

Vitamin D and colon cancer

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Abstract

Calcitriol, $1\alpha, 25$ -dihydroxyvitamin D_3 ($1,25$ (OH) $_2D_3$), the most active form of vitamin D, is a pleiotropic hormone with a wide range of biological activities. Due to its ability to regulate calcium and phosphate metabolism, $1,25D_3$ plays a major role in bone health. In addition, $1,25D_3$ binds to the vitamin D receptor and thereby regulates the expression of a number of genes which control growth, differentiation and survival of cancer cells. In agreement, the levels of vitamin D_3 appear to be an essential determinant for the development and progression of colon cancer and supplementation with vitamin D_3 is effective in suppressing intestinal tumorigenesis in animal models. Vitamin D_3 has been estimated to lower the incidence of colorectal cancer by 50%, which is consistent with the inverse correlation between dietary vitamin D_3 intake or sunlight exposure and human colorectal cancer. Several studies confirmed that increasing vitamin D_3 lowers colon cancer incidence, reduces polyp recurrence, and that sufficient levels of vitamin D_3 are associated with better overall survival of colon cancer patients. Vitamin D regulates the homeostasis of intestinal epithelium by modulating the oncogenic Wnt signaling pathway and by inhibiting tumor-promoting inflammation. Both activities contribute to the ability of $1,25D_3$ to prevent the development and progression of colon cancer.

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Key words: Colon cancer; Vitamin D; Wnt signaling; Inflammation; Chemoprevention

Core tip: Epidemiological studies suggest that deficiency of vitamin D increases the incidence of colon cancer and also has a negative impact on the survival of colon cancer patients. The ability of $1,25D_3$ to interfere with Wnt signaling and to ameliorate inflammation is likely to contribute to its anticancer activity.

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INTRODUCTION

The biologically active form of vitamin D_3 , $1\alpha, 25$ (OH) $_2D_3$ ($1,25D_3$), is obtained by 25-hydroxylation of vitamin D_3 in the liver and 1α -hydroxylation in the kidney, liver or other tissues. Hydroxylation of 25 (OH) D_3 by CYP27B1 yields the hormonally active form $1,25$ (OH) $_2D_3$, which is metabolized to less active metabolites by CYP24A1 (reviewed in^[1]). While the levels of CYP21B1 have been shown to be reduced in some cancers, the levels of CYP24A1 are increased in cancer cells, which may contribute to the resistance of some tumors to $1,25D_3$ ^[2].

$1,25D_3$ exerts most of its biological activity through binding to a specific vitamin D_3 receptor (VDR), a member of the nuclear receptor superfamily^[1]. VDR binds to retinoid X receptor (RXR), and the VDR-RXR heterodimers bind to a vitamin D response element (VDRE), activating or repressing gene expression, which contribute to the anti-neoplastic activity of vitamin D. VDR associates with other transcription factors, such as SP1 and β -catenin^[3] and thereby also regulates the expression of genes that do not harbor the consensus VDRE. A number of cancer cell lines, including colon cancer cell lines tested in our laboratory, display a limited response to vitamin D_3 *in vitro*^[4] and the expression of VDR is

