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Review Article

Vitamin D3 intervention can improve inflammatory response and immune function in colorectal cancer mouse model

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Abstract: To investigate the effect of vitamin D3 (VD3) on inflammatory factors and immune function of colorectal cancer (CRC) mice model. 125 SPF BALB/c mice were purchased and divided into model group, VD3 low dose group, VD3 medium dose group, VD3 high dose group and normal group on average. Tumorigenesis was induced in mice except the normal group by human CRC cell line HCT116. After administration for 4 weeks, tumor volume, thymus index, spleen index, survival time, cell cycle and apoptosis rate of CRC mice were recorded, and the effect of VD3 on inflammatory factors and immune indexes of CRC mice was detected. The intake of VD3 contributed to the reduction of tumor size, the improvement of thymus and spleen indexes, the extension of survival time, the stagnation of G0/G1 phase, the increase of apoptosis rate, the reduction of concentration of proinflammatory factors high sensitivity C-reactive protein (Hs-CRP), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), the increase of concentration of anti-inflammatory factor interleukin-4 (IL-4), the increase of phosphorylation level of inflammatory proteins SMAD2/3 and SMAD7, the decrease of transforming growth factor-β1 (TGF-β1) level, the increase of cellular immune index $CD4^+$, $CD4^+/CD8^+$ levels, the decrease of $CD8^+$ level and the increase of humoral immune index antibody-producing cells and hemolysin secretion in CRC mice. VD3 intervention can improve inflammatory response and immune function in CRC mouse model.

Keywords: Vitamin D3, colorectal cancer mouse, inflammatory factors, immune function

Introduction

Colorectal cancer (CRC) is a common fatal cancer in Europe, with therapeutic resistance and poor prognosis [1, 2]. According to global data, 2.2 million new cases and 1.1 million deaths are expected in 2030, and the morbidity and mortality in low and middle income countries are still rising rapidly [3]. The treatment strategy of CRC depends on the stage of the patient and the degree of metastasis. At present, the treatment schemes for CRC include endoscopic treatment, surgical treatment, chemotherapy and nutritional therapy [4, 5]. Endoscopic therapy, surgical therapy, chemotherapy and other treatment methods have potential side effects and chemical resistance, while nutritional therapy has a positive impact on the quality of life and functional status of CRC patients due to its own advantages [6, 7]. Therefore, it is of great value to explore the nutritional therapy of CRC for the treatment of CRC patients and the reduction of mortality.

Vitamin D3 (VD3) is a safe, economical and easily available nutrient, which exists in animal and plant organisms or skin synthesis assisted by ultraviolet rays. It can play an anti-cancer role by inducing cancer cell apoptosis or inhibiting proliferation [8, 9]. VD3 has been applied in the treatment of various cancer patients. When applied to breast cancer patients, it can help improve relapse-free survival and inhibit tumor growth. When applied to gastric cancer patients, it can improve cell apoptosis rate and further inhibit tumor progression [10, 11]. VD3 also has other effects in common diseases, such as regulating cells of patients with systemic lupus erythematosus to stagnate in G1 phase, participating in pain mechanism of patients with osteoarthritis and regulation of inflammatory factors, and relieving immuno-

suppression of patients with multiple sclerosis [12-14]. In Barry et al. [15] study, VD3 has certain curative effect in the treatment of patients with advanced colorectal adenoma, but there is genotypic heterogeneity of VD3 receptor, suggesting that VD3 may be a potential therapeutic scheme for CRC.

At present, there is little research on the mechanism of VD3 in CRC. We will establish a CRC mouse model to study the regulation mechanism of VD3 in CRC, hoping to provide reference for VD3 in the treatment of CRC.

Materials and methods

Experimental materials and reagents

There were SPF BALB/c male or female mice (JRDUN Biotechnogy (Shanghai) co., Ltd., China), with age of 5-6 weeks old and average weight of 14-18 g. There were human CRC cell line HCT116 (shanghai aolu biotechnology co., ltd., china, XB-3206), RPMI1640 complete medium (Shanghai Halling Biotechnology Co., Ltd., China, HL12052.4.1), VD3 (Shanghai Hengfei Biotechnology Co., Ltd., China, V8070), mouse Hs-CRP, IL-1β, IL-4, TNF-α ELISA kits (Shanghai Guangrui Biotechnology Co., Ltd., China, Mouse elisa371, Mouse elisa40, Mouse elisa165, Mouse elisa132), fully automatic enzyme-labelling measuring instrument (Tideradar Beijing Technology co., LTD., China, BIOTEK ELX800), TGF-β1, p-SMAD7 and β-Actin antibody (Shanghai Hengfei Biotechnology Co., Ltd., China, BM0291), p-SMAD2, p-SMAD3 (Shanghai Yubo Biotechnology Co., Ltd., China, IC195026, IC187103), BCA protein kit, ECL developer (Shanghai Yuanye Biotechnology Co., Ltd., China, R21250, R21313), flow cytometer (Shanghai Ranzhe Instrument Equipment Co., Ltd., NovoCyte, china), 10 μL fluorescein isothiocyanate (FITC)-labeled fluorescent monoclonal antibody (CD4-FITC/CD8-ECD) (Shanghai Haoran Biotechnology Co., Ltd., China, RM-25013, 737659), FBS, PBS (Shanghai Kemin Biotechnology Co., Ltd., China, DXT-10099141, DXT-130-070-525), Cell Quest software system (Becton Dickinson, Franklin Lakes, NJ, USA), mianyang erythrocyte (SRBC) (Shanghai Kemin Biotechnology Co., Ltd., China, ab99427), complement (Shanghai Rongweida Industry Co., Ltd., China, 6725-SE-010) and dushi liquid (Shanghai Yuduo Biotechnology Co., Ltd., YDSJ0928, China).

