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The effect of $1,25(OH)_2D_3$ on Dickkopf-1 methylation in colorectal cancer

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Abstract

Background Vitamin D is a fat-soluble vitamin that has a protective role in colorectal cancer. Several studies have identified the association between vitamin D and changes in DNA methylation in different types of tumours. Dickkopf-1 (DKK1) inhibits the Wnt/β-catenin signalling pathway, and $1,25(OH)_2D_3$ can induce DKK1 expression in colorectal cancer. However, whether $1,25(OH)_2D_3$ can affect DKK1 expression by regulating DNA methylation in colorectal cancer is not known.

Methods Fifty-seven colorectal cancer (CRC) patients and fifty-five healthy controls were included in this study. Serum DKK1 and 25(OH)D levels were measured via ELISA and liquid chromatography–tandem mass spectrometry, respectively, and the associations of DKK1 with clinicopathological characteristics and 25(OH)D were analysed. A DKK1 expression plasmid was transfected into cells to assess the functional significance of DKK1 in CRC progression via CCK8, wound healing and migration assays. BiSulphite Amplicon Sequencing (BSAS) and methylation-specific PCR were used to detect the DKK1 methylation status of colorectal cancer cells and tissues. The effect of $1,25(OH)_2D_3$ on DKK1 methylation was investigated by pyrosequencing. A dual-luciferase reporter assay was performed to investigate the influence of CpG island methylation on DKK1 transcriptional activity.

Results A decreased serum DKK1 level was closely associated with nerve infiltration and 25(OH)D status in patients with colorectal cancer. Overexpression of DKK1 reduced the proliferative and migratory capabilities of colorectal cancer cells. The methylation patterns of DKK1 (−195 to +231), including 31 CpG sites, were assayed via BSAS in CRC cells and tissues. Compared with those in adjacent normal tissues, the methylation levels of multiple CpG sites located in the promoter, 5′UTR and exon 1 were increased in tumour tissues. DKK1 hypermethylation was associated with decreased DKK1 expression in colorectal cancer cells and tissues. $1,25(OH)_2D_3$ induced DKK1 expression in colorectal cancer cells, and pyrosequencing revealed that $1,25(OH)_2D_3$ treatment induced demethylation of CpG sites located in the promoter (−97 to −32) and 5′UTR (+39 to +97). The dual-luciferase reporter assay further confirmed that CpG island methylation (-120 to +225) directly represses DKK1 transcription.

Conclusion DKK1 functions as a tumour suppressor in colorectal cancer, and $1,25(OH)_2D_3$ upregulates DKK1 expression by inducing demethylation of the DKK1 promoter and 5′UTR in specific colorectal cancer cell lines.

Keywords $1,25(OH)_2D_3$, DNA methylation, DKK1, Colorectal cancer

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Introduction

Colorectal cancer is the third most common malignancy globally, with the second highest mortality among malignant tumours [1]. Several prospective studies from various countries have shown that vitamin D deficiency is strongly associated with increased incidence and mortality of colorectal cancer [2]. Activation of the Wnt/β-catenin signalling pathway plays a crucial role in the pathogenesis and progression of colorectal cancer [3]. A number of studies have reported that $1,25(OH)_2D_3$ can inhibit the proliferation and promote the differentiation of colon cancer cells by inhibiting the abnormal activation of the Wnt/β-catenin signalling pathway [4]. In addition, $1,25(OH)_2D_3$ increased cellular differentiation. It reduced the levels of nuclear β-catenin and the Wnt target genes BCL-2, Cyclin D1, Snail1, CD44 and LGR5 in adenoma cells, highlighting its importance in protection against colorectal tumorigenesis [5].

Dickkopf-1 (DKK1) is a secretory antagonist of the Wnt/β-catenin signalling pathway [6]. DKK1 can be induced in colon cancer cells by $1,25(OH)_2D_3$ [7]. Rawson JB et al. reported that dietary vitamin D intake was strongly negatively associated with DKK1 methylation in a large cohort of Canadian CRC patients [8]. Since the DKK1 promoter region has no vitamin D response element sequence, active vitamin D may regulate the expression of DKK1 through an indirect pathway.

DNA methylation is an important epigenetic mechanism that regulates gene expression by causing changes in chromatin structure and DNA stability without altering the DNA sequence. The previous studies have focused mainly on the roles and mechanisms of CpG island methylation in the gene promoter. With the development of technologies such as genomic bisulphite sequencing and genome-wide DNA methylation mapping, an increasing number of studies have shown that DNA methylation is widespread in gene promoters and gene bodies [9]. Gene body methylation refers to the methylation of CpG sites in the transcribed regions of genes. Gene body methylation has been shown to regulate gene transcription and is closely related to the occurrence and progression of malignant tumours [10]. A CpG island was identified in the promoter and first exon region of DKK1 via the UCSC website. Galamb O et al. reported that hypermethylation of the DKK1 promoter was higher in colon carcinoma than in adenoma. Hypermethylation of the promoter region is an important reason for the downregulation of DKK1 expression in colorectal cancer [11]. However, the relationship between CpG island methylation in the gene body region and DKK1 expression has not been determined.