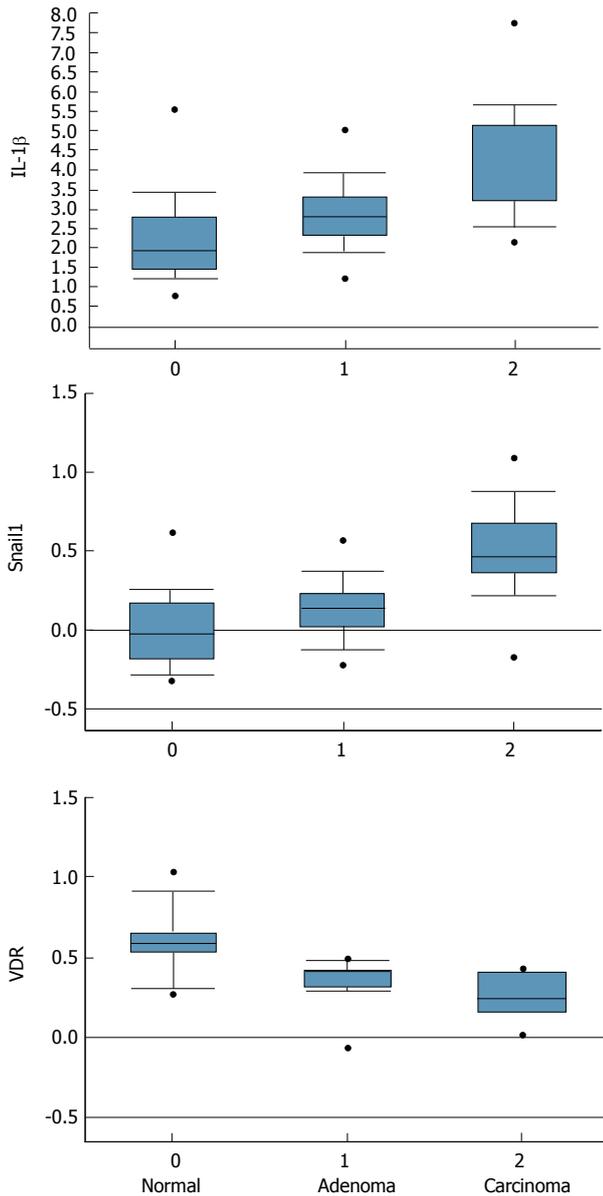


Figure 1 The expression levels of IL-1 β and Snail are increased and the levels of vitamin D receptor decreased in colon cancer patients (Skrypczak, *PLOS ONE* 2010^[71]). VDR: Vitamin D receptor.

downregulated in late stages of colon cancer^[5] (Figure 1), suggesting that vitamin D₃ may exert some of its biological activities in a VDR-independent manner, or that it targets cells in the tumor microenvironment. VDR^{-/-} mice display hyper-proliferation and have elevated levels of c-myc in both skin and colon, and VDR suppresses c-myc expression *in vitro* and *in vivo* in the absence of 1,25D₃^[6]. However, 1,25D₃ triggers association of VDR with c-myc and thereby promotes turnover of c-myc protein^[6], indicating that vitamin D signaling suppresses transcription of c-myc and also inhibits c-myc stability. In addition to its ability to inhibit c-Myc, 1,25D₃ induces the expression of its antagonist Mxd1/Mad1, suggesting that 1,25D₃ can exert its chemopreventive activity through regulation of the c-myc/Mxd1 network^[6].

The focus of this report is to discuss the role of vi-

tamin D in colon cancer, however the beneficial effects of vitamin D have been noted in other malignancies. Reduced serum levels of vitamin D were found in stage IV melanoma patients and it has been shown that melanoma patients with low serum levels of vitamin D developed metastasis earlier than patients with high levels of vitamin D^[7]. Similarly, chemopreventive activity of vitamin D has been observed in breast, ovarian, pancreatic and prostate cancer patients^[8].

In addition to its chemopreventive activity, 1,25D₃ or its analogues have been tested for their ability to improve the response to anticancer agents. Vitamin D and its derivatives have been shown to enhance the anticancer activity of 5FU, irinotecan and oxaliplatin both *in vitro* and *in vivo*^[9,10]. Although the therapeutic use of 1,25D₃ is restricted by its hypercalcemic activity, several 1,25D₃ analogues that retain the antitumor activity while being devoid of hypercalcemic effects, are currently being tested in clinical trials for a variety of malignancies.