Cells culture

The cells were cultured at 37°C, 5% CO_2 in RPMI1640 complete culture medium with 15% FBS. When cell attachment growth and fusion reached 85%, 0.2% pancreatin was added for digestion. After digestion, the cells were placed into culture medium for continuous culture and passage was completed.

Model establishment, grouping and medication

125 mice were divided into three groups: VD3 low, medium and high dose treatment groups (n=25, a total of 75 mice) were taken as V1, V2 and V3 groups respectively. Mice in V1, V2, V3 and model groups (n=25) received subcutaneous injection of 2×10^6/2.0 ml HCT116 cell suspension. Mice in the normal group (n=25) received normal saline injection. The fourth day after injection, the mice were given the drug by gavage for four weeks. Among them, mice in V1 group were given VD3 of 1.0 U/kg/d, mice in V2 group were given VD3 of 2.0 U/kg/d, mice in V3 group were given VD3 of 4.0 U/kg/d, and mice in the model group and normal group were given normal saline of 200 ml/kg/d. Except for the normal group, 10 mice in each group were given 25 mg/kg hydrocortisone daily by gavag for the determination of thymus and spleen indexes. On the fifth day before the end of treatment, 0.2 mL of 5% SRBC suspension and 50 mg/kg cyclophosphamide were injected intraperitoneally into the other 10 mice for two days for the determination of antibody-producing cells and hemolysin. The remaining 5 mice were used for survival experiments to observe their survival time.

Detection indexes

One day after the treatment, 20 mice that had received the additional drug were killed in each group. Peripheral blood of eyeball, thymus gland, spleen, lymph node and tumor tissue were taken. The maximum diameter (d_1) and minimum diameter (d_2) of tumor tissue, thymus weight and spleen weight were measured. The tumor volume, thymus index and spleen index were calculated. Tumor volume = $d_1d_2^2/2$. Thymus or spleen index = thymus or spleen weight (mg)/body weight (g). Except for the normal group, the survival time of the other 5 mice in each group was recorded. When the mice

suffered from body failure, the mice were killed and dissected. Tumor tissues were taken to make single cell suspension. Cell cycle and apoptosis were measured by flow cytometry.

The serum was centrifuged and separated at 1500× g, 4°C for 10 min. The levels of Hs-CRP, IL-1β, IL-4 and TNF-α in the serum were determined by enzyme-linked immunosorbent assay (ELISA). The detection was carried out with reference to the instruction manual of the kit. Blank hole (without any reagent), standard hole and sample hole to be tested were set up. 50 L of standard substance was added to the standard hole. 50 L of sample was added to the sample hole to be tested. 50 L of streptavidin-HRP was added to each hole, the plate was sealed, and then incubated at 37°C for 60 min. The liquid was discarded, and then washed with washing liquid and shook dry. The experiment was repeated five times. 50 μL of developer A and 50 L of developer B were added to each hole, and mixed well, and stood for 10 min at 37°C in the dark. 50 μL of terminal liquid was added to each hole. The blank wells were zeroed. The absorbance value (OD value) of each hole was measured sequentially at the wavelength of 450 nm with the fully automatic enzyme-labelling measuring instrument. The levels of Hs-CRP, IL-1β, IL-4, TNF-α were calculated.