Several studies have identified the association between vitamin D and DNA methylation changes [12]. $1,25(OH)_2D_3$ induces de novo E-cadherin expression in breast cancer cells via promoter demethylation [13]. Lai GR et al. reported that $1,25(OH)_2D_3$ treatment reduced the expression levels and activities of DNA methyltransferases 1 and 3B and that long-term $1,25(OH)_2D_3$ exposure induced hypomethylation of genes linked to the mTOR signalling pathway in prostate cancer cells [14]. In addition, vitamin D may induce DNA methylation changes in myeloid cells and dendritic cells [15, 16]. However, whether $1,25(OH)_2D_3$ can affect the expression of DKK1 by regulating DNA methylation in colorectal cancer is still unknown.

In this study, we evaluated the clinical significance of DKK1 in CRC, and the results revealed that decreased serum DKK1 levels were closely associated with nerve infiltration and the 25(OH)D status in CRC patients. Moreover, we investigated the effect of DKK1 in colorectal cancer and the mechanism by which vitamin D upregulates DKK1. Our results indicated that DKK1 plays a role as a tumour suppressor in colorectal cancer and that $1,25(OH)_2D_3$ upregulates DKK1 expression by inducing demethylation of the DKK1 promoter and 5'UTR in specific colorectal cancer cell lines.

Materials and methods

Patients and healthy controls

Fifty-seven CRC patients who underwent surgery at the China-Japan Union Hospital of Jilin University between 2021 and 2022 were enrolled in this study. The patient did not receive preoperative chemoradiotherapy or immunotherapy. The tissues were obtained directly after surgical resection and frozen in liquid nitrogen. Blood samples were collected before surgery, and the serum was isolated and frozen at −80 °C until detection. The demographic and clinicopathological information for 57 CRC patients were collected from each patient's medical records. In addition, 55 healthy controls were enrolled in this study. The healthy control group was selected from the physical examination participants in our hospital, and the inclusion criteria were as follows: (1) no personal or family history of colorectal cancer; (2) age and sex were matched with the case group; (3) blood samples for serum separation were available and (4) no osteoporosis, diabetes or other malignant tumours were present. All patients provided signed informed consent, and the Ethical Committee of the China-Japan Union Hospital of Jilin University approved this study.

Measurement of DKK1 and 25(OH)D

A human DKK1 ELISA Kit (ml057577V, mlbio) was used to measure DKK1 levels in both serum and tissue. Specifically, 50 μl serum and 20 mg tissue were used for detection. The tissues were homogenized in PBS supplemented

with protease inhibitors using a cryogenic homogenizer, the homogenates were then centrifuged to obtain the supernatant for detection. A parallel sample well was set to eliminate the background signal, and each sample was tested three times. DKK1 levels were calculated from the standard curve. Serum 25(OH)D levels were measured by liquid chromatography–tandem mass spectrometry to estimate vitamin D (VitD) status accurately. VitD status was defined by the serum 25(OH)D cut-off recommended by the United States-American Institute of Medicine as follows: deficient: < 30 nmol/L (12 ng/ml); insufficient: 30– < 50 nmol/L (12– < 20 ng/ml) and sufficient: ≥ 50 nmol/L (20 ng/ml) [17].

Cell proliferation assays

To explore the function of DKK1 in colorectal cancer, human full-length DKK1 cDNA was subcloned and inserted into the pcDNA3.1 vector, and the gene expression plasmid and control vector were transiently transfected into cells via Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cell proliferation was assessed with a Cell Counting Kit-8 (CCK8; Beyotime, Shanghai, China). The medium was changed at 24 h, 48 h and 72 h after plasmid transfection, and 10 μL of CCK8 reagent was added to each well. The plates were measured in a microplate reader (Bio-Tek, USA) at 450 nm after a 2 h incubation at 37 °C.

Wound healing and migration assays

DLD1 cells were seeded in a 6-well plate for the wound healing assay and transfected with the DKK1 expression plasmid or vector control. After transfection for 24 h, the cell layer was carefully wounded with a 200 μL sterile tip and then cultured in serum-free medium. Wound margins were photographed at 0 and 24 h in five randomly selected microscopic regions. The wound healing rate was calculated to determine the migration ability of different groups of cells.

Cell migration assays were performed using transwell chambers (pore size 8.0 μm; Costar, Corning, Switzerland). SW480 cells were transfected with the DKK1 expression plasmid or control vector for 24 h before seeding in the transwell chambers. Two hundred microlitres of serum-free medium containing 5×10^4 cells was added to each upper chamber, and 600 μL of medium containing 15% FBS was added to each lower chamber. After incubation for 48 h, the cells that migrated through the insert membrane were fixed with cold 100% methanol for 20 min, stained with 0.1% crystal violet solution for 20 min and rinsed with PBS. Then, the nonmigrating cells on the upper surface of the membrane were removed with cotton swabs. Finally, images were taken with an Olympus IX-53 microscope. Four fields within each transwell chamber were chosen to count the migrating cells.

