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Vitamin D Receptor Expression Is Associated with *PIK3CA* and *KRAS* Mutations in Colorectal Cancer

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Abstract

Vitamin D is associated with decreased risks of various cancers, including colon cancer. The vitamin D receptor (VDR) is a transcription factor, which plays an important role in cellular differentiation and inhibition of proliferation. A link between VDR and the RAS-MAPK or phosphatidylinositol 3-kinase (PI3K)-AKT pathway has been suggested. However, the prognostic role of VDR expression or its relationship with *PIK3CA* or *KRAS* mutation remains uncertain. Among 619 colorectal cancers in two prospective cohort studies, 233 (38%) tumors showed VDR overexpression by immunohistochemistry. We analyzed for *PIK3CA* and *KRAS* mutations and LINE-1 methylation by Pyrosequencing, microsatellite instability (MSI), and DNA methylation (epigenetic changes) in 8 CpG island methylator phenotype (CIMP)-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*] by MethyLight (real-time PCR). VDR overexpression was significantly associated with *KRAS* mutation [odds ratio (OR), 1.55; 95% confidence interval (CI), 1.11-2.16] and *PIK3CA* mutation (OR, 2.17; 95% CI, 1.36-3.47), both of which persisted in multivariate logistic regression analysis. VDR was not independently associated with body mass index, family history of colorectal cancer, tumor location (colon vs. rectum), stage, tumor grade, signet ring cells, CIMP, MSI, LINE-1 hypomethylation, *BRAF*, p53, p21, β-catenin or cyclooxygenase-2. VDR expression was not significantly related with patient survival, prognosis or clinical outcome. In conclusion, VDR overexpression in colorectal cancer is independently associated with *PIK3CA* and *KRAS* mutations. Our data support potential interactions between the VDR, RAS-MAPK and PI3K-AKT pathways, and possible influence by *KRAS* or *PIK3CA* mutation on therapy or chemoprevention targeting VDR.

Keywords

colon cancer; VDR; methylation; *PI3K*; *RAS*

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Introduction

The vitamin D receptor (VDR) is a member of the steroid hormone receptor superfamily, and regulates gene expression in a ligand-dependent manner (1). Plasma vitamin D level and estimated whole-body vitamin D level have been associated with decreased incidence and mortality in various cancers (1), including colorectal cancer (2,3). Vitamin D and VDR appear to play important roles in preventing tumor development and progression through induction of cellular differentiation and inhibition of proliferation (1,4-9). VDR is overexpressed or repressed in various human cancers (1,10-15). In colorectal cancer, VDR is expressed in early-stage neoplasias, but repressed in high-grade and metastatic cancers (12).

An activation of epidermal growth factor receptor (EGFR) triggers a chain of downstream signaling events that include the phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/MAPK pathways. Accumulating evidence suggests that VDR binds to PI3K and inhibits cell proliferation by inducing differentiation (1,16), and that the PI3K/AKT pathway is activated by 1,25$(OH)_2$D, the active form of vitamin D (17). In addition, VDR expression has been associated with RAS/MAPK pathway activation in leukemia cells (18). These observations suggest that VDR-expressing cells may need activation of the PI3K/AKT or RAS/MAPK pathway in order to acquire malignant characteristics. Activating mutations in *PIK3CA* and *KRAS* play important roles in colorectal carcinogenesis. Thus, we hypothesized that VDR expression is associated with *PIK3CA* and *KRAS* mutations in colorectal cancer. If this hypothesis is true, therapy or chemoprevention targeting VDR and downstream pathways may be influenced by *PIK3CA* or *KRAS* mutations. In addition, VDR-mediated action of 1,25 $(OH)_2$D can limit colon cancer cell growth particularly when induced by activation of EGFR (19). Thus, response to EGFR-targeted therapy may be modified by VDR status in tumor cells.

We therefore utilized 619 colorectal cancers identified in two prospective cohort studies, and examined the relation of VDR expression with patient survival, *PIK3CA* and *KRAS* mutations, and other related molecular features including *BRAF* mutation, microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP), which were potential confounders.

Materials and Methods

Study group

We utilized the databases of two independent prospective cohort studies; the Nurses' Health Study (N = 121,700 women followed since 1976) (20), and the Health Professional Follow-up Study (N = 51,500 men followed since 1986) (20). Data on height and weight were obtained by biennial questionnaire. A subset of the cohort participants developed colorectal cancers during prospective follow-up. Data on tumor location and TNM stage were obtained through medical record review by study physicians. We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumors. Up to 2002, there were 1834 incident colorectal cancer cases with adequate clinical information. We successfully retrieved formalin-fixed paraffin-embedded tumor tissue in 998 cases (54%). We have previously shown that there are no differences in demographic, nutritional or exposure features between patients with and without available tumor tissue (20). Among 998 cases with available tumor tissue, we were able to construct tissue microarrays (TMA) using 625 cases. Among them, we obtained valid VDR expression data in 619 cases, which were eligible for the current study (Table 1). In any of our previous studies, we have not examined VDR expression in colorectal cancers. This current analysis represents a new analysis of VDR on the existing colorectal cancer database that have been previously characterized for MSI, *KRAS*, *PIK3CA* and *BRAF* (21,22), which is analogous to novel analysis using the well-characterized cell lines or animal models. Patients were observed until death or June 30, 2006, whichever came first. Ascertainment of deaths included reporting by the family

or postal authorities. In addition, the names of persistent nonresponders were searched in the National Death Index. More than 98% of deaths in the cohorts were identified by these methods. The cause of death was assigned by physicians blinded to information on lifestyle exposures and molecular features in colorectal cancer. Written informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