100 mg of the right lung tissue of rats in each group was taken, and the supernatant was taken at 1500× g, 4°C and 10 min after suspension preparation. The protein concentration was detected by BCA. The protein concentration was adjusted to 4 μg/μL, separated by 12% SDS-PAGE electrophoresis and transferred to PVDF membrane after ionization. The PVDF membrane was then sealed with 5% skim milk powder for 2 h. TGF-β1 (1:500), p-SMAD2 (1:500), p-SMAD3 (1:500), p-SMAD7 (1:500) and β-Actin (1:1000) primary antibody were added and sealed overnight at 4°C. The first antibody was removed by washing the film, and horseradish peroxidase conjugated goat anti-rabbit second antibody (1:1000) was added, incubated at 37°C for 1 h, and rinsed 3 times with PBS for 5 min each time, and then developed in darkroom. The excess liquid on the film was dried with a filter paper. The ECL was illuminated and developed. The protein bands were scanned and the gray values were analyzed in the Quantity One. Relative expression level of its protein = the gray value of the target protein band/the gray value of the β-Actin protein band.

Flow cytometry (Beckman Coulter, Brea, CA, USA) was used to measure peripheral blood t lymphocyte subsets. The peripheral blood and CD4-FITC/CD8-ECD were mixed evenly, and kept in dark for 20 min at room temperature. 500 μL of erythrocyte lysate was added to lyse erythrocytes, and kept in dark for 15 min at room temperature. 500 μL PBS buffer was added and mixed well, and then kept in dark for 10 min at room temperature. Samples were detected on flow cytometer and $CD4^+$, $CD8^+$ and $CD4^{+1}/CD8^+$ data were read by Cell Quest software system.

Quantitative hemolysis spectrophotometry was used to determine antibody-producing cells. Spleen was made into cell suspension, which was filtered through a 200 mesh sieve, centrifuged at 1500× g, 4°C for 10 min. After separating the supernatant, 1 mL of distilled water was added to lyse erythrocytes and remove platelets. The cell density was adjusted to 5×10^6/mL by RPMI1640 complete culture solution. 1 mL of spleen cells were mixed with 0.5 mL of 10% SRBC and 1.0 mL of complement. After a water bath at 37°C for 30 min, the OD value of l mL supernatant was measured at 413 nm.

50-fold diluted 1 mL serum was mixed with 0.5 mL 10% SRBC and 1.0 mL complement. After a water bath at 37°C for 30 min, l mL supernatant was mixed with 3 mL dushi solution. The relevant OD value was determined at 540 nm after standing for 10 min. Hemolysin content was expressed as half hemolysis value (HC_{50}). HC_{50} = OD value of sample/half hemolysis OD value of/SRBC × dilution multiple.

Statistical analysis

SPSS22.0 (Beijing Baiao Yijie Technology Co., Ltd., China) was used for statistical analysis. The counting data was expressed by the number/percentage (n/%) of cases. The comparison of counting data in the two groups was conducted by Chi-square test. When the theoretical frequency in Chi-square test was less than 5, the continuity correction Chi-square test was used. The measuring data was expressed by mean ± SEM. The comparison of measuring

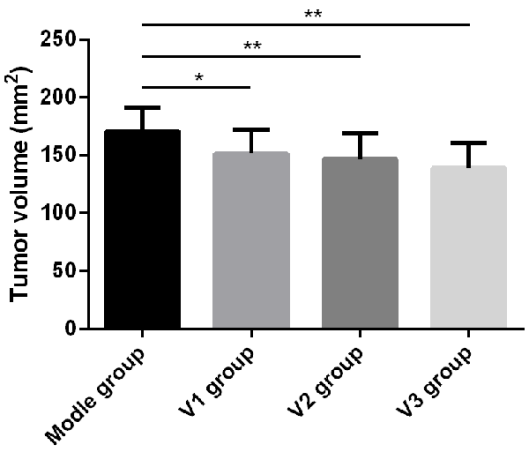

Figure 1. VD3 had a positive effect on the reduction of tumor volume in CRC mice in a dose-dependent manner. Note: *P<0.05, **P<0.01.

data in the two groups was conducted by independent sample t-test. The comparison before and after treatment within groups was conducted by paired t-test. The difference was statistically significant with P<0.05.

Results

Effect of VD3 on tumor size of CRC mice

The tumor volume in the model group was (170.85±20.38) mm^3, while the tumor volumes in V1, V2 and V3 groups were (151.62±20.73) mm^3, (146.97±22.06) mm^3 and (139.34±21.88) mm^3. VD3 can significantly reduce the tumor volume of CRC mice (P<0.05). With the increase of VD3 dose, the tumor volume showed a trend of reduction. More details are shown in **Figure 1**.

Effect of VD3 on thymus and spleen indexes of CRC mice

The index of thymus and spleen in model group was significantly lower than that in normal group, while VD3 could significantly increase the index of thymus and spleen in CRC mice (P<0.05). With the increase of VD3 dosage, the index of thymus and spleen in CRC mice could further increase, but it was still lower than that in normal group. More details are shown in **Figure 2**.