Quantitative real-time PCR

Total RNA from colorectal cancer tissues and cells was extracted with TRIzolTM reagent (Thermo Fisher Scientific). A PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, RR047A) was used to convert 500 ng of RNA into cDNA, and the mRNA expression levels were quantified via qPCR via TB GreenPremix Ex Taq™II (Tli RNaseH Plus) (TaKaRa, RR820A) in triplicate. The primers used were as follows: DKK1 forward primer, 5'- ATAGCACCTTGGATGGGTATTCC −3' and reverse primer, 5'- CTGATGACCGGAGACAAACAG −3'. GAPDH: forward primer 5'- AGAAGGCTGGGG CTCATTTG −3' and reverse primer 5'- AGGGGCCAT CCACAGTCTTC −3'. The reaction was performed with the ABI StepOnePlus Real-Time PCR System via a two-step amplification procedure. Relative target gene expression was determined by comparing average threshold cycles with the housekeeping gene GAPDH via the $2^{-\Delta\Delta Ct}$ method.

BiSulphite amplicon sequencing

BiSulphite Amplicon Sequencing (BSAS) was used for DNA methylation analysis [18]. Genomic DNA extracted from colorectal cells and tissues via a genomic DNA purification kit (A1120, Promega, Madison, WI, USA) was bisulphite treated with a bisulphite conversion kit (D5005, EZ DNA Methylation-Gold™, USA). PCR amplification was performed via Taq Plus DNA Polymerase (Biotech B600090, Sangon, Shanghai). The primers used were forwards, 5'-GGTAGTTTTTATTTYGAAGGT GAGT-3' and reverse, 5'-CAAAATAACRCTCACTCC CAAC-3'. The DNA methylation patterns of the targeted genomic regions were investigated via amplicon-based next-generation sequencing (NGS) via an Illumina sequencing system (Sangon, Shanghai).

Pyrosequencing

The DNA methylation levels of candidate CpG sites in the promoter and 5'UTR of DKK1 were analysed by pyrosequencing. Primers for the pyrosequencing assays were designed (Table S1). The pyrosequencing reactions were performed in a PyroMark Q96 ID pyrosequencer (Qiagen Sciences Inc.), and the pyrosequencing data were analysed with PyroMark Q96 software according to the manufacturer's instructions.

Methylation-specific PCR (MSP)

Genomic DNA extraction and bisulphite conversion were conducted as previously described in the BSAS. The primers used were as follows (Li and Dahiya, 2002): MSP

primers for unmethylated DKK1 were 5'-GGGGATTTAGGTTTGTAAAGTGAT-3' (forwards) and 5'-CACTCCCAACAAAAAATAACCAC-3' (reverse); MSP primers for methylated DKK1 were 5'-GGGGATTTAGGTTTGTAAAGTGAC-3' (forwards) and 5'-ACTCCCAACAAAAAATAACCG-3' (reverse), and 2 µL of bisulphite-treated genomic DNA was amplified via Zymo Taq™ Pre-Mix (E2003, ZYMO RESEARCH) under the following cycling conditions: 40 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. The PCR products were analysed via 2% agarose gel electrophoresis and photographed via a gel imaging system (UVP UVsolo2 touch, Germany).

Dual-luciferase reporter assay

To investigate the influence of CpG island methylation on DKK1 transcriptional activity, the CpG island of DKK1 (−120 to +225) was synthesized, cloned and inserted into pGL3-Basic. The enriched fragments were removed from pGL3-Basic and methylated in vitro via CpG methylase (M.SssI), which can completely methylate all cytosine residues in double-stranded, nonmethylated and hemimethylated DNA with the dinucleotide sequence 5'-CpG-3'. The methylated fragments were re-ligated into pGL3-Basic to assess differentially methylated promoter activity. Firefly and Renilla luciferase activities were separately measured via a dual-luciferase reagent kit (E1910, Promega).

Statistical analysis

Statistical analyses were performed via IBM SPSS Statistics for Windows, version 25.0 (IBM Corporation, Armonk, NY, USA). Normally distributed continuous variables were presented as the means ± SDs, and non-normally distributed data were presented as medians and quartile intervals. The differences between groups were assessed by independent sample t-test or Mann–Whitney U-test. A t-test was used when the data were normally distributed; otherwise, the Mann–Whitney U-test was used. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal serum DKK1 cut-off value. Correlations between DKK1 expression and clinicopathological factors were analysed via Pearson's $\chi 2$ test or Fisher's exact probability test. All tests were two-sided, and a P value less than 0.05 was considered statistically significant.

Results

Associations of serum DKK1 levels with 25(OH)D levels in CRC patients

Serum DKK1 and 25(OH)D levels were detected in 57 colorectal cancer patients and 55 healthy controls. The serum DKK1 level in colorectal cancer patients was 11.20 [8.25, 17.58] ng/ml, and in healthy controls, it was 15.26 [11.88, 20.21] ng/ml. The serum level of DKK1 was significantly decreased in CRC patients ($P = 0.002$, Fig. 1A).

