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Vitamin D Reduces the Helper T Cells 17 (Th17) Differentiation in Patients with Ulcerative Colitis by Targeting Long Non-coding RNA (lncRNA) OIP5-AS1/miR-26a-5p/IL-6 Axis

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ABSTRACT

Background: Vitamin D has anti-inflammatory efficacy against ulcerative colitis (UC), however, the mechanism is yet little understood.
Objective: To investigate the immunomodulatory effects of vitamin D against the UC, and to explore the potential downstream mechanisms.
Methods: Serum vitamin D, Interferon-γ (IFN-γ) and Interleukin (IL)-17 levels of the patients with UC were quantified using enzyme-linked immunosorbent assay (ELISA). Long non-coding RNAs (lncRNAs) levels were determined by using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Peripheral blood mononuclear cells (PBMCs) were collected from healthy control subjects, stimulated with CD4+ T lymphocytes or helper T cells 17(Th17) differentiation conditions, and then exposed to calcitriol (vitamin D active form) or certain lentiviral treatment, followed by subsequent molecular level testing. For in vivo assay, mice were given 3% dextran sulfate sodium (DSS) to induce colitis.
Results: Compared with the control group, vitamin D levels in the UCs were statistically lower, and there was a negative correlation between IL-17 and vitamin D in the UCs. The lncRNA OIP5-AS1 could decrease under calcitriol treatment in both CD4+ T cells and Th17 differentiation. The lncRNA OIP5-AS1 was a microRNA (miR)-26a-5p sponge and therefore modulated the Th17 cells and IL-6 expression. The lncRNA OIP5-AS1/miR-26a-5p/IL-6 axis mediated the regulation of calcitriol-induced Th17 differentiation. Calcitriol had therapeutic effects on the UC mouse models by regulating the lncRNA OIP5-AS1 related pathway.
Conclusion: Vitamin D might have anti-inflammatory potential in the treatment of the UC.
Keywords: lncRNA, Th17 Differentiation, Ulcerative Colitis, Vitamin D

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INTRODUCTION

As a gastrointestinal inflammatory disease, inflammatory bowel disease (IBD) has the characteristics of being idiopathic, recurrent, and chronic. Ulcerative colitis (UC) which is the main pathological type of IBD, is mainly manifested in mucosal inflammation, occasionally in the gastrointestinal tract, colon, and submucosa (1-2). The imbalance between immunosuppressive cells and pathogenic cells is closely related to the disease activity of the UC.

T lymphocytes are essential for this immune balance (3). Generally speaking, helper T (Th) cells included Th1 and Th2 subtypes. Th2 cells expressed IL-4, while Th1 cells expressed Interleukin (IL)-2 and Interferon-γ (IFN-γ) (4). Theoretically, the UC is the result of abnormal Th2 cell immunity (4). Coupled with this, another subset of T cells, namely, Th17 cells, is also likely to play a role in the UC (5). Th17 can secrete IL-17, which could induce inflammation and promote tissue damage. The elevation of inflammatory cytokines like IFN-γ or IL-6 was also observed in Th17 cells (6). Recent studies demonstrated that the inhibition of Th17 differentiation can effectively decrease the UC-related inflammations (6).

Vitamin D is being given more and more attention due to its immunoregulating properties. It has been confirmed that lower levels of vitamin D in remission increase the risk of the UC recurrence, which is not related to endoscopic or histological classification, as vitamin D is associated with anti-inflammatory serum cytokine profiles (7). Reports also demonstrated that Vitamin D treatment could alleviate the UC in the mouse models, and this function might be directly or indirectly mediated by T cells differentiation (8). However, it is still unknown whether and how Vitamin D affects Th17 differentiation to perform an anti-inflammatory function against the UC.

The change of long non-coding RNA (lncRNA) expression by Vitamin D may mediate the function of Vitamin D (9). We allocated the UC patients into Vitamin D high and low groups and found that the expression of the lncRNA OIP5-AS1 was negatively correlated with the amount of Vitamin D. It is shown that the lncRNA OIP5-AS1 facilitates Th17 differentiation and inflammatory severity in several diseases (10). We, therefore, conducted this study to investigate the effects of Vitamin D on Th17 differentiation in the UC and to explore the potential downstream mechanisms involving the lncRNA OIP5-AS1 related pathway.

MATERIALS AND METHODS

Patients

The inclusion criteria of the subjects were: 1) the patients were diagnosed with the UC; 2 the patients had to be over the age of 18.); 3) the patients had to have either mild and moderate UC or severe UC but did not need first aid; the exclusion criteria of the subjects were: 1) the patients were pregnant; 2) the patients had severe complications; 3) the patients had mental disorders or severe primary diseases. Based on the above-mentioned criteria, a total of 55 UC patients from our hospital, were included in this study. The normal control group comprised 55 healthy volunteers. The study was based on the Helsinki declaration. Written informed consent was given to all participants. The Ethics Committee at our hospital has given its approval.