Effect of VD3 on survival time of CRC mice

The survival time in the model group was (39.03±4.05) d, and that in the V1, V2, V3 groups was (43.51±3.37) d, (50.96±3.81) d and (63.29±2.54) d. VD3 can significantly improve the survival time of CRC mice (P<0.05). With the increase of VD3 dose, the survival time was prolonged. More details are shown in **Figure 3**.

Effect of VD3 on cell cycle and apoptosis rate of CRC mice

VD3 can significantly trap CRC mouse cells in G_0/G_1 phase and increase the apoptosis rate of cells (P<0.05). With the increase of VD3 dose, the apoptosis rate of G_0/G_1 phase and cells showed an increasing trend. More details are shown in **Figure 4**.

Effect of VD3 on serum inflammatory factors in CRC mice

VD3 can significantly reduce the concentrations of Hs-CRP, IL-1β, TNF-α and other pro-inflammatory factors (P<0.05). With the increase of VD3 dose, the concentration of these pro-inflammatory factors can be further reduced, but it was still higher than the normal group. However, VD3 had the opposite effect on anti-inflammatory factor IL-4. The concentration of IL-4 enhanced with the increase of dose, but it was still lower than that of the normal group. More details are shown in **Figure 5**.

Effect of VD3 on inflammatory proteins in CRC mice

VD3 can significantly reduce the concentration of TGF-β1 (P<0.05). With the increase of VD3 dose, the concentration of TGF-β1 can be further reduced, but it was still higher than the normal group. However, VD3 can significantly increase the concentrations of p-SMAD2/3 and p-SMAD7 (P<0.05). With the increase of VD3 dose, the concentrations of p-SMAD2/3 and p-SMAD7 can be further increased, but it was still lower than the normal group. More details are shown in **Figure 6**.

Effect of VD3 on cellular immunity of CRC mice

VD3 can significantly increase $CD4^+$ and $CD4^+/CD8^+$ levels (P<0.05). With the increase of VD3 dosage, the percentage of $CD4^+$ and $CD4^+/CD8^+$ levels can be further increased, but it was still lower than the normal group. However, VD3 can significantly reduce $CD8^+$ level (P<0.05). With the increase of VD3 dose, the concentra-

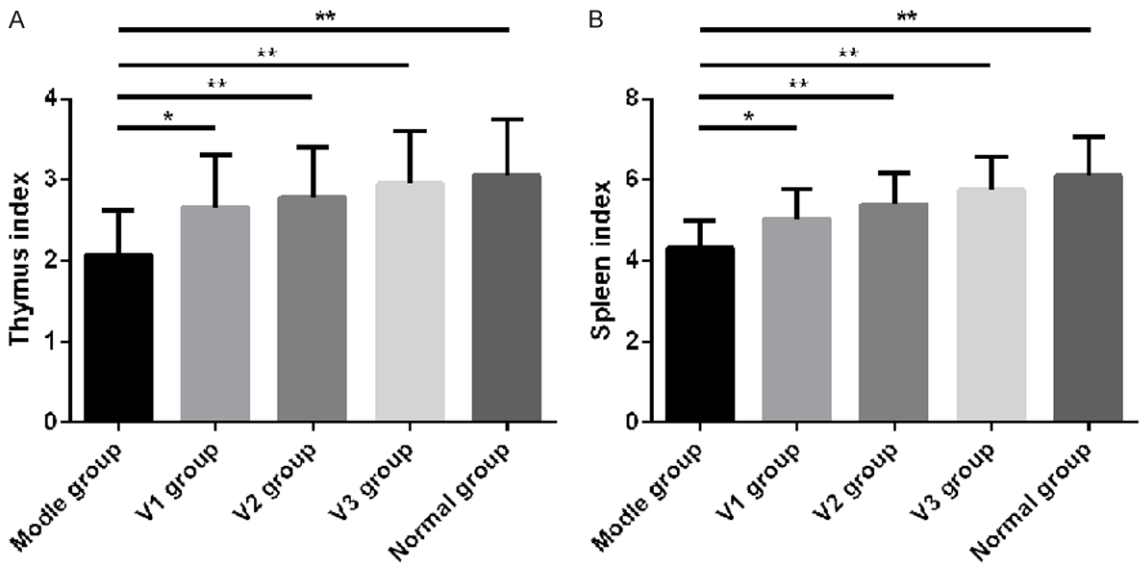

Figure 2. VD3 contributed to the increase of thymus and spleen indexes in CRC mice in a dose-dependent manner. A. VD3 had a promoting effect on the increase of thymus index of CRC mice in a dose-dependent manner. B. VD3 had a promoting effect on the increase of spleen index of CRC mice in a dose-dependent manner. Note: *P<0.05, **P<0.01.