The serum 25(OH)D level in colorectal cancer patients was 27.25 [20.13, 40.75] nmol/L. In contrast, it was 43.87 ± 17.26 nmol/L in healthy controls (Fig. 1B). The vitamin D deficiency, insufficient and sufficient rates were 56.14% (n = 32), 24.56% (n = 14) and 19.30% (n = 11), respectively, in CRC patients, and 23.64% (n = 13), 32.73% (n = 18) and 43.64% (n = 24), respectively, in healthy controls (Fig. 1C). There was a significant difference in the serum 25(OH)D concentration between colorectal cancer patients and healthy controls ($P = 0.000$).

The serum level of DKK1 was significantly lower in CRC patients with deficient vitamin D than in those with sufficient or insufficient vitamin D (10.53 [6.90, 15.60] ng/ml vs. 13.67 [9.14, 18.40], P = 0.044; Fig. 1D). Moreover, the optimal DKK1 cut-off value was 11.81, and the area under the curve (AUC) was 0.666 according to the receiver operating characteristic (ROC) curve (Fig. S1). The correlations between serum DKK1 levels and clinicopathological features in 57 CRC patients were further evaluated, and decreased serum DKK1 levels were closely associated with nerve infiltration and 25(OH)D status ($P = 0.017$) (Table 1).

Overexpression of DKK1 decreased the proliferation and migration of CRC cells

To determine the function of DKK1 in CRC, CRC cells were transfected with a DKK1 overexpression plasmid, and the effects of DKK1 on cell proliferation and migration were assayed. As shown in Fig. 2, overexpression of DKK1 decreased the proliferation of DLD1 and RKO cells in vitro (Fig. 2A, B). Compared with the control, overexpression of DKK1 significantly decreased the wound healing rate in DLD1 cells (35.03 ± 2.2% vs. 27.00 ± 1.65%, $P = 0.007$; Fig. 2C, D). Overexpression of DKK1 significantly decreased the migration capacity of SW480 cells (391.67 ± 11.01 vs. 343.67 ± 10.41, $P = 0.005$; Fig. 2C, E).

DKK1 expression is modulated by DNA methylation in colorectal cancer cells

A CpG island spanning from the promoter region to exon 1 (chr10:52,314,230–52,315,067) was identified in the DKK1 gene by using the University of California Santa Cruz Genome Browser (https://genome.ucsc.edu/). To explore whether DNA methylation of the CpG island silences the expression of DKK1, the DNA methylation status and DKK1 expression levels in the colorectal cancer cell lines DLD1, RKO and SW480 were detected. The methylation patterns of the region (−195 to +231), which included 31 CpG sites, were assayed in colorectal cell lines via BSAS (Fig. 3A). The results revealed that the proximal promoter, 5'UTR and exon 1 region of DKK1