Serum Vitamin D, IFN-γ, and IL-17 Measurement

In our study, 5mL fasting blood was taken, stored at room temperature for 0.5 hrs., and centrifuged at 2000rpm for 20 min. At last, we collected non-hemolytic serum. Following the manufacturer's guidelines, IL-17, IFN-γ, and vitamin D were measured by enzyme-linked immunosorbent assay (ELISA), using the ELISA kits purchased from Abcam, USA.

Grouping Human CD4+ T Cells

We collected the peripheral blood

mononuclear cells (PBMCs) and isolated them by Histopaque-1077 (Sigma, USA, 10771-100ML). The cells were then washed twice with Roswell Park Memorial Institute (RPMI) -1640 medium (Gibco, USA). MagniSort™ Human CD4 T cell Enrichment Kit (ThermoFisher, USA) was used to isolate CD4+ T cells.

Th17 Polarization Induction

CD4 + T cells were stimulated into Th17 cells by adding a series of antibodies and cytokines for 48 hrs, previously described in detail (10). The added antibodies included 1 µg/mL anti-CD28 antibody, 1 µg/mL anti-CD3 antibody, 2 µg/mL IFN-γ-neutralizing antibody and 2 µg/mL IL-4-neutralizing antibody. The added cytokines included 20 ng/mL IL-1β, 20 ng/mL IL-6 and 20 ng/mL IL-23.

Transfection of Oligonucleotides

Lentivirus packaged lncRNA OIP5-AS1 over-expression vector and negative control (NC) vector, together with the lentivirus packaged miR-26a-5p mimics, inhibitor and NC, were used for CD4+ T cell transfection.

Luciferase Reporter Determination

pGL3 vector (Promega, USA) was used to construct a wide-type lncRNA OIP5-AS1 luciferase reporter (WT-OIP5-AS1). The sequence of the lncRNA OIP5-AS1 binding to IL-6 3'untranslated regions (UTRs) were predicted by bioinformatics analysis software and were mutated. The lncRNA OIP5-AS1 with the mutated sequence was also constructed into a pGL3 vector (MUT-OIP5-AS1). The indicated reporter plasmid and microRNAs were transfected into HEK293 cells. The fluorescence intensity was measured after the cells were transfected for 48 hrs.

RNA Extraction and Reverse-transcription Quantitative Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated from tissues or cells using the TRIzol reagent (Invitrogen, USA). Reverse transcription of the total RNA was performed using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). A PCR assay was carried out using SYBR Green Realtime PCR Master Mix (TaKaRa, Dalian, China). Thermocycling was performed as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 min, and 60 °C for 30 min. To determine microRNA's levels, an All-in-One miRNA First-Strand cDNA Synthesis Kit and a qRT-PCR Detection Kit (GeneCopoeia, USA) were used. The $2^{-\Delta\Delta Cq}$ method, which is well defined and extensively employed, was utilized in this work for the normalization and calculation of the expression level of both the lncRNAs and miRNAs (11).

Flow Cytometric Analysis (FACS)

Mouse anti-human CD4-FITC, CD3-PE, and IL17-PE-Cy7 were purchased from Becton Dickinson Biosciences (San Diego, USA). The mouse anti-human IFN-γ-Percp-Cy5 antibodies were obtained from eBioscience (San Diego, USA). Cells were stained and run on a FACS Calibur cytometer (BD Bioscience, USA), and the data were analyzed using FACS Diva software (BD Bioscience, USA).

Animals and Treatment

C57BL/6 mice (male, 6–8 wk old) were maintained according to the national guideline and were approved by the Committee for Animal Research of Eighth Medical Center of Chinese PLA General Hospital. The acute UC mouse models was constructed by adding 3% DSS into their drinking water for 6 days. Each mouse received 100 µg/mL calcitriol in the abdomen every 2 days. Indicated lentivirus was injected via the tail vein. Lentivirus was injected twice, the first on the first day ahead of DSS intake, and the second on the fourth day during DSS intake. The mice were then sacrificed and the inflammatory indexes related to the UC were subsequently detected.

Statistical Analyses

We checked the normality of all the tested

data by using D'Agostino Pearson analysis or Shapiro-Wilk analysis before statistical analyses. Differences would be tested with a two-tailed Student's t-test between the groups if the results conformed to the normal distribution with homogeneous variance, otherwise, the Kruskal Wallis test was used. Logistic regression was used to test the associations of Vitamin D levels with the indicated cytokine levels or the lncRNA OIP5-AS1 levels. All the statistical analyses were performed by SPSS (IBM, Armonk, NY, USA). The judgment basis with statistical significance is $P<0.05$.