Histopathologic evaluations

Hematoxylin and eosin (H&E) stained tissue sections were examined by a pathologist (S.O.) unaware of other data. The tumor grade was categorized as low (≥50% gland formation) vs. high (<50% gland formation). The presence and extent of extracellular mucin and signet ring cells were categorized as 0% (no mucin or signet ring cells), or ≥1% of the tumor volume.

Sequencing of *KRAS*, *BRAF* and *PIK3CA*, and analyses for MSI

DNA was extracted from paraffin-embedded tumor tissue sections, and PCR and Pyrosequencing targeted for *KRAS* (codons 12 and 13) (23), *BRAF* (codon 600) (24) and *PIK3CA* (exons 9 and 20) were performed as previously described (25). Microsatellite instability (MSI) analysis was performed, using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487) (26). MSI-high was defined as the presence of instability in ≥30% of the markers. MSI-low was defined as instability in 10-29% of the markers, and “microsatellite stable (MSS)” tumors were defined as tumors with no unstable marker.

Real-time PCR for CpG island methylation and Pyrosequencing to measure LINE-1 methylation

Sodium bisulfite treatment on genomic DNA and subsequent real-time PCR (MethyLight) were validated and performed as previously described (27). We quantified DNA methylation in 8 CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*] (28-30). CIMP-high was defined as the presence of ≥6 of 8 methylated promoters, CIMP-low as the presence of 1/8-5/8 methylated promoters, and CIMP-0 as the absence (0/8) of methylated promoters, according to the previously established criteria (30). In order to accurately quantify relatively high methylation levels in LINE-1 repetitive elements, we utilized Pyrosequencing (31).

Immunohistochemistry for p53, p21, β-catenin, COX-2 and VDR

Tissue microarrays (TMAs) were constructed as previously described (20). Methods of immunohistochemical procedures and interpretations were previously described for p53, p21 (32,33), β-catenin (34) and COX-2 (20,26). Appropriate positive and negative controls were included in each run of immunohistochemistry for all markers. For VDR immunohistochemistry, antigen retrieval was performed, and deparaffinized tissue sections in Antigen Retrieval Citra Solution (Biogenex Laboratories, San Ramon, CA) were treated with microwave for 15 min. Tissue sections were incubated with 3% H_2O_2 (10 min) to block endogenous peroxidase (Dako Cytomation, Carpinteria, CA), with 10% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (10 min). Primary antibody against VDR [polyclonal rabbit anti-VDR (C-20), 1:200 dilution; Santa Cruz Biotechnology, San Diego, CA] was applied, and the slides were maintained for 1 hours at room temperature. Next, we applied Envision System HRP labeled polymer anti rabbit (Dako) for 30 min, followed by visualizing signal with diaminobenzidine (5 min) and methyl-green counterstain. Appropriate positive and negative controls were included in each run of VDR immunohistochemistry. VDR overexpression (Figure 1) was evaluated by one of the investigators (K.No.) unaware of other data. Cytoplasmic VDR expression was recorded as

no, weak, or moderate/strong expression, and the number of cells with expression was recorded. Among the 619 tumors, 82 showed strong cytoplasmic expression, 139 showed moderate cytoplasmic expression, 12 showed ≥50% of tumor cells with weak cytoplasmic expression, 53 showed 30-49% of tumor cells with weak cytoplasmic expression, 112 showed 1-29% of tumor cells with weak cytoplasmic expression and 221 tumors showed no staining in tumor cells. VDR positivity (i.e., overexpression) was defined as moderate/strong cytoplasmic expression in any fraction of tumor cells or at least 50% of tumor cells with weak cytoplasmic expression. Either the absence of staining or the presence of weak cytoplasmic expression in <50% of tumor cells was interpreted as negative. This cut point was based on the frequency of *PIK3CA* mutation in colorectal cancer groups categorized by VDR status. The frequency of *PIK3CA* mutation was; 21% (40/191) in tumors with moderate or strong cytoplasmic expression; 36% (4/11) in tumors with weak cytoplasmic expression in ≥50% of tumor cells, 11% (5/47) in tumors with weak cytoplasmic expression in 30-49% of tumor cells, 12% (12/103) in tumors with weak cytoplasmic expression in 1-29% of tumor cells and 11% (23/202) in tumors with no staining.

