pathological epidemiology of epigenetics: emerging integrative science to analyze environment, host, and disease. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2013; 26:465–84.
50. Ogino S, Lochhead P, Giovannucci E, Meyerhardt JA, Fuchs CS, Chan AT. Discovery of colorectal cancer PIK3CA mutation as potential predictive biomarker: power and promise of molecular pathological epidemiology. Oncogene. 2013
51. Reimers MS, Zeestraten EC, Kuppen PJ, Liefers GJ, van de Velde CJ. Biomarkers in precision therapy in colorectal cancer. Gastroenterology report. 2013; 1:166–83. [PubMed: 24759962]
52. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World journal of gastroenterology: WJG. 2014; 20:4230–43. [PubMed: 24764661]
53. Bardhan K, Liu K. Epigenetics and colorectal cancer pathogenesis. Cancers. 2013; 5:676–713. [PubMed: 24216997]
54. Zoratto F, Rossi L, Verrico M, Papa A, Basso E, Zullo A, et al. Focus on genetic and epigenetic events of colorectal cancer pathogenesis: implications for molecular diagnosis. Tumor Biology. 2014:1–12.
55. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, et al. Genome-wide association study of circulating vitamin D levels. Human molecular genetics. 2010; 19:2739–45. [PubMed: 20418485]
56. Schernhammer ES, Giovannucci E, Kawasaki T, Rosner B, Fuchs CS, Ogino S. Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. Gut. 2010; 59:794–9. [PubMed: 19828464]
57. Solomon C, Kremer R, White JH, Rhim JS. Vitamin D resistance in RAS-transformed keratinocytes: mechanism and reversal strategies. Radiation research. 2001; 155:156–62. [PubMed: 11121228]
58. Fleet JC. Molecular actions of vitamin D contributing to cancer prevention. Molecular aspects of medicine. 2008; 29:388–96. [PubMed: 18755215]

Abbreviations

CI	confidence interval
EGFR	epithermal growth factor receptor
HPFS	Health Professionals Follow-up Study
HR	hazard ratio
MAPK	mitogen-activated protein kinase
NHS	Nurses' Health Study
PI3K	phosphatidylinositol-4,5-bisphosphonate 3-kinase
25(OH)D	25-hydroxyvitamin D
VDR	vitamin D receptor

Table 1

Age-standardized characteristics[a] of participants by quintiles (Q) of predicted 25(OH)D score in the Health Professionals Follow-up Study (HPFS) and the Nurses' Health Professional Study (NHS) in 1998 (median follow-up time)[b]

Population characteristics	Predicted 25(OH)D score					
	HPFS			NHS		
	Q1	Q3	Q5	Q1	Q3	Q5
Predicted 25(OH)D score, median (IQR), ng/mL	20 (19-21)	24 (23-24)	28 (27-28)	23 (20-24)	28 (27-28)	32 (31-33)
Age, years	64.0 (8.8)	65.3 (9.4)	66.3 (9.7)	64.2 (7.0)	64.3 (7.2)	65.0 (7.1)
Region[c]						
Northeast, %	45	33	17	66	61	45
Midwest, %	20	20	12	24	20	12
South, %	34	47	71	10	19	43
Height, inches	69.7 (3.3)	70.2 (2.7)	70.4 (2.6)	64.3 (2.5)	64.5 (2.4)	64.7 (2.4)
Body mass index, kg/m^2	28.8 (4.4)	25.9 (3.0)	24.1 (2.5)	32.4 (6.4)	25.8 (3.9)	23.4 (3.1)
Physical activity, MET-h per week	18.2 (26.0)	32.7 (36.5)	53.2 (47.4)	8.9 (12.9)	15.5 (17.2)	26.2 (22.9)
Pack years smoking before age 30, years	5.3 (7.4)	5.0 (7.0)	4.5 (6.6)	3.4 (5.2)	3.6 (5.2)	4.0 (5.4)
Current multivitamin use, %	41	57	76	43	60	80
Endoscopy experience, %	23	25	25	16	20	23
Family history of colorectal cancer, %	14	13	14	16	17	17
Aspirin use, %	44	48	47	46	44	46
Dietary intake						
Total calorie intake, kcal per day	1930 (623)	1995 (621)	2041 (602)	1700(560)	1705 (531)	1767 (520)
Total vitamin D intake, IU per day	352 (272)	467 (305)	607 (319)	323 (258)	420 (267)	555 (271)
Vitamin D intake without supplement, IU per day	193 (131)	237 (149)	280 (161)	154 (101)	180 (112)	220 (126)
Total calcium intake, mg per day	779 (285)	931 (335)	1075 (386)	842 (312)	998 (340)	1205 (393)
Total folate intake, μg per day	613 (299)	712 (319)	845 (339)	546 (262)	628 (266)	740 (265)
Red meat intake, servings per day	1.4 (1.0)	1.2 (0.8)	1.0 (0.8)	1.0 (0.5)	0.9 (0.4)	0.8 (0.4)
Fruit and vegetable intake, servings per day	5.2 (2.4)	5.8 (2.5)	6.5 (2.7)	4.9 (2.0)	5.2 (2.0)	5.7 (2.1)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; MET, IQR, interquartile range; metabolic equivalents; IU, international units

[a] Values are means (SD) or percentages, otherwise specified. All data except age and predicted 25(OH)D score are standardized to the age distribution of the study population

[b] Availability of predicted vitamin D score varies by each time periods. In this table, we present the population characteristics at the median follow-up time to represent the study population.