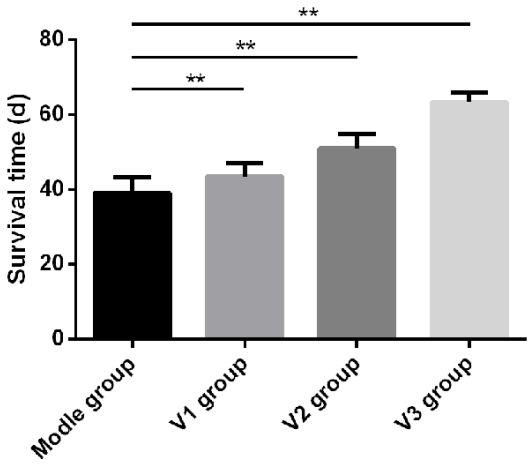

Figure 3. VD3 had a positive effect on prolonging the survival time of CRC mice in a dose-dependent manner. Note: **P<0.01.

tion of $CD8^+$ can be further reduced, but it was still higher than the normal group. More details are shown in **Figure 7**.

Effect of VD3 on humoral immunity of CRC mice

VD3 can significantly improve the formation of antibody-producing cells and the secretion of hemolysin (P<0.05). With the increase of VD3 dosage, antibody-producing cells and hemolysin can further increase, but they were still lower than the normal group. More details are shown in **Figure 8**.

Discussion

Our research results indicated that VD3 was dose-dependent on the reduction of tumor size and the extension of survival time in CRC mice, suggesting that VD3 is helpful to the good progress of CRC tumors and may have chemoprevention effect. In Li [16] and other studies, VD3 can be used for chemical prevention of various cancers. VD3 and metformin can inhibit protein expression of proto-oncogene c-Myc and cyclin D1 by activating vitamin D receptor/β-catenin pathway, and also reduce S6P expression by activating AMPK (IGFI)/mTOR signal, thus preventing early formation of CRC tumors in cooperation with chemistry. It is beneficial to reduce the number of CRC abnormal crypt foci and tumors, suggesting VD3 can play a synergistic role with metformin through complex mechanisms to inhibit malignant progression of CRC tumors. 25416412 wolfberry polysaccharide is also an anti-cancer drug of CRC, which can play an anti-cancer role by blocking cells in G0/G1 phase and increasing cell apoptosis rate [17]. In our study, CRC mice receiving VD3 intervention significantly increased the number of cells stagnating in the G_0/G_1 phase and the rate of

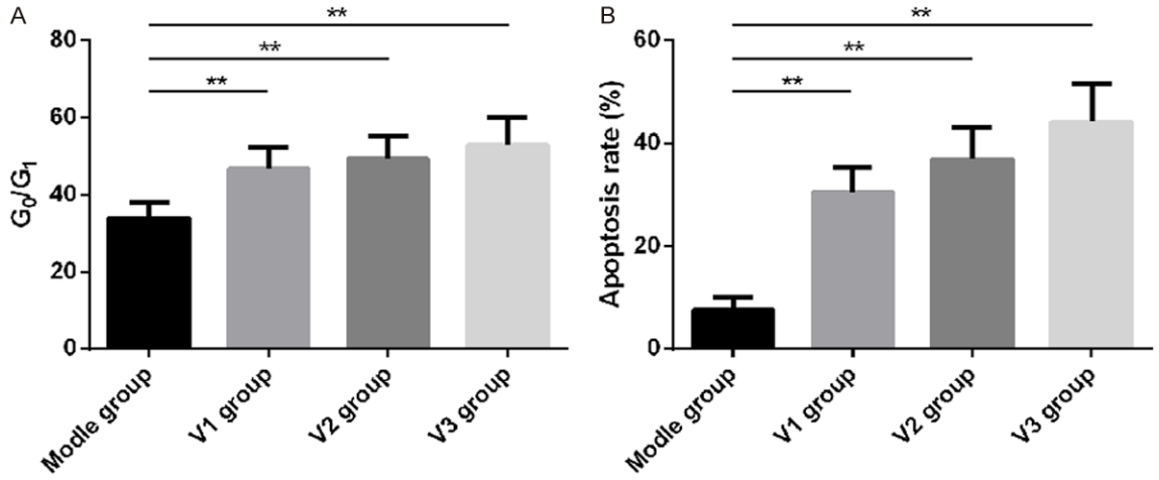

Figure 4. VD3 had a positive effect on cell cycle and apoptosis rate of CRC mice in a dose-dependent manner. A. VD3 can significantly induce CRC mice to suffer G_0/G_1 phase. B. VD3 can significantly increase the apoptosis rate of CRC mouse cells. Note: **P<0.01.