A random selection of 139 cases was examined for VDR by a second observer (Y.B.) unaware of other data, and concordance between the two observers was 0.81 (κ=0.62, p<0.0001), indicating substantial agreement. Each of the other immunohistochemical markers was interpreted by a pathologist unaware of other data (p53, p21 and COX-2 by S.O.; β-catenin by K.No.). For each of the other immunohistochemical markers, a second observer (S.O. for β-catenin; K.S. for p21; K.No. for p53; R. Dehari, Kanagawa Cancer Center, for COX-2) examined a random sample of 108-402 tumors, unaware of other data. The κ coefficient between the two observers was 0.65 for β-catenin (p<0.0001; N=402), 0.62 for p21 (p<0.0001; N=179), 0.75 for p53 (p<0.0001; N=118), and 0.62 for COX-2 (p<0.0001; N=108), indicating substantial agreement.

Statistical analysis

For all statistical analyses, we used SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided, and statistical significance was set at $p \leq 0.05$; however, whenever appropriate, p values were conservatively interpreted considering multiple hypotheses testing. For categorical data, the chi-square test (or Fisher's exact test when any expected cell count was less than 5) was performed. To assess independent relations of VDR with a number of variables, a multivariate logistic regression analysis was performed. Odds ratio (OR) was adjusted for age at diagnosis (<65 vs. ≥65-year-old), sex, tumor location (proximal vs. distal), body mass index (BMI, ≥30 vs. <30 kg/m^2), tumor stage (I-II vs. III-IV), grade (low vs. high), family history of colorectal cancer in any first degree relative (present vs. absent), mucinous component (present vs. absent), CIMP status (high vs. low/0), MSI status (high vs. low/MSS), LINE-1 methylation (continuous), β-catenin, COX-2, p53, p21, *BRAF*, *KRAS* and *PIK3CA*. For cases with missing data on *KRAS* (0.3% missing) and *PIK3CA* (11% missing), we assigned separate ("missing") indicator variables, and included those cases in the multivariate model. For cases with missing information in other variables [BMI (5.8% missing), tumor location (1.1%), tumor stage (11%), tumor grade (3.2%), mucinous component (13%), CIMP (2.6%), MSI (0.5%), LINE-1 (3.7%), *BRAF* (2.9%), β-catenin (11%), p53 (0.8%), p21 (2.3%) and COX-2 (0.6%)], we included those cases in a majority category, in order to minimize the number of indicator variables. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown).

For survival analysis, Kaplan-Meier method was used and log-rank test was used to test significance of a deviation from the null hypothesis. For analyses of colorectal cancer-specific mortality, death as a result of colorectal cancer was the primary end point and deaths as a result of other causes were censored. To assess independent effect of VDR status on mortality, we

constructed a multivariate, stage-matched (stratified) Cox proportional hazards model to compute a hazard ratio (HR) according to VDR status, adjusted for age at diagnosis, sex, BMI, year of diagnosis, tumor grade, tumor location, mucinous component, family history, CIMP, MSI, *KRAS*, *BRAF*, *PIK3CA*, p53, β-catenin, p21, COX-2 and LINE-1 methylation. Tumor stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) was used as a matching variable using the "strata" option in the SAS "proc phreg" command to minimize residual confounding and overfitting. We also used stage-matched Cox model including only stage as a stratifying variable without any other covariate in the model. The proportionality of hazards assumption was satisfied by evaluating time-dependent variables, which were the cross-product of the VDR variable and survival time (p=0.62 for colorectal cancer-specific mortality; p=0.85 for overall mortality). For cases with missing data in any of the covariates were dealt as in the multivariate logistic regression analysis described above, except for stage, *KRAS* and *PIK3CA*. For cases missing data on *KRAS* (or *PIK3CA*), we included those cases in the wild-type category. An interaction was assessed by including the cross product of the VDR variable and another variable of interest (excluding data-missing cases) in a multivariate Cox model, and the Wald test was performed.

Results

Vitamin D receptor (VDR) expression in colorectal cancer

We examined VDR overexpression by immunohistochemistry in 619 colorectal cancers identified in two independent prospective cohort studies, and detected VDR overexpression in 233 (38%) tumors. Table 1 shows the frequencies of VDR expression according to various clinical and pathologic features. VDR overexpression was significantly less common among high grade tumors [odds ratio (OR), 0.41; 95% confidence interval (CI), 0.20-0.81; p=0.008].

Relationship of VDR expression with *KRAS* and *PIK3CA* mutations

Table 1 shows the frequencies of VDR overexpression according to various molecular features in colorectal cancer. VDR overexpression was significantly more common among *KRAS*-mutated tumors (OR, 1.55; 95% CI, 1.11-2.16; p=0.010) and *PIK3CA*-mutated tumors (OR, 2.17; 95% CI, 1.36-3.47; p=0.001). In order to examine combined effect of *KRAS* and *PIK3CA* mutations on VDR overexpression, we classified tumors into 4 subtypes according to *KRAS* and *PIK3CA* status (Table 1). VDR overexpression was significantly more common in *KRAS*-mutated/*PIK3CA*-mutated tumors (58%=29/50; OR, 2.97; 95% CI, 1.61-5.47; p=0.0003) than in *KRAS*-wild-type/*PIK3CA*-wild-type tumors (32%=99/312). We also examined the frequency of *PIK3CA* or *KRAS* mutation according to VDR staining intensity in tumor cells (Table 2). The frequencies of *PIK3CA* and *KRAS* mutations increased as intensity of VDR staining increased, and reached plateau at moderate intensity of staining.