VITAMIN D AND COLON CANCER

Recent case-controlled studies have established that there is an inverse correlation between serum levels of vitamin D and the incidence of polyps and adenomas in the colon^[11-13], consistent with the inverse correlation between dietary vitamin D₃ intake or sunlight exposure and human colorectal cancer^[14-17]. This is significant because a large segment of the human population suffers from vitamin D₃ insufficiency or deficiency^[18], which is particularly prevalent among colon cancer patients. Indeed, numerous studies have suggested that higher vitamin D₃ levels are associated with lower colon cancer incidence, reduced polyp recurrence and better overall survival of colon cancer patients^[19-22].

Vitamin D and its analogues reduce the growth of colon cancer xenografts and inhibit tumorigenesis in several genetic models of intestinal cancer. In agreement, dietary initiation of colon cancer in rodents, a model of sporadic colon cancer, has been shown to be prevented by supplementation with vitamin D₃ and Ca^[23,24].

Despite the established chemopreventive activity of vitamin D₃, its targets and the molecular basis for its antitumor activity remain poorly understood. 1,25D₃ inhibits growth of tumor cells by inducing the expression of cyclin-dependent kinase inhibitors, such as p21, p27, and cystatin D, and by inhibiting the expression of pro-proliferative genes, including c-myc and cyclin D1. In addition, 1,25D₃ has been shown to upregulate miR-627, which targets the histone demethylase jumonji domain containing protein 1A, and thereby inhibits proliferation of colon cancer cells *in vitro* and *in vivo* through epigenetic regulation^[25]. By increasing the expression of alkaline phosphatase, maltase, E-cadherin and cell adhesion proteins, vitamin D promotes differentiation. In a cell-type specific manner, vitamin D promotes apoptosis by regulating the expression of B-cell lymphoma 2 family members. Thus, due to its ability to affect multiple signaling pathways and to regulate many target genes, 1,25D₃

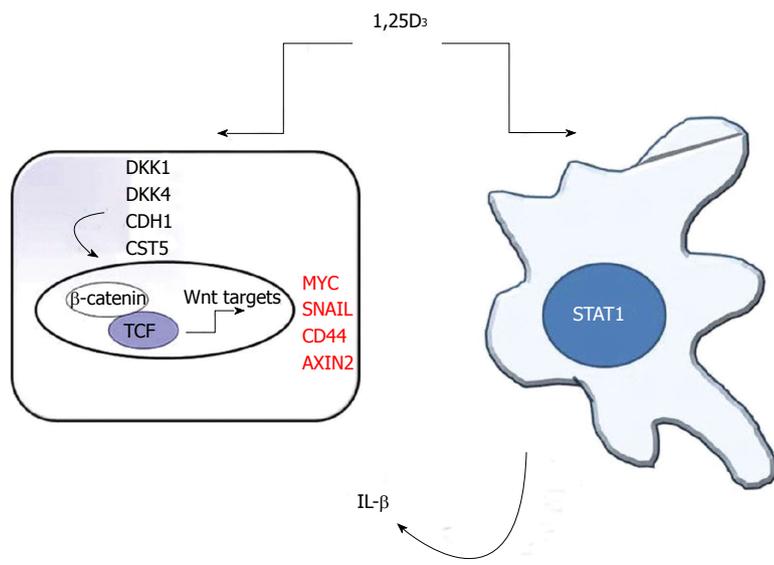


Figure 2 The multiple mechanisms whereby vitamin D inhibits Wnt signaling: 1,25D₃ acts on both tumor cells and tumor-associated macrophages (and potentially on other stromal cells). In tumor cells, 1,25D₃ promotes VDR/β-catenin binding and thus inhibits nuclear translocation of β-catenin. It also induces the expression of E-cadherin (CDH1), Dickkopf1 (DKK1), Dickkopf4 (DKK4) and cystatin 5 (CST5), antagonizing β-catenin/TCF transcriptional activity. As a result, the expression of several Wnt target genes, such as Snail, CD44, Myc, Axin2 (in red) is downregulated by 1,25D₃. These activities require VDR expression in tumor cells. In addition, vitamin D also acts on cells in the tumor microenvironment. We demonstrated that 1,25D₃ inhibits STAT1 activity in tumor-associated macrophages and prevents the release of IL-1β, which in a paracrine manner promotes Wnt signaling in cancer cells. 1,25D₃ can thereby regulate Wnt signaling in tumor cells that do not respond directly to 1,25D₃. VDR: Vitamin D receptor.