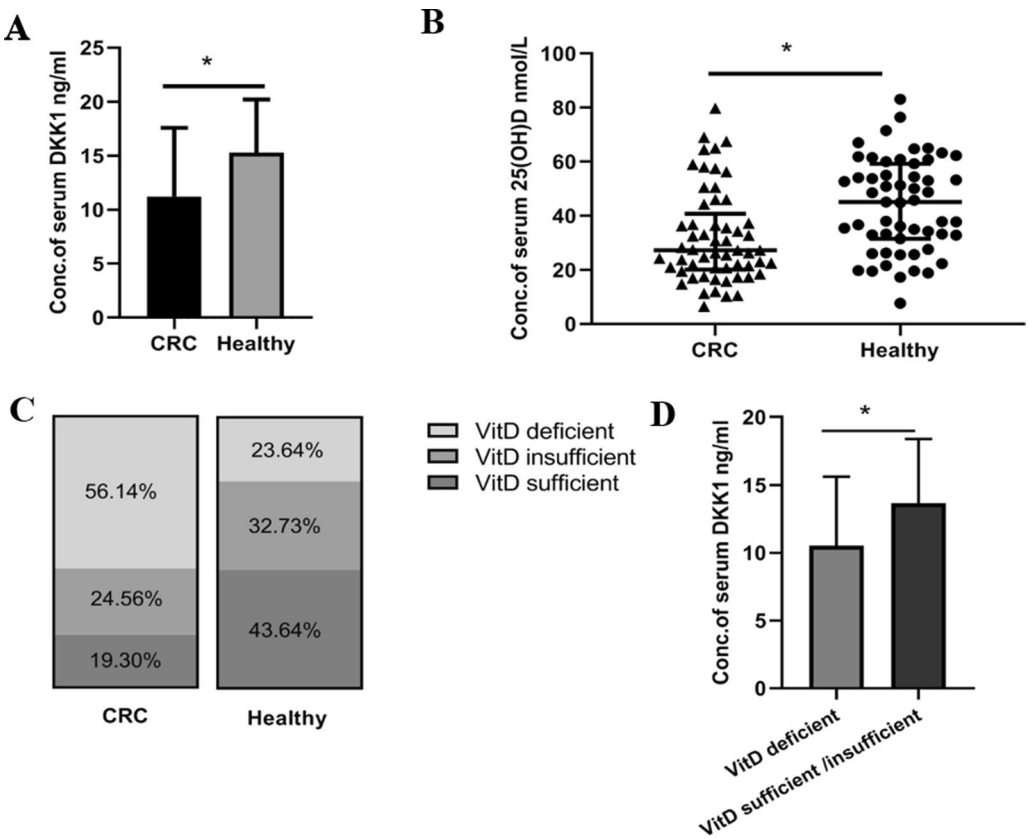

Fig. 1 Associations of serum dkk1 levels with 25(OH)D levels in CRC patients. **A** The serum DKK1 level in colorectal cancer patients and healthy control. **B** The serum 25(OH)D level in colorectal cancer patients and healthy control. **C** Vitamin D status in colorectal cancer patients and healthy control. **D** Comparison of serum DKK1 level in colorectal cancer patients in normal/insufficient vitamin D group and deficient group. *$p < 0.05$

were highly methylated in DLD1 and RKO cells but nonmethylated in SW480 cells (Fig. 3B). DKK1 mRNA expression in SW480 and DLD1 was 491.7 ± 143.49 times and 6.84 ± 0.34 times higher than that in RKO, respectively (SW480 vs. RKO, $P = 0.040$; DLD1 vs. RKO, $P = 0.0017$; Fig. 3C, D), indicating that DKK1 expression was associated with DNA methylation.

To further investigate the role of methylation in regulating DKK1 expression, DLD1, RKO and SW480 cells were treated with 10 μM DAC, and DKK1 expression was then detected via RT-PCR. The results revealed no significant difference in DKK1 mRNA expression in SW480 cells (Fig. 3E). However, treatment with DAC induced DKK1 mRNA expression in DLD1 and RKO cells (Fig. 3F and G).

Correlation of DKK1 expression in human colorectal tissues with DNA methylation status

We further detected the DNA methylation status and mRNA expression level of DKK1 in colorectal cancer tissues. First, the methylation status of the region (− 195 to + 231), which included 31 CpG sites, was assayed in five matched human colorectal cancer and adjacent colorectal tissues. The methylation levels of multiple CpG sites were increased in tumour tissues compared with adjacent normal tissues. The CpG sites with increased methylation levels were located in the promoter, 5'UTR and exon 1 of DKK1, mainly in the exon 1 region (Fig. 4A). DKK1 expression was decreased in CRC tissues compared with corresponding noncancerous tissues (Fig. 4B). We detected the methylation status of another ten matched human colorectal cancer and adjacent colorectal tissues (Fig. 4C, D), and the methylation rate was 70% in colorectal cancer and 30% in adjacent colorectal tissues. We further detected DKK1 expression, which was significantly lower in cancer tissues than in adjacent normal tissues ($P = 0.045$, Fig. 4E). DKK1 expression in human colorectal tissues is associated with DNA methylation status.

Table 1 Associations of serum DKK1 level with clinicopathological characteristics of CRC patients

Variable	No. of cases	Serum DKK1		*p* values
		Low (≤11.81 ng/ml)	High (>11.81 ng/ml)	
Age(year)				0.359
<60	28	14	14	
≥60	29	18	11	
Gender				0.907
Male	36	20	16	
Female	21	12	9	
BMI				0.790
18.5–23.9	40	22	18	
>23.9	17	10	7	
Tumour size (cm)				0.249
≥5	27	13	14	
<5	30	19	11	
Tumour location				0.962
Colon	34	19	15	
Rectum	23	13	10	
Histology differentiation			0.847	
Well and moderate	35	20	15	
Poor	22	12	10	
Depth of invasion				0.409
T1–T2	13	6	7	
T3–T4	44	26	18	
Lymph node metastasis			0.554	
Yes	23	14	9	
No	34	18	16	
Venous permeation			0.366	
Yes	22	14	8	
No	35	18	17	
Perineural invasion				**0.017***
Yes	16	13	3	
No	41	19	22	
MMR				0.227
Deficient	18	8	10	
Competent	39	24	15	
25(OH) D				**0.048***
Deficient	32	22	10	
Insufficient	14	5	9	
Sufficient	11	4	7	

*statistically significant

1,25(OH)$_2$D$_3$ upregulated DKK1 expression by inducing DKK1 demethylation

As shown in the first part of the results, the serum level of DKK1 was significantly decreased in CRC patients with deficient vitamin D status. We investigated whether active vitamin D induces DKK1 expression in colorectal cancer cells. SW480, DLD1 and RKO cells were treated with 100 nM 1,25(OH)$_2$D$_3$ for 48 h, and DKK1 mRNA expression was detected by RT–PCR. The results revealed that 1,25(OH)$_2$D$_3$ induced DKK1 mRNA expression in SW480, DLD1 and RKO cells (Fig. 5A).