Relationship of VDR expression with other molecular variables

We determined CpG island methylator phenotype (CIMP) status using MethyLight assays on a panel of 8 CIMP-specific promoters (*CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3* and *SOCS1*) (29,30). VDR overexpression was not significantly associated with CIMP (Table 1). On the other hand, VDR overexpression was more common in microsatellite instability (MSI)-low tumors (51%=30/59; OR, 1.80; 95% CI, 1.05-3.11; p=0.032) than in microsatellite stable (MSS) tumors (36%=168/461); nonetheless, this could be a chance association given multiple hypothesis testing on multiple tumor markers (excluding *PIK3CA* and *KRAS*).

VDR expression is independently associated with *PIK3CA* and *KRAS* mutations

We performed multivariate logistic regression analysis, to confirm independent relations between VDR and mutations of *KRAS* and *PIK3CA* after adjusting for clinical, pathologic and other molecular variables (Table 3). VDR overexpression was significantly associated with *PIK3CA* mutation (adjusted OR, 2.36; 95% CI, 1.43-3.91; p=0.0008) and *KRAS* mutation (adjusted OR, 1.53; 95% CI, 1.04-2.23; p=0.029).

VDR expression and patient survival

Utilizing our cohort database, we previously demonstrated that molecular features in colon cancer such as *BRAF* mutation, *PIK3CA* mutation and LINE-1 hypomethylation were significantly associated with inferior prognosis (21,22,35). We assessed the influence of VDR overexpression on survival of patients with stage I-IV colorectal cancers. During follow-up of 599 patients who were eligible for survival analysis, there were 260 deaths, including 158 colorectal cancer-specific deaths. In Kaplan-Meier analysis, VDR status was not significantly associated with colorectal cancer-specific survival (log rank p=0.57) or overall survival (log rank p=0.31) (Figure 2).

We performed Cox regression analysis to assess mortality according to VDR status (Table 4). VDR status was not significantly related with patient survival in univariate analysis, stage-matched analysis, or multivariate analysis in colorectal cancer. We also examined whether any of the clinical, pathologic and molecular variables significantly modified the effect of VDR overexpression on patient survival. There was no evidence for a significant interaction between VDR overexpression and any of the variables examined, including *KRAS* and *PIK3CA* (all $P_{interaction} > 0.05$).

Discussion

We conducted this study to examine the relationship between vitamin D receptor (VDR) expression and mutations in *PIK3CA* and *KRAS* in colorectal cancer. Accumulating evidence indicate a substantial role of vitamin D in prevention of various forms of human cancer (1,2, 17,36-38). A potential link between VDR and the PI3K/AKT or RAS/MAPK pathway has been suggested (17,18). Thus, examining VDR in colorectal cancer may shed lights on biological mechanisms of vitamin D action and its failure. We have found that VDR overexpression in colorectal cancer is significantly associated with both *PIK3CA* and *KRAS* mutations, independent of other clinical and molecular features. Our data support the hypothesis that the VDR pathway interact with the PI3K/AKT and RAS/MAPK pathways in colonic neoplastic cells.

Our resource of a large number of colorectal cancers derived from the two prospective cohort studies has enabled us to precisely estimate the frequency of colorectal cancers with a specific molecular feature (such as VDR expression, *KRAS* mutation, *PIK3CA* mutation, etc.). The large number of cases has also provided a sufficient power in our multivariate logistic regression analysis and survival analysis.

Studying risk modifying factors or molecular changes is important in cancer research (39-44). Previous studies have consistently reported the preventive effect of vitamin D on colorectal cancers (1,2,36-38), and that higher plasma levels of vitamin D confer a greater reduction in the risk of colorectal cancer (2). Studies have reported that $1,25(OH)_2D$ potentiates the effects of many cytotoxic and anti-proliferative drugs (1,17), and that higher plasma levels of 25(OH)D is associated with a significant reduction in colon cancer mortality (2). Thus, accumulating evidence indicates important roles of vitamin D in preventing the development and progression of colorectal cancer. Interestingly, VDR and 1-α-hydroxylase (encoded by

CYP27B1), which converts 25(OH)D into $1,25(OH)_2D$, are frequently overexpressed in colon cancer cells (1,4-6,8,9). The anti-proliferative action of $1,25(OH)_2D$ appears to depend on VDR expression level and differentiation status of tumor cells (45,46). Therefore, it is possible that the effect of serum vitamin D level, which is protective against cancer incidence and mortality, may differ according to VDR expression status in colorectal cancer. Additional studies are necessary to address this intriguing hypothesis.