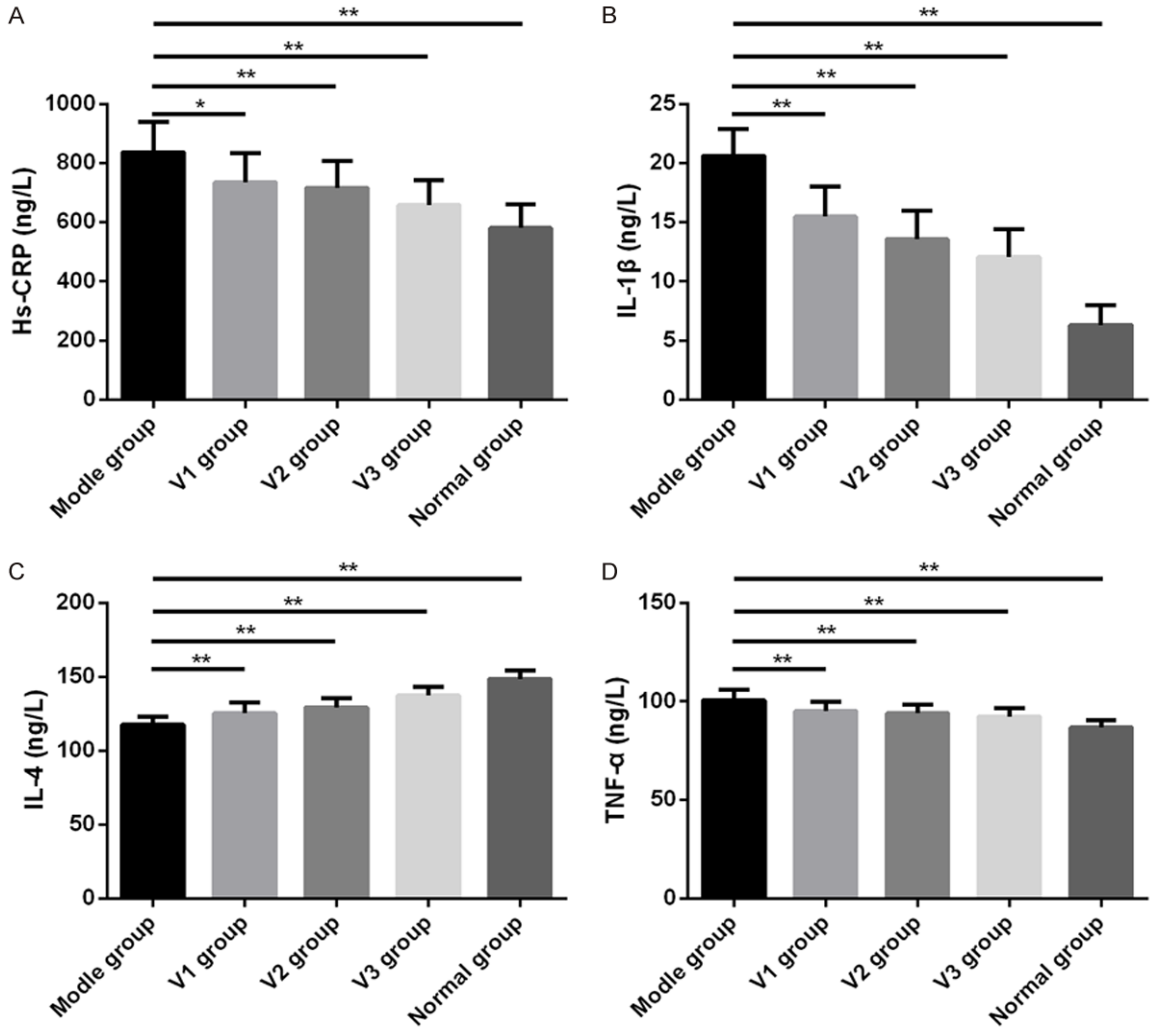

Figure 5. VD3 had a regulatory effect on serum inflammatory factors in CRC mice in a dose-dependent manner. A. VD3 had an inhibitory effect on Hs-CRP in CRC mice in a dose-dependent manner. B. VD3 had an inhibitory effect

on IL-1β in CRC mice in a dose-dependent manner. C. VD3 had a promoting effect on IL-4 in CRC mice in a dose-dependent manner. D. VD3 had an inhibitory effect on TNF-α in CRC mice in a dose-dependent manner. Note: *P<0.05, **P<0.01.