RESULTS

Vitamin D and Cytokines Levels in the UC Subjects

A total of 55 patients aged 21-62 years were diagnosed with the UC. The controls (55 cases) ranged in age from 20 to 65 years. According to Table 1, there was no significant difference in drinking, smoking, weight and age between the control group and UC group. The serum vitamin D level in the UC group significantly decreased (Figure 1A), coupled with the serum IFN-γ, and IL-17 levels which increased in the UC cases (Figure 1B-C). In addition, there is a negative correlation between serum IL-17 and serum vitamin D (Figure 1D, P=0.007, R=-0.506). The correlation between serum IFN-γ and serum vitamin D levels was not statistically significant (Figure 1E, P=0.055, R=-0.223). The lncRNA OIP5-AS1 in PBMCs enhanced in the UC subjects (Figure 1F), and there is a negative correlation between the lncRNA OIP5-AS1 and serum vitamin D (Figure 1G, P=0.001, R=-0.60).

Vitamin D Reduces Th17 Differentiation by Targeting the lncRNA OIP5-AS1

CD4+ T cells were then isolated from the healthy volunteers and treated with calcitriol (vitamin D active form). As shown in Figure 2A, in these cells, IL-17 significantly down-regulated under calcitriol treatment. Then, CD4+ T cells were induced into Th17 polarization, followed by calcitriol treatment for 48 hrs. The percentage of Th17 significantly up-regulated in the induced group than that of the non-induced group, and decreased under calcitriol treatment in Th17 induced group, but did not alter in the non-induced group (Figure 2B). Meanwhile, the lncRNA OIP5-AS1 expressions enhanced in Th17 differentiation conditions, while calcitriol treatment decreased the lncRNA OIP5-AS1 expressions significantly in both Th17 induced group and the non-induced group (Figure 2C). Next, we found that lenti-OIP5-AS1 co-treatment could reverse Th17 differentiation regulated by calcitriol, indicating that Vitamin D might reduce Th17 differentiation by targeting the lncRNA OIP5-AS1 (Figure 2D).

lncRNA OIP5-AS1 Interacts with miR-26a-5p

We next explored the downstream-regulated genes of the lncRNA OIP5-AS1. We found that the 3'UTR of the lncRNA OIP5-AS1 formed a complementary base pairing with miR-26a-5p (Figure 3A). Subsequently, we carried out a luciferase reporter gene assay in HEK293 cells. miR-26a-5p mimics could inhibit the WT-OIP5-AS1 luciferase activity, while it did not affect the MUT-

Table 1. Characteristics of the participants in UC and control groups

	UC group (N=55)	Control group (N=55)	P value
Age	41.3±8.84	39.6±10.21	0.353
BIM (Kg/m^2)	23.9±2.44	24.1±2.65	0.681
Smoking amount (sticks/day)	7.67±4.31	7.02±3.37	0.380
Drinking amount (tael/day)	1.57±0.84	1.49±0.43	0.531

UC: Ulcerative Colitis; All the data here conformed to the normal distribution.