A potential link between VDR and the PI3K/AKT or RAS/MAPK pathway has been suggested (16-18,47-51). In these pathways, *PIK3CA* or *KRAS* mutation plays an important role in the progression of colorectal cancer. Mutant *PIK3CA* stimulates the downstream AKT pathway, and promotes cell growth in various cancers, including colorectal cancer. The PI3K/AKT pathway has been known to mediate signals from growth factors, which is influenced by the state of energy balance. In addition, PI3K/AKT signaling is influenced by *KRAS* (52). In fact, *PIK3CA* mutation is positively associated with *KRAS* mutation in colorectal cancer (25,53). Our data support a potential link between *PIK3CA* and VDR and suggest that VDR expression may affect the regulation of PI3K/AKT pathway in colorectal cancer. In myeloid leukemia cells, VDR activated by $1,25(OH)_2D$ can inhibit tumor cell proliferation by inducing differentiation, which depends on the formation of activated VDR and PI3K complexes (1, 16). A combination of $1,25(OH)_2D$ with AKT pathway inhibitors is strongly anti-proliferative and should be considered for differentiation therapy of myeloid leukemia (17). These previous observations (16,17) and our data suggest that VDR-expressing cells may need the activation of PI3K/AKT pathway in order to acquire malignant characteristics, and that therapy or chemoprevention targeting VDR and downstream pathways may be influenced by *PIK3CA* mutation in colon cancer cells.

The vitamin D pathway may also interact with RAS signaling. A recent case-control study has reported that the *VDR* poly A, rs10735810 (so-called “FokI SNP”) and rs11568820 (so-called “CDX2 SNP”) polymorphisms are associated with *KRAS* mutation in colon cancer (54). VDR protein expression has been shown to be down-regulated in *KRAS*-mutated colon cancer cells (47). In contrast, VDR expression has been associated with the activation of the RAS/MAPK pathway in leukemia cells (18), which is in agreement with our current data. In addition, RAS-transformed human keratinocytes are shown to be resistant to the growth-inhibitory effects of $1,25(OH)_2D$ (48-50). Further analysis is needed to clarify how vitamin D and its downstream pathway influence colorectal cancer development in relation to *KRAS* mutation.

We did not observe a significant relation between VDR expression and microsatellite instability (MSI)-high or the CpG island methylator phenotype (CIMP) in colorectal cancer. A molecular classification of colorectal cancer based on MSI and CIMP is increasingly important (55), because MSI and CIMP status represent genomic and epigenomic alterations, respectively, in tumor cells and largely determine clinical, pathologic and molecular characteristics (55). Nonetheless, our data do not support a link between VDR and CIMP or MSI in colorectal cancer. A recent study has reported that the *VDR* rs10735810 (so-called “FokI SNP”) polymorphism is significantly associated with CIMP-high and inversely with MSI-high (54); however, either of these could be a chance association given multiple hypothesis testing.

In the current study, we have shown that VDR expression is inversely associated with high grade tumors in univariate analysis, but not in multivariate analysis. Thus, our data are not incompatible with the inverse association between VDR expression and high tumor grade in the previous study (12), but do not support a mechanistic link between VDR loss and high tumor grade. With regard to disease stage, the previous study (12) has shown that VDR expression in colon cancer cells is commonly lost in a metastatic focus, but not in a primary lesion. Therefore, the absence of the relation between loss of VDR expression in primary tumor

and advanced disease stage in the current study is not incompatible with the previous data (12).

As one limitation in our cohort studies, data on cancer treatment were limited. Nonetheless, it is unlikely that chemotherapy use differed according to tumoral VDR status, since such data were not available to patients or treating physicians. In addition, beyond cause of mortality, data on cancer recurrences were not available in these cohorts. Nonetheless, given the median survival for metastatic colorectal cancer was approximately 10 to 12 months during much of the time period of this study, colorectal cancer-specific survival should be a reasonable surrogate for colorectal cancer-specific outcomes.

As another limitation of this study, there are currently no standardized methods to assess VDR overexpression in colorectal cancer. Thus, our current study is exploratory by nature, and our data and method to determine a cutpoint for VDR overexpression need to be validated and confirmed by independent datasets.

In summary, this large cohort of colorectal cancers has shown that VDR expression is significantly associated with *PIK3CA* and *KRAS* mutations, independent of clinical, pathological and molecular features. On the other hand, VDR expression is not significantly related with patient survival. Our data support the hypothesis that *PIK3CA* and/or *KRAS* mutations may influence biological effect of VDR and its downstream pathway. Thus, targeting VDR for chemoprevention or cancer therapy likely needs to consider the effect of *KRAS* or *PIK3CA* mutation. Likewise, therapy targeting EGFR or the downstream RAS or PI3K pathway may be influenced by VDR status. Considering that VDR regulates the transcription of various genes involved in cellular differentiation and inhibition of proliferation, our findings may have considerable clinical implications. Further studies are necessary to elucidate exact roles of vitamin D and VDR in prevention of colorectal neoplasias, as well as to examine a potential mechanistic link between VDR and the RAS/MAPK and PI3K/AKT pathways.