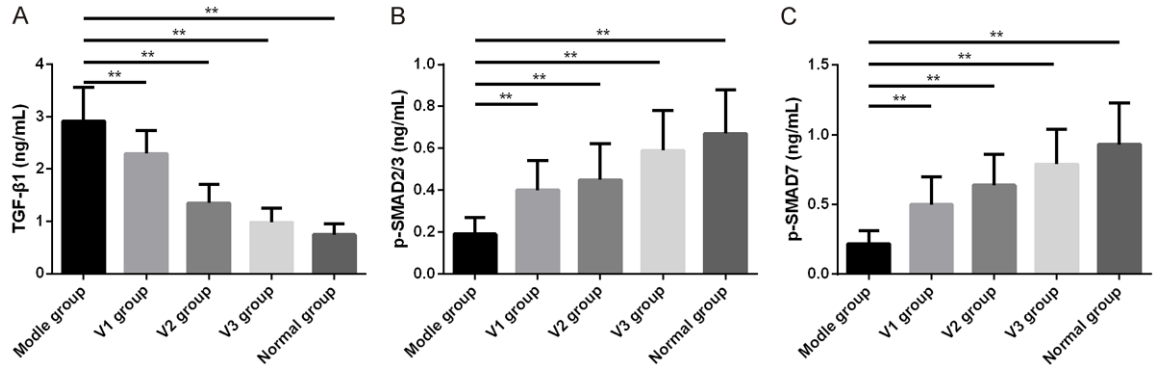

Figure 6. VD3 had a regulatory effect on inflammatory proteins in CRC mice in a dose-dependent manner. A. VD3 had a inhibitory effect on TGF-β1 in CRC mice in a dose-dependent manner. B. VD3 had a promoting effect on p-SMAD2/3 in CRC mice in a dose-dependent manner. C. VD3 had a promoting effect on p-SMAD7 in CRC mice in a dose-dependent manner. Note: **P<0.01.

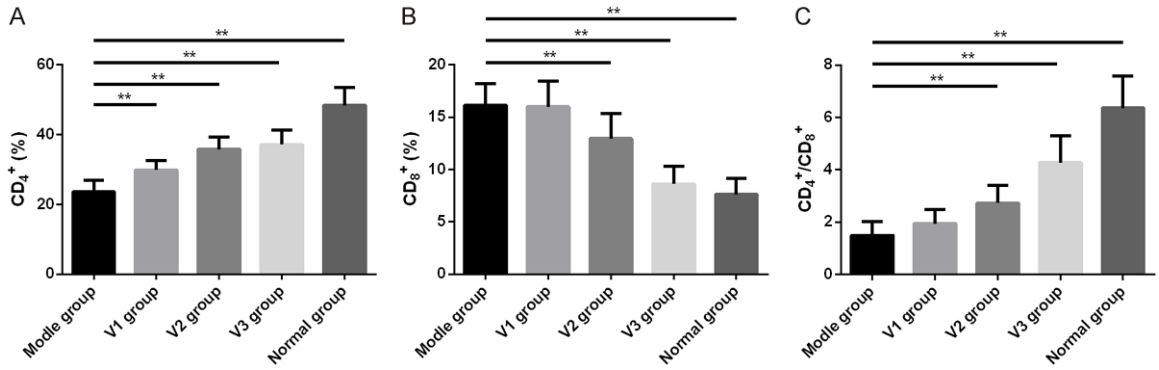

Figure 7. VD3 had a regulatory effect on cell immune indicators in CRC mice in a dose-dependent manner. A. VD3 had a promoting effect on $CD4^+$ in CRC mice in a dose-dependent manner. B. VD3 had an inhibitory effect on $CD8^+$ in CRC mice in a dose-dependent manner. C. VD3 had a promoting effect on $CD4^+/CD8^+$ in CRC mice in a dose-dependent manner. Note: **P<0.01.

apoptosis, suggesting that CRC can also exert tumor inhibition effect by blocking cells in G_0/G_1 phase and increasing the cell apoptosis rate.

The increase of secretion of pro-inflammatory cytokines in CRC patients and the changes of immune system is related to the decrease of quality of life [18]. Studies have shown that VD3 can play an anti-inflammatory role, inhibit the secretion of pro-inflammatory cytokines Hs-CRP, IL-1β, TNF-α, etc., and increase the expression of anti-inflammatory cytokine IL-4 [19] by reducing toll-like receptor protein 2/4, repressing the activation of phosphorylated signal transductant and transcription activator 5 and the production of reactive oxygen species. In this study, the intake of VD3 significantly reduced the concentrations of Hs-CRP, IL-1β, TNF-α and increased the concentration of IL-4, suggesting that VD3 is helpful to inhibit the excessive inflammatory reaction of CRC mouse model and improve the inflammatory imbalance of the body. In Huang et al. [20] research, SMAD2/3, SMAD7 and TGF-β1 can participate in TGF-β signaling pathway mediated tumor regulatory network, which plays a key role in the early stage of tumor development. In this study, VD3 plays a regulatory role in the increase of phosphorylation level of SMAD2/3, SMAD7 and the decrease of transforming growth factor-β1