Since DKK1 expression is modulated by DNA methylation, the effect of 1,25(OH)$_2$D$_3$ on the methylation of DKK1 was further investigated by pyrosequencing. The DNA methylation levels of 22 candidate CpG sites in the promoter and 5'UTR of DKK1 were analysed. The pyrosequencing regions are shown in Fig. 5B (Fig. 5B). There are five CpG sites in region 1, six in region 2, five in region 3 and six in region 4. The results of pyrosequencing indicated that there was no significant difference in the methylation rates of CpG sites in regions 1 and 3 after 1,25(OH)$_2$D$_3$ treatment (Fig. 6A, C). However, methylation rates at the 2rd to 6th CpG sites of region 2 were lower in the 1,25(OH)$_2$D$_3$-treated DLD1 cells than in the control cells (C_pG2, 39.51 ± 7.73 vs. 28.65 ± 6.63, $P=0.046$; C_pG3, 92.04 ± 1.56 vs. 84.31 ± 1.44, $P=0.007$; C_pG4, 88.77 ± 0.17 vs. 81.54 ± 0.42, $P=0.016$; C_pG5, 94.77 ± 0.99 vs. 84.95 ± 0.74, $P=0.012$ and C_pG6, 92.27 ± 1.92 vs. 81.98 ± 1.34, $P=0.025$), methylation rates at the 4th and 5th of region 2 also decreased in RKO cells after 1,25(OH)$_2$D$_3$ treatment (C_pG4, 91.98 ± 2.82 vs. 80.62 ± 1.57, $P=0.049$; C_pG5, 92.12 ± 1.44 vs. 82.43 ± 0.66, $P=0.036$; Fig. 6B). Compared with the control group, the methylation rate of the first two C_pG sites in region 4 was lower in the 1,25(OH)$_2$D$_3$-treated DLD1 cells (C_pG1, 90.95 ± 0.69 vs. 77.83 ± 1.81, $P=0.039$; C_pG2, 93.63 ± 9.01 vs. 82.51 ± 9.52, $P=0.021$; Fig. 6D). Methylation rates at the 2nd and 5th C_pG sites of region 4 also decreased in RKO cells after 1,25(OH)$_2$D$_3$ treatment (C_pG2, 95.37 ± 0.09 vs. 83.36 ± 1.26, $P=0.044$; C_pG5, 84.73 ± 0.70 vs. 69.22 ± 0.93, $P=0.047$; Fig. 6D).

To examine whether CpG island methylation in region 2 and region 4 directly represses DKK1 transcription, we cloned the DKK1 exon 1 region (−120 to +225), containing 27 CpG sites, into pGL3-Basic. The cloned inserts were then methylated via M.SssI and re-ligated into pGL3-Basic. The transcriptional activity of the differentially methylated DKK1 promoter and exon region was assessed via the transfection of luciferase reporter constructs into SW480 cells. The results revealed that DKK1 transcriptional activity was repressed after methylation (Fig. 5C).

Discussion

Vitamin D is a fat-soluble vitamin that is protective in colorectal cancer, and DKK1 is an inhibitor of the Wnt/β-catenin signalling pathway. In this study, we found that