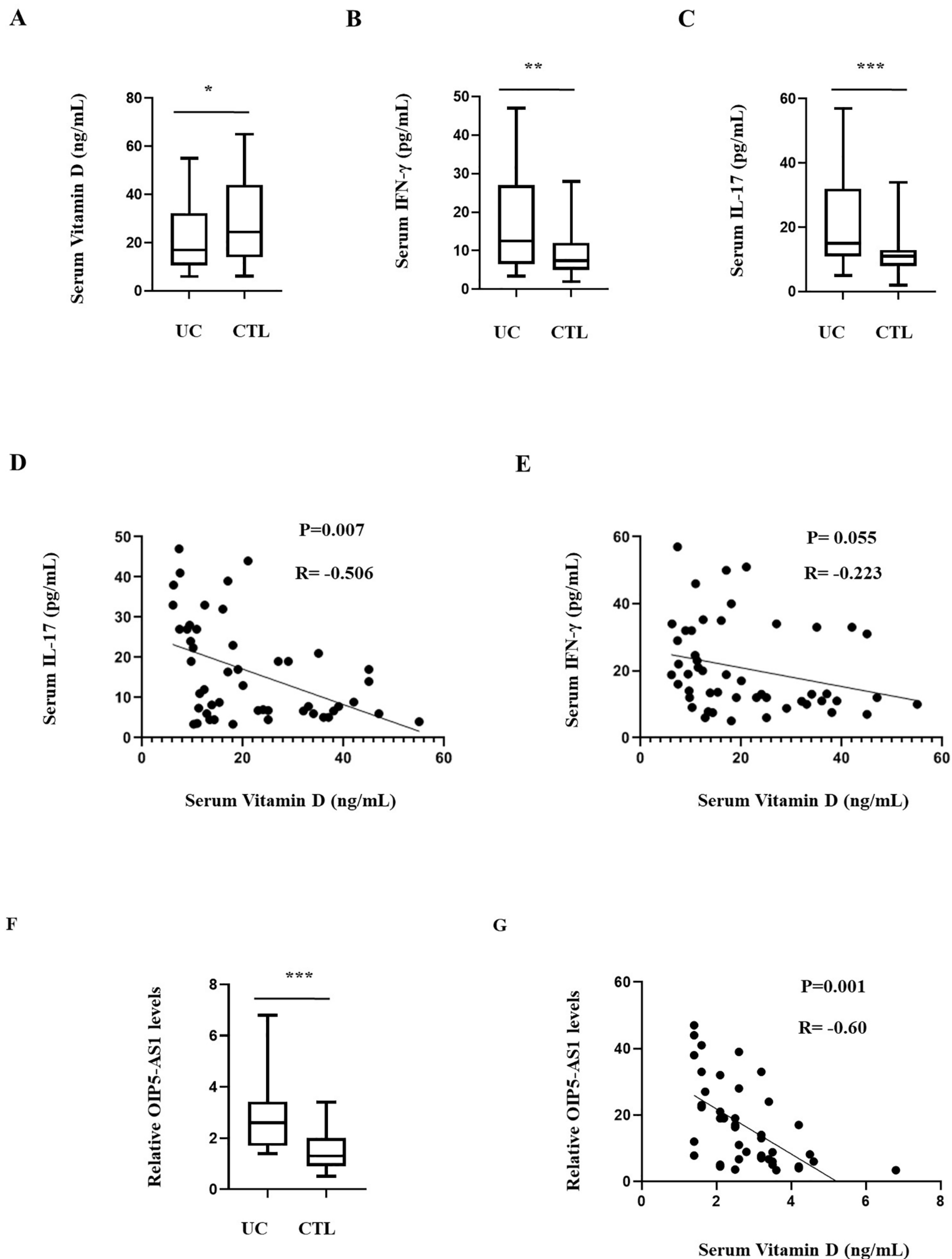

Figure 1. Vitamin D and cytokines levels in the UC subjects. (A-C) Comparing the serum (A) Vitamin D (Normal distribution, student t-test); (B) IFN-γ (not normal distribution, KW test); and (C) IL-17 (not normal distribution, KW test) levels among the UC patients and the healthy controls. (D-E) Correlation between serum vitamin D contents and serum (D) IL-17; and (E) IFN-γ contents in the UCs. (F) Comparing the PBMC lncRNA OIP5-AS1 contents among the UC patients and CTLs by qRT-PCR (Normal distribution, student t-test). (G) Correlation between serum vitamin D levels and lncRNA OIP5-AS1 levels in PBMCs in the UC patients. UC: Ulcerative colitis; CTL: the healthy controls. OIP5-AS1: lncRNA OIP5-AS1; KW: Kruskal-Wallis

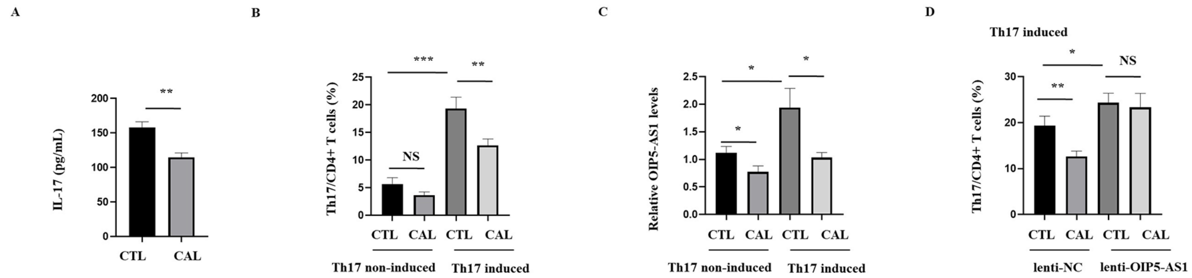

Figure 2. Vitamin D reduces Th17 differentiation by targeting the lncRNA OIP5-AS1. (A) IL-17 contents in CD4+ T cells treated with 100 nM calcitriol. (B-C) CD4+ T cells from CTLs were polarized to Th17 for 48hrs. Together with or without 100 nM calcitriol treatment. (B) The percentage of Th17 cells was determined; (C) The relative lncRNA OIP5-AS1 levels were determined by qRT-PCR. (D) Th17 polarizing cells were treated with or without 100 nM calcitriol, together with lenti-OIP5-AS1 or lenti-NC treatment. The percentage of Th17 cells was determined. CTL: negative controls; CAL: calcitriol treatment; OIP5-AS1: lncRNA OIP5-AS1; All the data in this figure conformed to the normal distribution.