controls a variety of biological processes. Although 1,25D₃ has also been shown in preclinical studies to inhibit invasiveness of tumor cells and to reduce their ability to metastasize, clinical trials suggest that while vitamin D is effective in early stages of cancer, it appears to have limited activity in advanced, aggressive malignancies.

Important mechanisms whereby 1,25D₃ regulates the homeostasis of intestinal epithelium and exerts its anti-neoplastic activity is through its ability to interfere with Wnt/β-catenin signaling^[3,26,27] and to inhibit inflammation. Because inflammation can fuel Wnt signaling in colon cancer cells, the two activities may be coupled, suggesting that 1,25D₃ might exert chemopreventive activity by interrupting the link between inflammation and cancer. However, large clinical trials are required to firmly establish the preventive and therapeutic value of vitamin D in colon cancer. Such trials are complicated by the necessity of maintaining and monitoring vitamin D levels as well as clinical outcome in a large number of patients over a long period of time.

INHIBITION OF WNT SIGNALING BY VITAMIN D

The Wnt/β-catenin signaling pathway regulates the intracellular levels of β-catenin and controls the expression of β-catenin/TCF4 target genes. In normal cells, β-catenin is sequestered in a large cytoplasmic protein complex, called the β-catenin destruction box, which includes Axin and Apc and the GSK3β and CK1 kinases^[28,29]. Due to mutations in the tumor suppressor Apc, or less frequently in Axin or β-catenin, the oncogenic Wnt/β-catenin signaling pathway is abnormally activated in over 90% of colon cancers^[30].

The β-catenin destruction complex promotes β-catenin phosphorylation and its subsequent degradation. Wnt activation of its receptors, Frizzled and LRP5/6, inhibits the destruction complex and results in accumulation of β-catenin, both in the cytoplasm and in

the nucleus, where it acts as a co-activator of LEF/TCF and regulates the expression of a variety of genes. Wnt/β-catenin signaling activates genes, such as c-myc and cyclin D and thereby promotes proliferation of tumor cells. Activation of Wnt signaling also induces the expression of COX2 and survivin which increases the survival of intestinal epithelial cells. Wnt signaling has been shown to promote transcription, protein stability and to regulate nuclear localization of Snail, a transcription factor that mediates epithelial mesenchymal transition^[31,32]. In turn, Snail interacts with β-catenin and increases the expression of Wnt target genes^[33]. We showed that inflammation-induced stabilization of Snail contributes to Wnt signaling in colon cancer cells and creates a positive feedback loop initiated, and propagated, by macrophage-derived IL-1β^[34]. IL-1β was sufficient to increase the levels of Snail in colon cancer cells^[35], and the levels of both IL1β and Snail are increased in colon cancer patients (Figure 1). Importantly, Snail1 and Slug (Snail2) have been shown to inhibit the expression of VDR and to inhibit the activity of 1,25D₃^[5,36-38]. Wnt-dependent stabilization of Snail is likely to contribute to reduced expression of VDR in colon cancer patients (Figure 1).

1,25D₃ has been shown, in a VDR-dependent manner, to antagonize Wnt signaling through a variety of mechanisms. These include sequestration of β-catenin through a direct VDR/β-catenin interaction and induction of nuclear export of β-catenin. 1,25D₃ also enhances the expression of DKK1, which is an endogenous inhibitor of Wnt signaling. Furthermore, cystatin D, whose expression is strongly upregulated by 1,25D₃, inhibits Wnt signaling and the expression of its target genes, including Snail (Figure 2). Cystatin D inhibits migration and anchorage-independent growth of colon cancer cells and its silencing abrogates the anti-proliferative activity of 1,25D₃ and increases the expression of c-Myc^[39]. A comprehensive review of the mechanisms whereby vitamin D represses Wnt signaling has been published recently^[40].