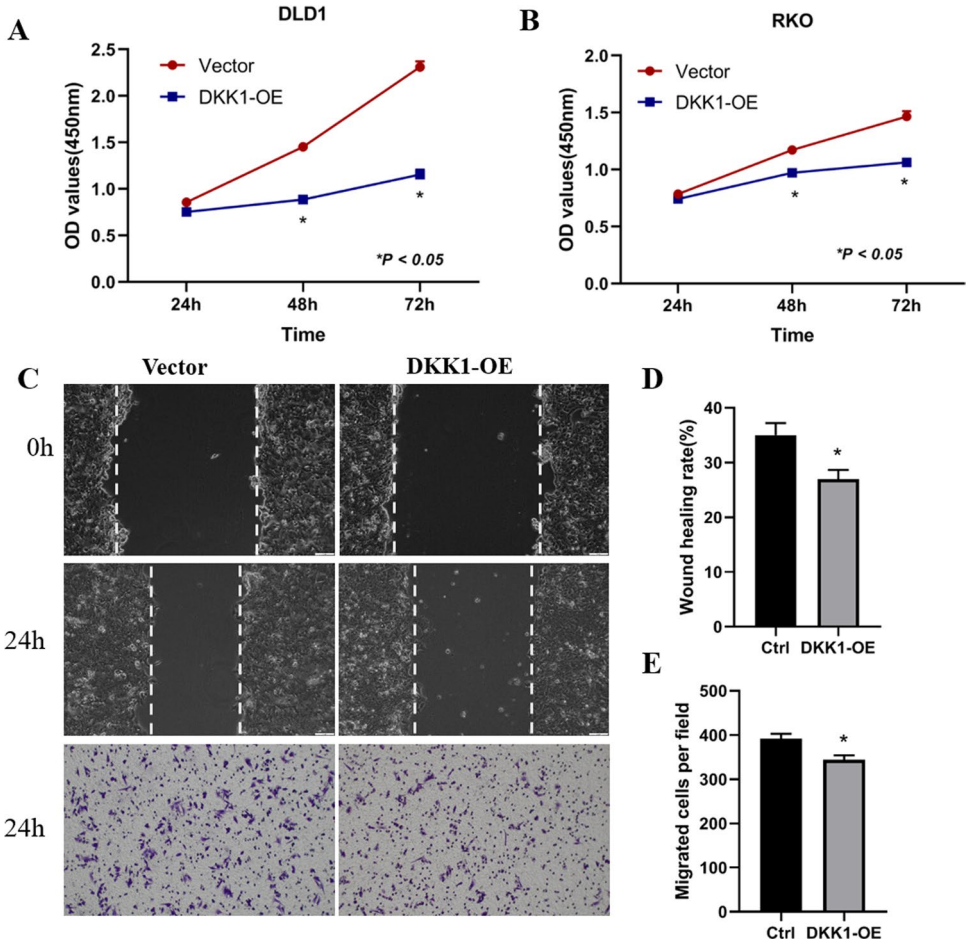

Fig. 2 Overexpression of DKK1 decreased proliferation and migration capacities of colorectal cancer cells. **A** Overexpression of DKK1 significantly decreased cell proliferation of DLD1 cells. **B** Overexpression of DKK1 significantly decreased cell proliferation of RKO cell. **C**, **D**. Overexpression of DKK1 significantly decreased the wound healing rate compared to control in DLD1 cell. **C**, **E**. Overexpression of DKK1 significantly decreased the migration capacities of SW480 cells. *$p < 0.05$

the level of circulating vitamin D was positively correlated with serum DKK1 levels in patients with colorectal cancer. We first reported that $1,25(OH)_2D_3$ could upregulate DKK1 expression in specific colorectal cancer cell lines by inducing demethylation of the DKK1 promoter and 5'UTR. Our findings further support the role of vitamin D in regulating DNA methylation in colorectal cancer.

25(OH)D is the major circulating form of vitamin D, and the measurement of blood circulation 25(OH)D is considered the best indicator of vitamin D status. The prevalence of vitamin D deficiency has increased in recent years. A systematic review of 195 studies conducted in forty-four countries involving more than 168,000 participants showed that 37.3% of the studies reported mean values of 25(OH)D less than 50 nmol/L [19]. In this study, vitamin D deficiency rates were as high as 56.14% in patients with colorectal cancer. Zhou et al. reported that higher concentrations of serum 25(OH)D are associated with a lower incidence and improved survival of CRC based on the UK Biobank data [20]. A recent pooled study of 17 cohorts worldwide revealed that vitamin D deficiency was associated with increased colorectal cancer risk, and the optimal 25(OH)D concentration for reducing colorectal cancer risk was 75–100 nmol/L, which is higher than the current Institute of Medicine (IOM) recommendations [21]. The findings of a recent and the most extensive systematic review and meta-analysis of case–control and prospective cohort studies supported an inverse association between circulating vitamin D levels and CRC risk [2]. These findings suggest the importance of maintaining sufficient blood circulating vitamin D levels to prevent CRC progression.

Increasing evidence has shown that DKK1 has different effects on the progression of different tumours and has dual functions of inhibiting and promoting cancer