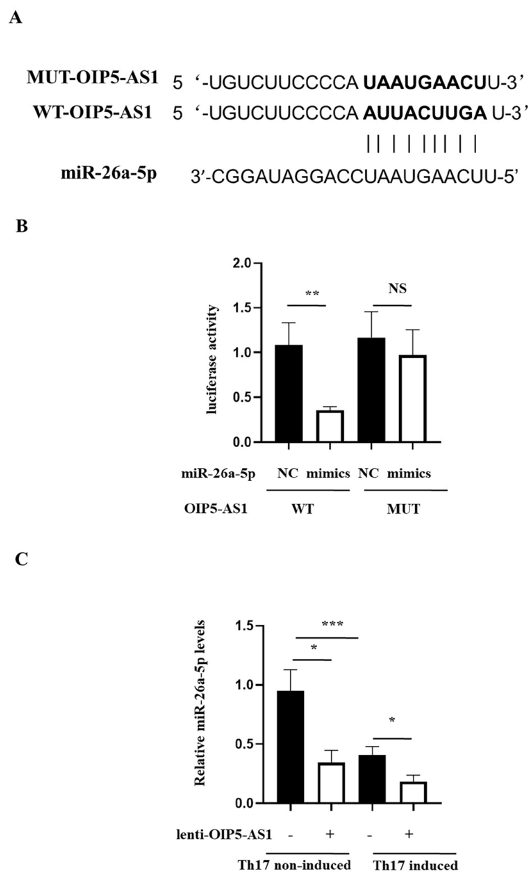

Figure 3. The lncRNA OIP5-AS1 interacts with miR-26a-5p. (A) Bioinformatics software predicted the sequence alignment between the lncRNA OIP5-AS1 and miR-26a-5p. (B) The WT-OIP5-AS1 and MUT-OIP5-AS1 luciferase reporter gene vectors, together with miR-26a-5p mimics or NC were transfected into HEK293, and then the luciferase activity in each group was determined. (C) CD4+ T cells from CTLs were polarized to Th17 for 48hrs. together with certain lentivirus treatment, and then the relative miR-26a-5p levels were determined. All the data here conformed to the normal distribution.

OIP5-AS1 (Figure 3B). Subsequently, we found that miR-26a-5p expressions decreased in Th17 differentiation conditions, and lenti-OIP5-AS1 treatment in both CD4+ T cells and Th17 differentiation cases inhibited the miR-26a-5p expressions significantly (Figure 3C). The above results demonstrated that the lncRNA OIP5-AS1 could regulate the miR-26a-5p expressions in both CD4+ T cells and Th17 differentiation cases.

miR-26a-5p Targets the Th17-type Cytokine IL-6

Using the online software targetscan (Http://targetscan.org/mamm_31/), we identified IL-6, which is one of the important Th17-type cytokines, as the target for miR-26a-5p (Figure 4A). Luciferase reporter assay confirms that miR-26a-5p can bind the wide type 3’ UTR of IL-6, but cannot bind the mutant 3’ UTR of IL-6 (Figure 4B). Meanwhile, lenti-miR-26a-5p-mimics treatment in both CD4+ T cells and Th17 differentiation cases inhibited IL-6 expressions significantly, together with IL-17 and IFN-γ decreasing (Figure 4C-E). The above results demonstrated that miR-26a-5p targets IL-6 and reduces Th17 differentiation.

Vitamin D Reduces Th17 Differentiation by Regulating the lncRNA OIP5-AS1/ miR-26a-5p Axis

Calcitriol treatment could induce the miR-26a-5p levels in both CD4+ T cells and Th17