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Abbreviations and the HUGO Gene Nomenclature Committee-approved official gene symbols

BMI

body mass index

CI
: confidence interval

CIMP
: CpG island methylator phenotype

COX-2
: cyclooxygenase-2 (*PTGS2*)

EGFR
: epidermal growth factor receptor

HR
: hazard ratio

MSI
: microsatellite instability

MSS
: microsatellite stable

OR
: odds ratio

VDR
: vitamin D receptor

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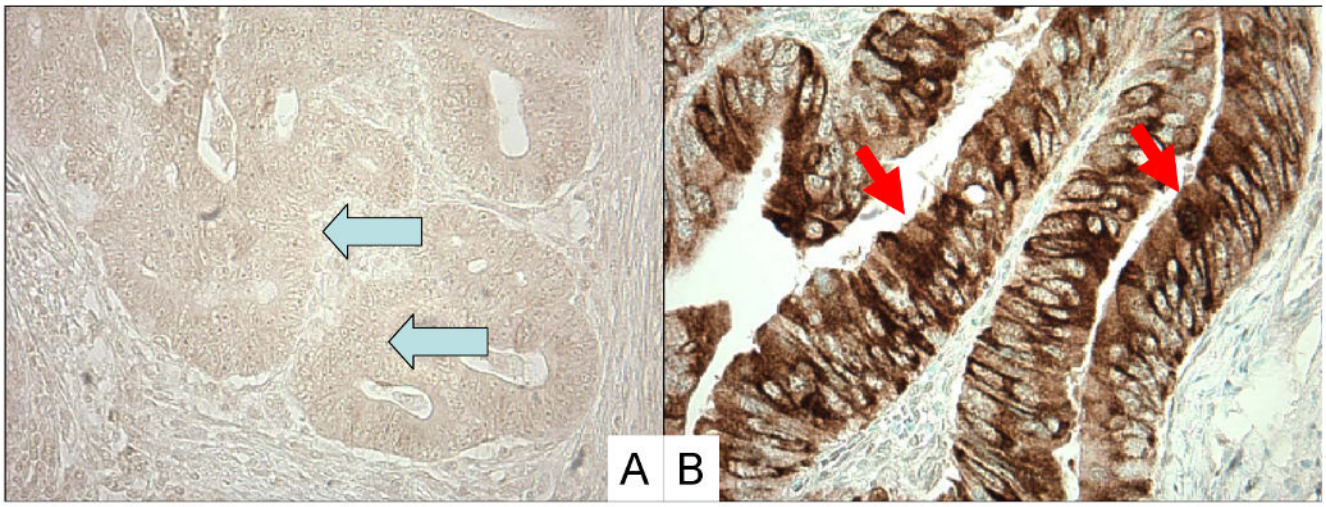

Figure 1.
Vitamin D receptor (VDR) expression in colon cancer. A. No overexpression of VDR in cancer cells (arrows). B. Overexpression of VDR in cytoplasm of cancer cells (arrows).