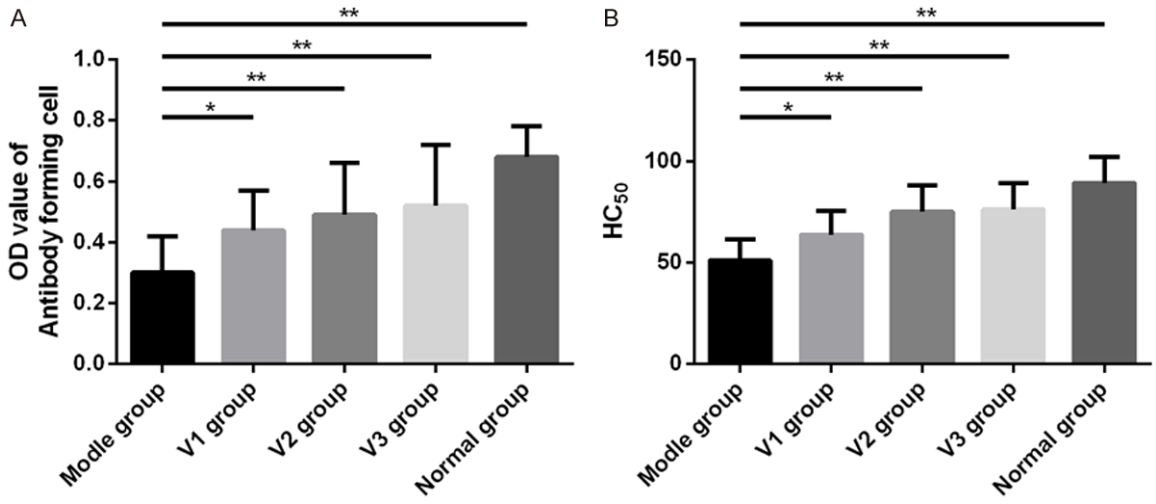

Figure 8. VD3 had a regulatory effect on humoral immunity indicators in CRC mice in a dose-dependent manner. A. VD3 had a promoting effect on OD value of antibody-producing cells of CRC mice in a dose-dependent manner. B. VD3 had a promoting effect on HC_{50} of CRC mice in a dose-dependent manner. Note: *P<0.05, **P<0.01.

(TGF-β1) in a dose-dependent manner, suggesting that VD3 may actively respond to the regulation of TGF-β signal pathway on early tumor development. In the research of Szymczak et al. [21] VD3, as a bone homeostasis regulator, also has the function of regulating adaptive immunity and innate immunity. It can be mediated by endocrine mechanism of circulating calcitriol, and can also exert immune regulation function through paracrine and endocrine mechanism. Spleen and thymus are immune organs that regulate the key pathogenesis of CRC microenvironment and play a key role in the prevention of immune escape. It is suggested that paying attention to the situation of spleen and thymus is helpful for us to speculate the status of immune organs after medication in CRC [22, 23]. Studies have shown that preoperative cellular immune parameters $CD3^+$, $CD4^+$, $CD8^+$ are related to survival of CRC and gastric cancer patients at phase III. The absolute number of lymphocyte subsets at a higher level has a positive impact on the good prognosis of patients, which indicates that these cellular immune parameters may help us to measure the prognosis of CRC [24]. In this study, the intake of VD3 significantly increased the indexes of thymus and spleen and the cellular immune indexes $CD4^+$, $CD4^+/CD8^+$ and significantly reduced the level of $CD8^+$, suggesting that VD3 was beneficial to the development of immune organs and the recovery of cellular immune function in CRC mice. In Bi et al. [25] studies on the treatment of hepatitis b infected mice with VD3, the intake of VD3 was beneficial to the increase of thymus and spleen index, $CD4^+$ and $CD4^+/CD8^+$ levels, and the decrease of $CD8^+$, suggesting that VD3 has an improving effect on the immune function of hepatitis b infected mice. It is similar to the results of this study. Studies have shown that bacterial toxin is a new therapeutic scheme for CRC. Thermal stability direct hemolysin secreted by vibrio parahaemolyticus can induce calcium to flow into cells to achieve the purpose of increasing calcium level in intestinal epithelial cells, thus exerting CRC antiproliferative effect, suggesting that the increase of hemolysin level is helpful to inhibit the malignant proliferation of CRC tumors [26]. In this study, the antibody-producing cells and secretion of hemolysin in CRC mouse model under the intervention of VD3 significantly increased, suggesting that VD3 can improve the humoral immune function of CRC mouse model by increasing the antibody-producing cells and secretion of hemolysin.

To sum up, VD3 intervention in CRC mouse model is helpful to balance excessive inflammation and immune homeostasis, but the research remains to be improved. We can increase the research on the specific regulatory mechanism of VD3 in CRC mouse model, or supplement the specific therapeutic effect analysis of VD3 combined with metformin on CRC, etc. The research results will be gradually improved from the above perspective in the future.

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Disclosure of conflict of interest

None.

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