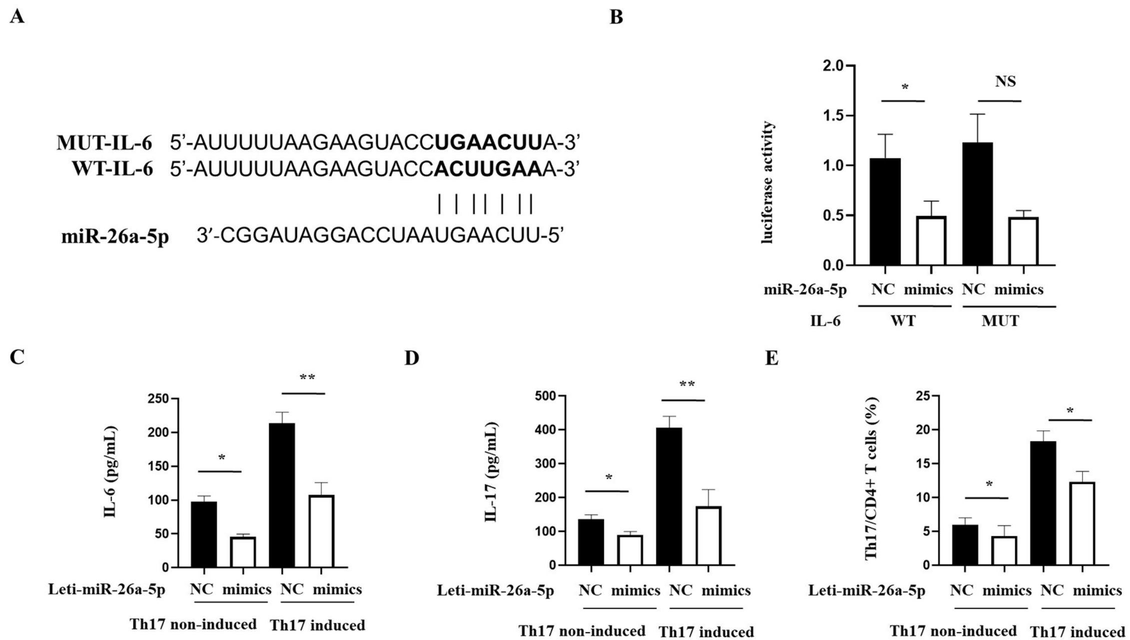

Figure 4. miR-26a-5p targets Th17-type cytokine IL-6. (A) Bioinformatics software predicted the miR-26a-5p targeted to 3′UTR of IL-6. (B) Wide type IL-6 and mutant IL-6 luciferase reporter gene vectors, together with miR-26a-5p mimics or NC were transfected into HEK293, and then the luciferase activity in each group was determined. (C-E) CD4+ T cells from CTLs were polarized to Th17 for 48hrs. together with certain lentivirus treatment, and then (C) IL-6 contents; and (D) IL-17 contents were determined by ELISA; (E) The percentage of Th17 cells was determined. All the data here conformed to the normal distribution.

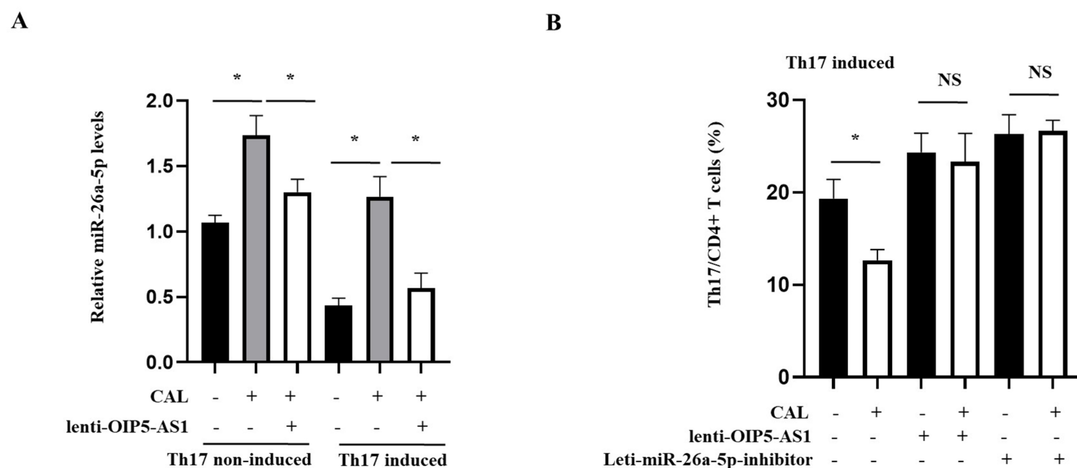

Figure 5. Vitamin D reduces Th17 differentiation by regulating the lncRNA OIP5-AS1/miR-26a-5p axis. (A) CD4+ T cells from CTLs were polarized to Th17 for 48hrs. together with the indicated treatment, and the relative miR-26a-5p levels were determined. (B) CD4+ T cells from CTLs were polarized to Th17 for 48hrs. together with the indicated treatment, and the percentage of Th17 cells was determined. CAL: calcitriol treatment; all the data here conformed to the normal distribution.

differentiation cases, and these impacts could be reversed by lenti-OIP5-AS1 co-treatment (Figure 5A), indicating that Vitamin D could regulate the lncRNA OIP5-AS1/miR-26a-5p axis. Further, we confirmed that calcitriol could reduce Th17 differentiation, and this impact could be reversed by co-treatment with lenti-OIP5-AS1 or lenti-miR-26a-5p-inhibitor (Figure 5-D). These results demonstrated that vitamin D reduces Th17 differentiation by regulating the lncRNA OIP5-AS1/miR-26a-5p/ axis.