Wnt activity in primary human tumors is heterogeneous, and it has been demonstrated that its activity is

regulated by factors from the tumor microenvironment. Although loss of *Apc* occurs early in adenoma development in the colon, *in vivo* progression from micro-adenomas to macroscopic tumors in *Apc*^{Min/+} mice is associated with further elevation of canonical Wnt signaling and increased expression of Wnt target genes^[41]. This suggests that enhancement of Wnt signaling beyond a threshold level sufficient for tumor initiation may be required for tumor progression and metastatic spread. Often factors from the tumor microenvironment provide signals that regulate the extent of oncogenic signaling in tumor cells. We and others have demonstrated that tumor-associated macrophages promote Wnt signaling in colon cancer cells *via* IL-1 β and TNF^[34,42]. Fibroblasts have also been shown to enhance Wnt signaling through hepatocyte growth factor^[43], confirming the role of inflammatory factors in Wnt signaling and in maintenance of cancer stem cells (see below). Leukotriene D4, which can be produced and secreted by stromal cells in the local tumor microenvironment, promotes the expression and nuclear translocation of β -catenin and thus enhances the growth of colon cancer cells^[44]. Indeed, β -catenin translocation is often detected at the invasive front of tumors^[45,46], consistent with the interpretation that stromal tissue at the invasion front provides signals to tumor cells that promote nuclear translocation of β -catenin and thus drive tumor progression. It is therefore likely that 1,25D₃ regulates Wnt signaling by targeting both the tumor microenvironment as well as the tumor cells themselves. Indeed, we have shown that vitamin D interrupts signaling between tumor cells and macrophages and thereby decreases the intensity of Wnt signaling in HCT116 colon cancer cells which are themselves unresponsive to direct effect of vitamin D^[34]. We demonstrated that this mechanism involved 1,25D₃ inhibition of STAT1 activity in macrophages, blocking the release of IL-1 and thereby restoring the sensitivity of colon cancer cells to TRAIL-induced apoptosis^[35]. This is in line with the concept that the tumor microenvironment represents an important target of chemopreventive and chemotherapeutic agents^[47].

The ability of vitamin D to regulate Wnt signaling has been confirmed in animal models. Vitamin D and its analogues reduced the number of tumors in *Apc*^{Min/+} mice^[48], associated with decreased nuclear β -catenin and reduced expression of β -catenin target genes^[49]. Likewise, dietary induction of colon tumors in mice, a model of sporadic colon cancer, accompanied by functional enrichment of Wnt signaling, is reversed by supplementation with vitamin D and Ca^[24]. *Apc*^{Min/+} mice lacking VDR have an increased number of aberrant crypt foci (ACF) and both ACFs and tumors in *Apc*^{Min/+}/*VDR*^{-/-} mice display increased nuclear β -catenin and elevated expression of β -catenin/TCF target genes^[50]. While the number of adenomas and carcinomas was not affected by the inactivation of VDR, tumors that developed in the *Apc*^{Min/+}/*VDR*^{-/-} mice were significantly larger, consistent with increased growth due to enhanced Wnt signaling. We recently confirmed that while targeted inactivation of VDR in intestinal cells did not alter tumor multiplicity in

Apc^{Min/+} mice, inactivation of VDR in macrophages substantially reduced *Apc*^{Min/+} tumors (submitted), confirming the important role of VDR signaling in the tumor microenvironment.

Consistent with these *in vitro* data and with studies in mice, dietary supplementation with 1,25D₃ decreased the levels of β -catenin and increased the expression of E-cadherin in normal mucosa of colon cancer patients^[51].