[c] Region is categorized as Northeast, Midwest, and South to reflect low, middle and high average levels (respectively) of UV-B radiation, a major source of vitamin D. Census Bureau Regions and Divisions with State FIPS codes from the U.S. Census Bureau was used to categorize the regions.

Table 2

Multivariate[a] hazard ratios (HR) and 95% confidence intervals (CIs) of colorectal cancer defined by VDR expression level according to quintile of predicted plasma 25(OH)D[b] score in the Health Professionals Follow-up Study (HPFS) and the Nurses' Health Study (NHS)

Molecular marker		Quintiles of predicted 25(OH)D score					*P*, trend[c]	*P*, heterogeneity by tumor subtype [d]
		Q1	Q2	Q3	Q4	Q5		
All colorectal cancer								
Pooled (HPFS+NHS)		270	222	216	192	159		
	Age-adjusted (95%CIs)	1 (ref)	0.78 (0.65-0.93)	0.76 (0.64-0.91)	0.65 (0.54-0.78)	0.51 (0.42-0.62)	<.001	
	Model 1[a] (95%CIs)	1 (ref)	0.77 (0.64-0.92)	0.76 (0.63-0.91)	0.65 (0.53-0.78)	0.52 (0.42-0.64)	<.001	
	Model 2[e] (95%CIs)	1 (ref)	0.78 (0.64-0.94)	0.78 (0.64-0.95)	0.67 (0.54-0.83)	0.55 (0.43-0.71)	<.001	
Nuclear VDR expression status								
Pooled (HPFS+NHS)								
VDR (−)								
	N. of cases	48	45	41	53	25		
	Age-adjusted (95%CIs)	1 (ref)	0.79 (0.53-1.20)	0.84 (0.56-1.26)	0.79 (0.52-1.18)	0.46 (0.29-0.74)	0.002	
	Model 1[a] (95%CIs)	1 (ref)	0.78 (0.52-1.18)	0.84 (0.56-1.27)	0.79 (0.53-1.19)	0.48 (0.30-0.78)	0.004	0.75
	Model 2[e] (95%CIs)	1 (ref)	0.79 (0.52-1.20)	0.87 (0.58-1.33)	0.84 (0.55-1.29)	0.53 (0.32-0.88)	0.02	
VDR(+)								
	N. of cases	123	115	103	102	88		
	Age-adjusted (95%CIs)	1 (ref)	0.77 (0.59-0.99)	0.76 (0.59-0.98)	0.69 (0.53-0.89)	0.54 (0.41-0.71)	<.001	
	Model 1[a] (95%CIs)	1 (ref)	0.76 (0.58-0.98)	0.76 (0.58-0.99)	0.69 (0.53-0.90)	0.56 (0.42-0.75)	<.001	
	Model 2[e] (95%CIs)	1 (ref)	0.77 (0.59-1.00)	0.79 (0.59-1.04)	0.73 (0.55-0.99)	0.62 (0.44-0.86)	0.01	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio; CI, confidence interval; VDR, vitamin D receptor

[a] Adjusted for family history of colorectal cancer (yes, no), history of endoscopy (yes, no), aspirin use (yes, no), pack years of smoking before 30 years old (continuous), height (inches, quintiles), and intakes of total fruit and vegetables (serving/d, quintiles), red meat (serving/d, quintiles), calcium (mg/d, quintiles) and total calories (kcal/d, quintiles). Age in months and year of questionnaire return were included as stratification variables. In the analyses of the combined cohorts, sex (cohort) was included as stratification variable. All statistical tests were two-sided.

[b] Predicted 25(OH)D score was cumulatively averaged up to the end of follow-up.

[c] *P*, trend was calculated by using the Wald test statistic.

[d] *P*, test for heterogeneity by subtypes was calculated conducting the log likelihood ratio test comparing the model fit that produces separate associations with different tumors to the model fit that assumed a common effect.

[e] Based on model 1, model 2 was further adjusted for body mass index, physical activity, and alcohol intake.