Vitamin D Treatment Attenuates DSS-induced UC in Mice

Previous reports demonstrated that 100 μg/mL calcitriol could ameliorate DSS-induced UC in mice (8). In the current study, while proving the efficacy of calcitriol in this concentration, we try to explore whether or not calcitriol functions by regulating the lncRNA OIP5-AS1/miR-26a-5p axis in the UC mice. To inhibit or overexpress the lncRNA OIP5-AS1 or miR-26a-5p in mice, we used recombinant lentivirus vectors for tail vein injection. The UC mouse models were constructed by adding 3% DSS, and then, we allocated the modeled UC mice into the following groups: A) PBS treatment group; B) calcitriol treatment group; C) calcitriol+lenti-OIP5-AS1 co-treatment group; D) calcitriol+lenti-miR-26a-5p-inhibitor co-treatment group. We found that calcitriol can improve some indicators of the UC, including bodyweight loss, disease activity index (DAI) score, colon length, colon histopathological scoring, and lenti-OIP5-AS1 co-treatment and lenti-miR-26a-5p-inhibitor co-treatment reversed this function (Figure 6A-D). We next isolated lymphocytes and assessed Th17 percentage. It was shown that calcitriol can reduce the numbers of Th17 by the lncRNA OIP5-AS1/miR-26a-5p axis (Figure 6E-F).

DISCUSSIONS

In this study, we confirmed that vitamin D levels negatively correlated with cytokines IL-17 and IFN-γ in the UC patients. This finding is similar to several prior findings, but there are some distinctions as well. Gubatan et al. have reported a positive correlation between vitamin D levels and the anti-inflammatory factor IL-10 in the UC patients (12). Meckel et al. indicated that the increase in vitamin D is related to the decrease of pro-inflammatory cytokine, TNF-α, and IL-8 in the UC patients (13). These publications

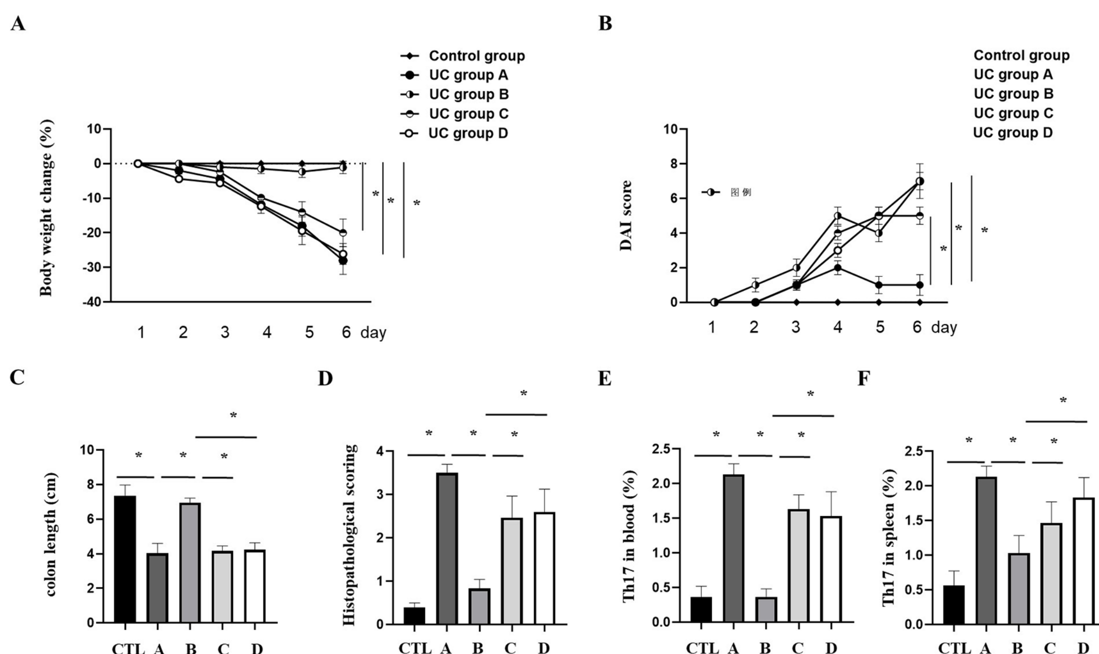

Figure 6. Vitamin D treatment has a protective effect on the UC mouse models. (A-B) The UC mouse models were constructed with 3% DSS, together with the indicated treatment. The UC mice were divided into the following groups: group A, PBS treatment group; group B, calcitriol treatment group; group C, calcitriol + lenti-OIP5-AS1 co-treatment group; group D, calcitriol + lenti-miR-26a-5p-inhibitor co-treatment group. (A) Bodyweight loss and (B) DAI scores were monitored during the 6 days of treatment. (C-F) At the end of the 6 days of treatment, the (C) colon length; (D) histopathological scoring; (C) Th17 in blood, and (D) Th17 in spleen were determined in the mice described in (A-B). All the data here conformed to the normal distribution.

and our data all indicated that vitamin D may inhibit inflammation and therefore contribute to the treatment of the UC. This is also confirmed by some clinical intervention trials. Sharifi et al demonstrated that vitamin D injection can significantly reduce the levels of TNF-α, IFN-γ, and IL12p70 in the UC patients (14). Ahamed et al. indicated that oral administration of nano vitamin D in the UC patients can reduce disease activity and severity (15).