ANTI-INFLAMMATORY PROPERTIES OF VITAMIN D

Chronic inflammation has been shown to predispose to development of tumors, a striking example being inflammatory bowel disease, which is associated with elevated risk of colon cancer^[52]. Moreover, it appears that colon cancers that are not linked to inflammatory bowel disease are also driven by inflammation; it has been shown that regular use of NSAIDs lowers the mortality from sporadic colon cancer and inhibits adenomas in FAP patients, who inherit a mutation in the *Apc* gene^[53]. The mechanisms whereby anti-inflammatory agents inhibit progression of tumors that are not associated with overt inflammation are not fully understood. However, it has been established that cancer and several other chronic diseases are associated with para-inflammation, a low grade inflammation that is coupled to a persistent activation of the DNA damage response^[54] and the induction of DNA damage-induced soluble factors, including major pro-inflammatory cytokines, chemokines and growth factors. It is possible that anti-inflammatory agents exert their chemopreventive activity by ameliorating the pro-tumorigenic activity of para-inflammation that is associated with aging and that is observed in colon cancer patients.

Inflammatory bowel disease (IBD) is among the three most prevalent high risk conditions for colon cancer^[52]. The risk for colorectal cancer increases with the duration and the extent of the disease, consistent with a direct connection between inflammation and colon cancer development. Patients with intestinal inflammatory conditions such as ulcerative colitis (UC) and Crohn's disease (CD) have a high incidence of vitamin D insufficiency and deficiency^[55] and show reduced levels of VDR in intestinal epithelium^[56]. Likewise, higher levels of vitamin D have been shown to lower the risk of Crohn's disease^[57]. Overexpression of VDR in intestinal cells inhibits the colitis-associated increase in proinflammatory cytokines, such as TNF, IL-1 and CCL2, and protects mice from developing colitis^[56]. Finally, a vitamin D analogue has been shown to inhibit colon carcinogenesis in the azoxymethane/dextran sodium sulphate (AOM/DSS) model of ulcerative colitis^[58], suggesting that VDR signaling may avert the conversion of the inflammatory stimuli into a tumor promoting signal.

VDR knock-out mice exhibit a proinflammatory phenotype associated with increased NF- κ B activity in intestine, consistent with the ability of VDR signaling to inhibit NF- κ B activation^[59]. TNF- α is a major proin-

flammatory cytokine that activates the NF- κ B signaling pathway in tumor cells and thereby regulates their growth and survival. Human colon cancers are infiltrated by inflammatory cells which secrete a variety of proinflammatory factors, including TNF- α ^[60]. Likewise, polyps arising in Apc ^{Δ 468} mice, a genetic model for intestinal cancer, showed infiltration with mast cells, and depletion of mast cells or anti-TNF- α treatment significantly suppressed polyposis in Apc ^{Δ 468} mice^[60]. Etanercept, a specific antagonist of TNF- α , also reduced the number and the size of tumors in the AOM/DSS model, confirming a role of TNF- α in inflammation-promoted intestinal tumorigenesis. More intriguing was the observation that inhibition of TNF- α blocks the accumulation of β -catenin mutations in intestinal cells, suggesting a mutagenic role of TNF- α ^[61]. Pharmacological inhibition of TNF- α by neutralizing TNF- α antibodies is very effective in alleviating inflammation in IBD patients^[62] and inhibitors of TNF- α have also been tested as potential agents for the treatment of colon cancer. Unfortunately, TNF- α inhibitors have been linked to a broad range of infections and to the development of lymphomas and skin and lung cancer, limiting their clinical utility.