Table 3

Multivariate[a] hazard ratios (HR) and 95% confidence intervals (CIs) of colorectal cancer defined by mutation status of *KRAS*, *BRAF* and *PIK3CA* according to quintile of predicted plasma 25(OH)D[b] score in the Health Professionals Follow-up Study (HPFS) and the Nurses' Health Study (NHS)

Molecular marker		Quintiles of predicted 25(OH)D score					*P*, trend[c]	*P*, heterogeneity by tumor subtype [d]
		Q1	Q2	Q3	Q4	Q5		
***KRAS* mutation status**								
Pooled (HPFS+NHS)								
KRAS wild								
	N. of cases	169	145	124	145	96		
	Age-adjusted (95%CIs)	1 (ref)	0.73 (0.59-0.91)	0.65 (0.51-0.81)	0.68 (0.54-0.85)	0.45 (0.35-0.58)	<.001	
	Model 1[a] (95%CIs)	1 (ref)	0.72 (0.57-0.90)	0.64 (0.51-0.81)	0.68 (0.54-0.86)	0.46 (0.35-0.60)	<.001	0.22
	Model 2[e] (95%CIs)	1 (ref)	0.72 (0.57-0.91)	0.65 (0.51-0.84)	0.70 (0.55-0.90)	0.48 (0.36-0.65)	<.001	
KRAS mutant								
	N. of cases	72	93	78	59	64		
	Age-adjusted (95%CIs)	1 (ref)	0.94 (0.69-1.29)	1.06 (0.78-1.43)	0.58 (0.41-0.82)	0.67 (0.48-0.94)	0.002	
	Model 1[a] (95%CIs)	1 (ref)	0.93 (0.68-1.28)	1.06 (0.78-1.44)	0.58 (0.40-0.83)	0.70 (0.50-0.98)	0.006	
	Model 2[e] (95%CIs)	1 (ref)	0.94 (0.68-1.30)	1.09 (0.79-1.49)	0.60 (0.41-0.87)	0.73 (0.51-1.06)	0.020	
***BRAF* mutation status**								
Pooled (HPFS+NHS)								
BRAF wild								
	N. of cases	204	207	173	175	134		
	Age-adjusted (95%CIs)	1 (ref)	0.83 (0.68-1.00)	0.77 (0.63-0.94)	0.68 (0.55-0.83)	0.50 (0.40-0.63)	<.001	
	Model 1[a] (95%CIs)	1 (ref)	0.82 (0.67-0.99)	0.77 (0.63-0.94)	0.68 (0.55-0.84)	0.52 (0.41-0.65)	<.001	0.88
	Model 2[e] (95%CIs)	1 (ref)	0.82 (0.67-1.01)	0.79 (0.63-0.98)	0.71 (0.56-0.89)	0.55 (0.42-0.71)	<.001	
BRAF mutant								
	N. of cases	38	33	26	29	25		
	Age-adjusted (95%CIs)	1 (ref)	0.57 (0.35-0.92)	0.70 (0.44-1.11)	0.49 (0.29-0.81)	0.55 (0.34-0.90)	0.01	
	Model 1[a] (95%CIs)	1 (ref)	0.56 (0.34-0.91)	0.70 (0.44-1.11)	0.49 (0.29-0.82)	0.58 (0.35-0.94)	0.01	

Molecular marker		Quintiles of predicted 25(OH)D score					*P*, trend[c]	*P*, heterogeneity by tumor subtype [d]
		Q1	Q2	Q3	Q4	Q5		
	Model 2[e] (95%CIs)	1 (ref)	0.57 (0.35-0.93)	0.71 (0.44-1.14)	0.51 (0.30-0.86)	0.60 (0.36-1.00)	0.02	
***PIK3CA* mutation status**								
Pooled (HPFS+NHS)								
*PIK3CA*wild								
	N. of cases	190	186	156	162	120		
	Age-adjusted (95%CIs)	1 (ref)	0.86 (0.70-1.05)	0.77 (0.63-0.95)	0.70 (0.56-0.86)	0.50 (0.40-0.63)	<.001	
	Model 1[a] (95%CIs)	1 (ref)	0.84 (0.69-1.03)	0.76 (0.62-0.94)	0.69 (0.55-0.86)	0.51 (0.40-0.65)	<.001	0.98
	Model 2[e] (95%CIs)	1 (ref)	0.85 (0.68-1.05)	0.78 (0.62-0.97)	0.71 (0.56-0.90)	0.53 (0.40-0.70)	<.0001	
PIK3CA mutant								
	N. of cases	30	38	36	30	22		
	Age-adjusted (95%CIs)	1 (ref)	0.71 (0.44-1.15)	0.90 (0.57-1.41)	0.57 (0.34-0.95)	0.53 (0.32-0.88)	0.01	
	Model 1[a] (95%CIs)	1 (ref)	0.70 (0.43-1.13)	0.89 (0.57-1.40)	0.55 (0.33-0.92)	0.54 (0.32-0.90)	0.009	
	Model 2[e] (95%CIs)	1 (ref)	0.70 (0.43-1.14)	0.90 (0.57-1.43)	0.57 (0.34-0.97)	0.56 (0.33-0.95)	0.02	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio; CI, confidence interval

[a] Multivariate model was adjusted for the multivariate model covariates listed in the footnote to Table 2.

[b] Predicted 25(OH)D score was cumulatively averaged up to the end of follow-up.

[c] *P*, trend was calculated by using the Wald test statistic.

[d] *P*, test for heterogeneity by subtypes was calculated conducting the log likelihood ratio test comparing the model fit that produces separate associations with different tumors to the model fit that assumed a common effect.

[e] Based on model 1, model 2 was further adjusted for body mass index, physical activity, and alcohol intake.