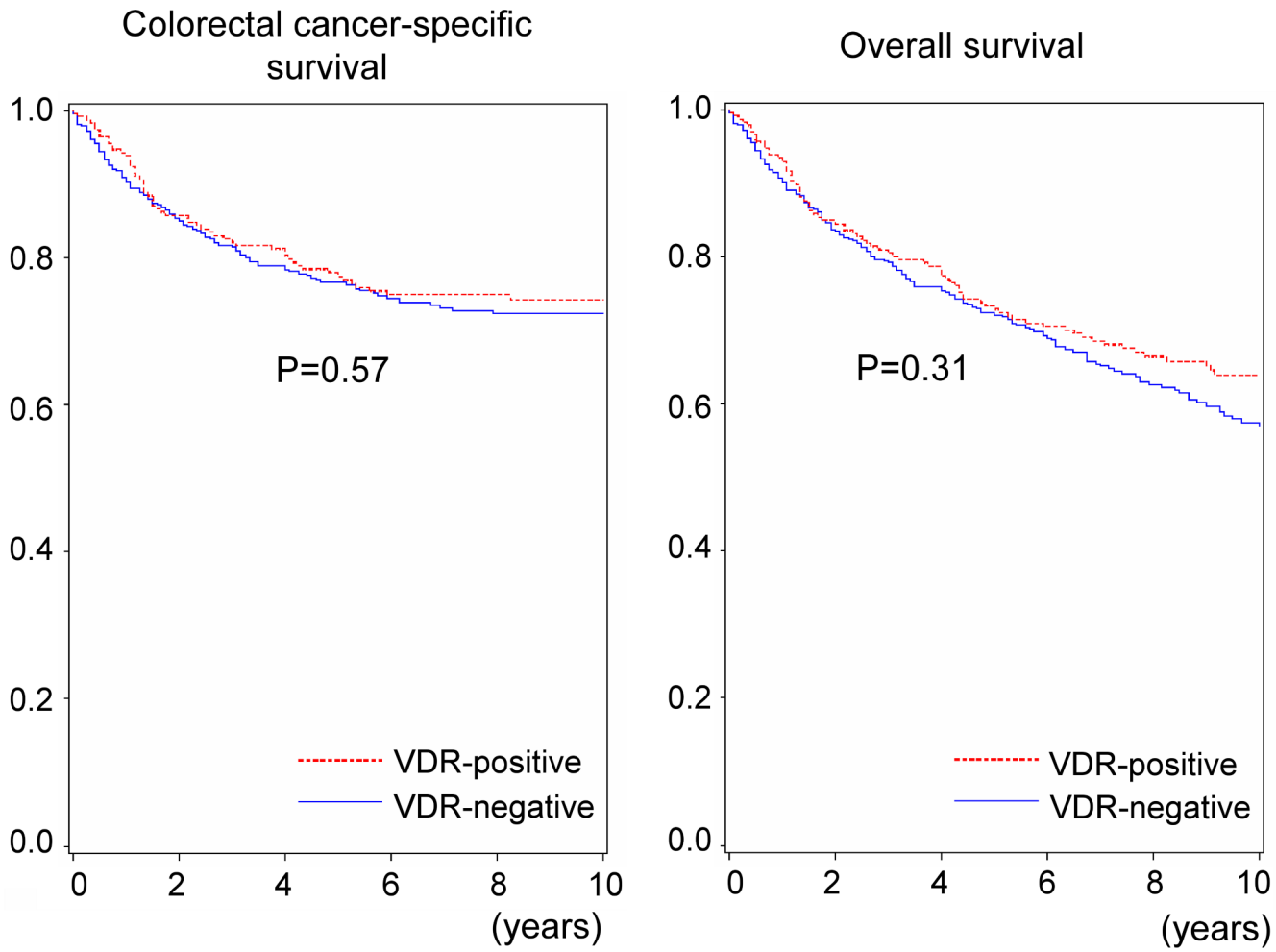

Figure 2.
Kaplan-Meier curves for colorectal cancer-specific survival (left panel) and overall survival (right panel) according to tumoral vitamin D receptor (VDR) status.

Table 1

Frequency of vitamin D receptor (VDR) expression in colorectal cancer

Clinical, pathologic or molecular feature	Total N	VDR+	Univariate OR (95% CI)	P value
All cases	619	233 (38%)		
Gender				
Men	244	83 (34%)	1	Reference
Women	375	150 (40%)	1.29 (0.92-1.81)	0.13
Age at diagnosis (years)				
≤59	141	60 (43%)	1	Reference
60-69	268	104 (39%)	0.86 (0.57-1.30)	0.46
≥70	210	69 (33%)	0.66 (0.43-1.03)	0.065
Body mass index (BMI, kg/m^2)				
<30	483	178 (37%)	1	Reference
≥30	100	38 (38%)	1.05 (0.67-1.64)	0.83
Family history of colorectal cancer				
(-)	472	180 (38%)	1	Reference
(+)	147	53 (36%)	0.91 (0.62-1.34)	0.65
Tumor location				
Distal (splenic flexure to rectum)	325	131 (56%)	1	Reference
Proximal (cecum to transverse)	287	98 (34%)	0.77 (0.55-1.07)	0.12
Stage				
I	135	47 (35%)	1	Reference
II	189	69 (37%)	1.08 (0.68-1.71)	0.75
III	174	79 (45%)	1.56 (0.98-2.47)	0.060
IV	84	27 (32%)	0.89 (0.50-1.58)	0.68
Tumor grade				
Low	546	214 (39%)	1	Reference
High	53	11 (21%)	0.41 (0.20-0.81)	0.008
Mucinous component				
0%	317	124 (39%)	1	Reference
≥1%	221	74 (33%)	0.78 (0.55-1.12)	0.18
Signet ring cell component				
0%	461	179 (39%)	1	Reference
≥1%	41	10 (24%)	0.51 (0.24-1.06)	0.068
CIMP status (No. of methylated CIMP markers)				
CIMP-0 (0)	266	100 (38%)	1	Reference
CIMP-low (1-5)	246	100 (41%)	1.14 (0.80-1.62)	0.48
CIMP-high (6-8)	91	27 (30%)	0.70 (0.42-1.17)	0.17
MSI status				
MSS	461	168 (36%)	1	Reference
MSI-low	59	30 (51%)	1.80 (1.05-3.11)	0.032
MSI-high	96	32 (33%)	0.87 (0.55-1.39)	0.56
MSI/CIMP status				
CIMP-low/0 MSI-low/MSS	478	186 (39%)	1	Reference
CIMP-high MSI-low/MSS	30	8 (27%)	0.57 (0.25-1.31)	0.18
CIMP-low/0 MSI-high	32	12 (38%)	0.94 (0.45-1.97)	0.87
CIMP-high MSI-high	61	19 (31%)	0.71 (0.40-1.26)	0.24
BRAF mutation				
(-)	515	194 (38%)	1	Reference
(+)	86	28 (33%)	0.80 (0.49-1.30)	0.36
KRAS mutation				
(-)	388	131 (34%)	1	Reference
(+)	229	101 (44%)	1.55 (1.11-2.16)	0.010
PIK3CA mutation				
(-)	470	158 (34%)	1	Reference
(+)	84	44 (52%)	2.17 (1.36-3.47)	0.001
KRAS/PIK3CA mutation				
KRAS(-)/PIK3CA(-)	312	99 (32%)	1	Reference
KRAS(-)/PIK3CA(+)	34	15 (44%)	1.70 (0.83-3.48)	0.14
KRAS(+)/PIK3CA(-)	157	59 (38%)	1.30 (0.87-1.94)	0.21
KRAS(+)/PIK3CA(+)	50	29 (58%)	2.97 (1.61-5.47)	0.0003
LINE-1 methylation				
≥70%	95	33 (35%)	1	Reference
50-69%	424	160 (38%)	1.14 (0.71-1.81)	0.58
≤49%	77	30 (39%)	1.20 (0.64-2.24)	0.57
p53 expression				
(-)	355	133 (37%)	1	Reference
(+)	259	97 (37%)	1.00 (0.72-1.39)	0.99
p21				
Expressed	115	36 (31%)	1	Reference
Lost	490	191 (39%)	1.40 (0.91-2.16)	0.13
Nuclear β-catenin expression				
(-)	345	136 (39%)	1	Reference
(+)	207	78 (38%)	0.93 (0.65-1.32)	0.68
COX-2 expression				