Previous research has identified a number of pathways that may underlie Vitamin D's anti-inflammatory impact. One view is that vitamin D is related to DNA methylation of CPGs, and may therefore regulate certain immune-related genes (16, 17). Besides, Rao et al. found that vitamin D receptors (VDR) can physically bind important inflammatory factors, such as NLRP3, and regulate their ubiquitination levels in macrophages (18). With an in-depth study of the lncRNAs, it has been proposed that the lncRNAs regulated by vitamin D may be the key factor in mediating vitamin D function (9). Our clinical study identified a negative correlation between vitamin D levels and the lncRNA OIP5-AS1 expressions in the UC patients, and experimental data indicated that vitamin D treatment could decrease IL-17 and suppress Th17 polarization by regulating the lncRNA OIP5-AS1 levels both in vitro and in vivo.

Th17 cells mediated tissue inflammation and participated in the pathogenesis of the UC (6). The main function of activated Th17 cells is to secrete cytokines, such as IL-17, IL-6, and IL-22 (19). It was thought that IL-17 family cytokines had a pro-inflammatory effect, and anti-IL-17 and anti-IL-6 antibodies had significant therapeutic effects on the UC mice (20). The highlight of this study is that we identified that the lncRNA OIP5-AS1 was the promising molecular factor mediating the regulation of Th17 by vitamin D. The lncRNA OIP5-AS1 is a newly identified lncRNA (21, 22). Recently, it has been reported that the lncRNA OIP5-AS1 can regulate the occurrence and development of tumors (23), diabetes (24), and many other autoimmune diseases (25). Similar to many lncRNAs, the lncRNA OIP5-AS1 interacts with certain miRNAs and affects the miRNAs levels (26). In this study, we found that miR-26a-5p could be directly targeted by the lncRNA OIP5-AS1, and vitamin D treatment significantly up-regulated the miR-26a-5p expressions by the lncRNA OIP5-AS1 inhibition.

This work demonstrated that miR-26a-5p could target Th17-type cytokine IL-6 to inhibit Th17 differentiation, which is consistent with some previous findings. He et al. indicated that miR-26a-5p, IL-6, and IL-17 form an axis, which ensures that miR-26a-5p can inhibit Th17 differentiation (27). A report by Honardoost et al. also showed a promising role of miR-26a-5p in Th17 differentiation (28). Here, lenti-OIP5-AS1 or lenti-miR-26a-5p-inhibitor treatment can decrease IL-6 and IL-17 levels, and reverse the vitamin D role in Th17 differentiation, indicating that the lncRNA OIP5-AS1/miR-26a-5p/-IL6 axis mediates vitamin D functions.

Vitamin D treatment was reported to protect the UC in the mouse models by suppressing inflammatory mediators like TNF-α and IFN-β (8). We observed the similar effect of vitamin D on DSS-induced UC mice, including improving mice weight, DAI scores, colon length, and hematoxylin-eosin (HE) scores, and inhibiting Th17 differentiation in both blood and spleen from the UC mice. It has been proposed that vitamin D could impact the macrophage M1/M2 polarization switch to suppress inflammasome activation, partly by inhibiting IL-1β secretion and caspase-1 cleavage (8). Our results proved that the lncRNA OIP5-AS1/miR-26a-5p axis is another promising way to mediate vitamin D functions in the treatment of the UC because as we use exogenous plasmids to eliminate the regulatory effect of vitamin D on the lncRNA OIP5-AS1/miR-26a-5p axis, the therapeutic effect of vitamin D on the UC is significantly inhibited.

From the mouse models data and the cell-based molecular mechanism results, we

hypothesize a link between vitamin D and the pathogenesis of the UC. A high level of vitamin D can reduce Th17 differentiation by regulating the lncRNA OIP5-AS1/miR-26a-5p/-IL6 axis, and therefore has a protective effect on the occurrence of the UC. Conversely, vitamin D deletion could activate Th17 and therefore promote the UC.

CONCLUSION

In conclusion, vitamin D plays a prominent role in inhibiting Th17 cells differentiation. Thus, vitamin D has a therapeutic potential for treatment the UC.

Conflict of Interest: None declared.

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