An alternative approach to targeting TNF/NF- κ B-mediated inflammation and interrupting the link between inflammation and cancer may be offered by vitamin D. 1,25D₃ inhibits the interaction of peripheral blood mononuclear cells and colon cancer cells and inhibits the production of TNF^[63] and blocks NF- κ B signaling, a major TNF signaling pathway. VDR physically interacts with IKK β ^[59] and vitamin D downregulates the expression of NF κ B target genes, such as Puma^[56], which play a major role in the survival of cancer cells. In addition, 1,25D₃ has been shown to downregulate the expression of Toll-like receptors 2 and 4 (TLR2 and TLR4) on human monocytes, resulting in hyporesponsiveness to TLR activating ligands^[64,65]. Inhibition of TLR signaling by vitamin D₃ has been suggested to reduce AOM/DSS-induced colon cancer^[66], pointing to a convergence of the chemopreventive and anti-inflammatory properties of vitamin D₃.

NF- κ B is not the only oncogenic signaling pathway activated in tumor cells by inflammatory factors. We have shown that TNF enhances Wnt signaling in β -catenin mutant colon cancer cells^[34], and established that macrophage-derived factors activate Wnt signaling in colon cancer cells through NF- κ B signaling^[42]. Oguma *et al*^[67] demonstrated that TNF- β promotes Wnt signaling also in gastric cancer cells, which was independent of NF- κ B in this tissue.

The HCT116 colon cancer cells have a functional VDR, but do not respond to 1,25D₃ treatment with growth arrest, apoptosis or differentiation. However, we demonstrated that in the presence of macrophages, 1,25D₃ reduced Wnt signaling in these seemingly vitamin D unresponsive cells by interrupting signaling between tumor cells and macrophages. 1,25D₃ inhibits STAT1 activity and prevents tumor cell-induced release of IL1 from macrophages and thereby prevents inflammation-induced Wnt signaling in colon cancer cells^[34] (Figure 2). Accordingly, 1,25D₃ inhibits the ability of macrophages to increase prolifera-

tion and survival of colon cancer cells. Among genes that were repressed by 1,25D₃ in tumor cells in a macrophage-dependent manner were cyclin D1 and c-myc, consistent with the finding that 1,25D₃ prevented macrophage-induced clonogenic growth of HCT116 cells. Therefore, 1,25D₃ can exert its tumor-preventive activity by normalizing the tumor microenvironment, and it can inhibit inflammation through a variety of mechanisms.

Diet-induced obesity, a risk factor for colon cancer, is also associated with increased expression of TNF- β in the intestine. In this settings, TNF- β has also been shown to be coupled to inactivation of GSK3- β and increased expression of β -catenin and c-myc, suggesting that obesity increases the risk of colorectal cancer by promoting inflammation^[68]. Indeed, western style diet (WSD), sufficient to initiate intestinal tumorigenesis in mice^[24], has been shown to trigger an inflammatory response in mice, accompanied by the accumulation of macrophages in intestinal mucosa and increased levels of circulating proinflammatory cytokines, including IL-1 β , CCL5 and CCL2^[69]. Importantly, dietary supplementation with vitamin D and Ca prevents WSD-induced increases in inflammatory markers and inhibits intestinal tumorigenesis^[24,69]. Dietary supplementation with 1,25D₃ reduced markers of inflammation, including C-reactive protein (CRP), TNF, IL-1 β , IL-6 and IL-8 also in colon cancer patients^[70], strongly suggesting that 1,25D₃ protects from colon cancer, at least in part, by decreasing inflammation.

CONCLUSION

Calcitriol, the most active form of vitamin D₃, acts as a potent steroid hormone that binds to VDR and thereby alters the expression of a variety of genes that regulate growth, differentiation and survival of epithelial cells. Epidemiological studies suggest that deficiency of vitamin D increases the incidence of colon cancer and also has a negative impact on the survival of colon cancer patients. The ability of 1,25D₃ to interfere with Wnt signaling and to ameliorate inflammation is likely to contribute to its anticancer activity. The optimal form and adequate concentration of vitamin D that have cancer preventive activity should be established, and randomized clinical trials are needed to confirm that 1,25D₃ alone, or in combination with other cytotoxic agents, offers therapeutic benefits.

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