Clinical, pathologic or molecular feature	Total N	VDR+	Univariate OR (95% CI)	P value
(-)	104	31 (30%)	1	Reference
(+)	511	199 (39%)	1.50 (0.95-2.37)	0.079

CI, confidence interval; OR, odds ratio; CIMP, CpG island methylator phenotype; COX-2, cyclooxygenase-2; MSI, microsatellite instability; MSS, microsatellite stable

Table 2

Intensity of vitamin D receptor (VDR) expression in colorectal cancer and mutations in *PIK3CA* and *KRAS*

	Total N	VDR expression			
		No	Weak	Moderate	Strong
PIK3CA mutation					
(-)	470 (85%)	179 (89%)	140 (87%)	98 (80%)	53 (78%)
(+)	84 (15%)	23 (11%)	21 (13%)	25 (20%)	15 (22%)
KRAS mutation					
(-)	388 (63%)	154 (70%)	110 (62%)	79 (57%)	45 (56%)
(+)	229 (37%)	66 (30%)	67 (38%)	60 (43%)	36 (44%)

A percentage number indicates the frequency of *PIK3CA* or *KRAS* mutation among colorectal cancers in a specific category of VDR expression intensity.

Table 3

Multivariate analysis to examine the relations of *PIK3CA* and *KRAS* mutations with vitamin D receptor (VDR) expression in colorectal cancer

Variable independently associated with VDR	Multivariate OR (95% CI)	P value
PIK3CA mutation	2.36 (1.43-3.91)	0.0008
KRAS mutation	1.53 (1.04-2.23)	0.029
Other variable		
Mucinous component	0.64 (0.43-0.96)	0.029

Multivariate logistic regression analysis assessing the relations of *PIK3CA* and *KRAS* mutations with VDR included age at diagnosis, sex, BMI, tumor location, tumor stage, grade, mucinous component, family history of colorectal cancer, microsatellite instability, CpG island methylator phenotype, LINE-1, β-catenin, COX-2, p53, p21 and *BRAF*. Only variables with $p<0.05$ are listed.

CI, confidence interval; OR, odds ratio.

Table 4

Vitamin D receptor (VDR) expression and patient mortality in colorectal cancer

	Total N	Colorectal cancer-specific mortality				Overall mortality			
		Deaths / person-years	Univariate HR (95% CI)	Stage-matched HR* (95% CI)	Multivariate HR^ (95% CI)	Deaths / person-years	Univariate HR (95% CI)	Stage-matched HR* (95% CI)	Multivariate HR^ (95% CI)
VDR (-)	374 (62%)	101/2284	1 (reference)	1 (reference)	1 (reference)	164/2284	1 (reference)	1 (reference)	1 (reference)
VDR (+)	225 (38%)	57/1540	0.91 (0.66-1.26)	0.88 (0.63-1.23)	0.88 (0.61-1.26)	96/1540	0.88 (0.68-1.13)	0.85 (0.65-1.10)	0.87 (0.66-1.14)

* The stage-matched (stratified) Cox model included stage categories (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) as a stratifying variable without any other covariate in the model.

^ The multivariate, stage-matched (stratified) Cox model included the VDR variable stratified by age at diagnosis, year of diagnosis, sex, body mass index, family history of colorectal cancer, tumor location, tumor grade, mucinous component, *KRAS*, *PIK3CA*, *BRAF*, β-catenin, COX-2, p53, p21, LINE-1 methylation, microsatellite instability, and the CpG island methylator phenotype.

CI, confidence interval; HR, hazard ratio.