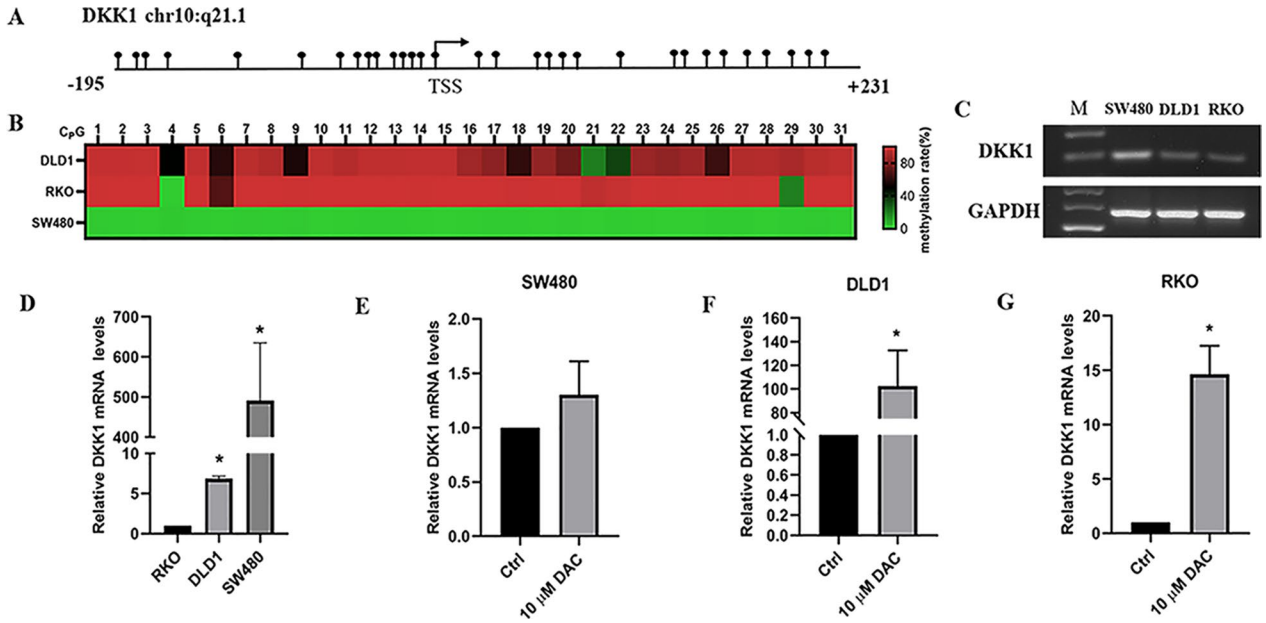

Fig. 3 DKK1 expression is modulated by DNA methylation in colorectal cancer cells. **A** A region rich in CpG sites which was assayed in this study. **B** The methylation status of different colorectal cancer cells. **C** A gel electrophoresis image showing DKK1 expression of different colorectal cancer cells. **D** DKK1 mRNA expression of different colorectal cancer cells. **E** Treatment with DAC did not induce changes in DKK1 expression in SW480 cells. **F** Treatment with DAC induced DKK1 expression in DLD1 cells. **G** Treatment with DAC induced DKK1 expression in RKO cells. *$p < 0.05$

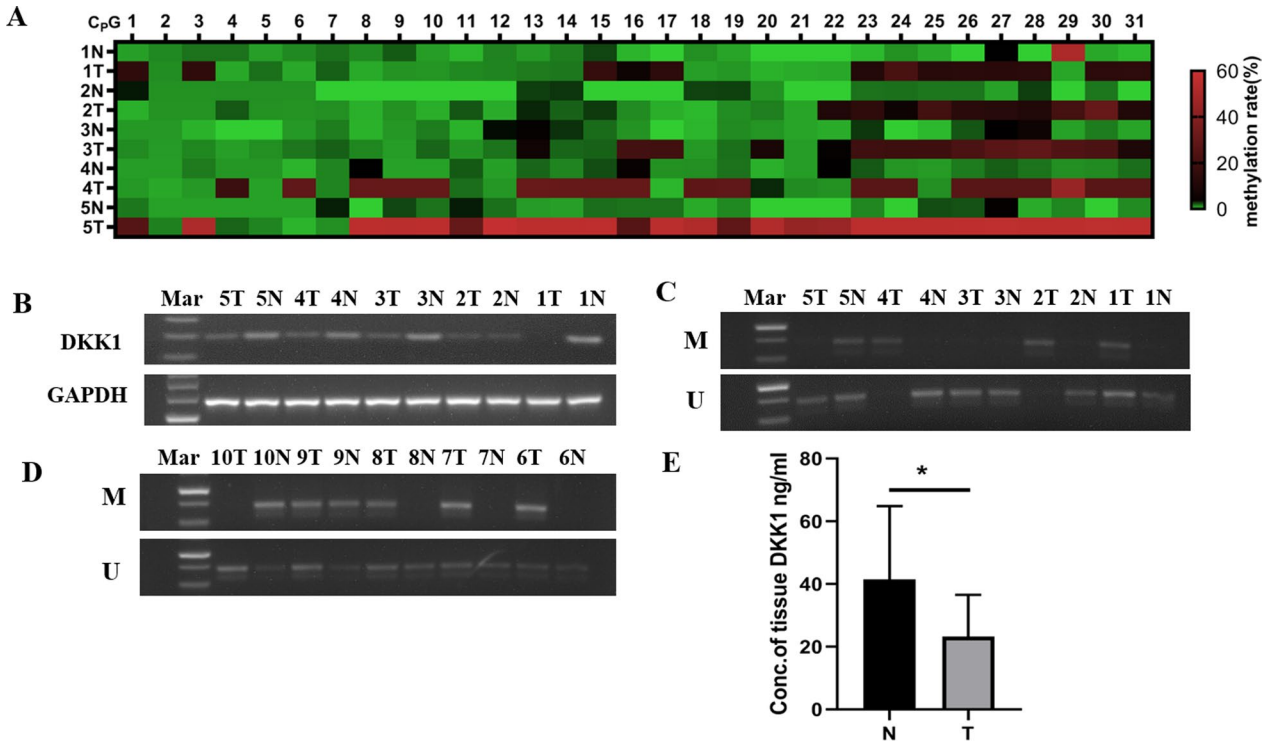

Fig. 4 DKK1 expression in human colorectal tissues was associated with DNA methylation status. **A** The methylation status of five matched human colorectal cancer and adjacent colorectal tissues. **B** DKK1 mRNA expression in five matched human colorectal cancer and adjacent colorectal tissues. **C** and **D** Methylation status of another 10 matched human colorectal cancer and adjacent colorectal tissues were detected by MSP. **E** The DKK1 protein level in human colorectal cancer and adjacent colorectal tissues. N: adjacent colorectal tissues, T: colorectal cancer tissue, M: methylated bands, U: unmethylated bands and Mar: DL500 marker

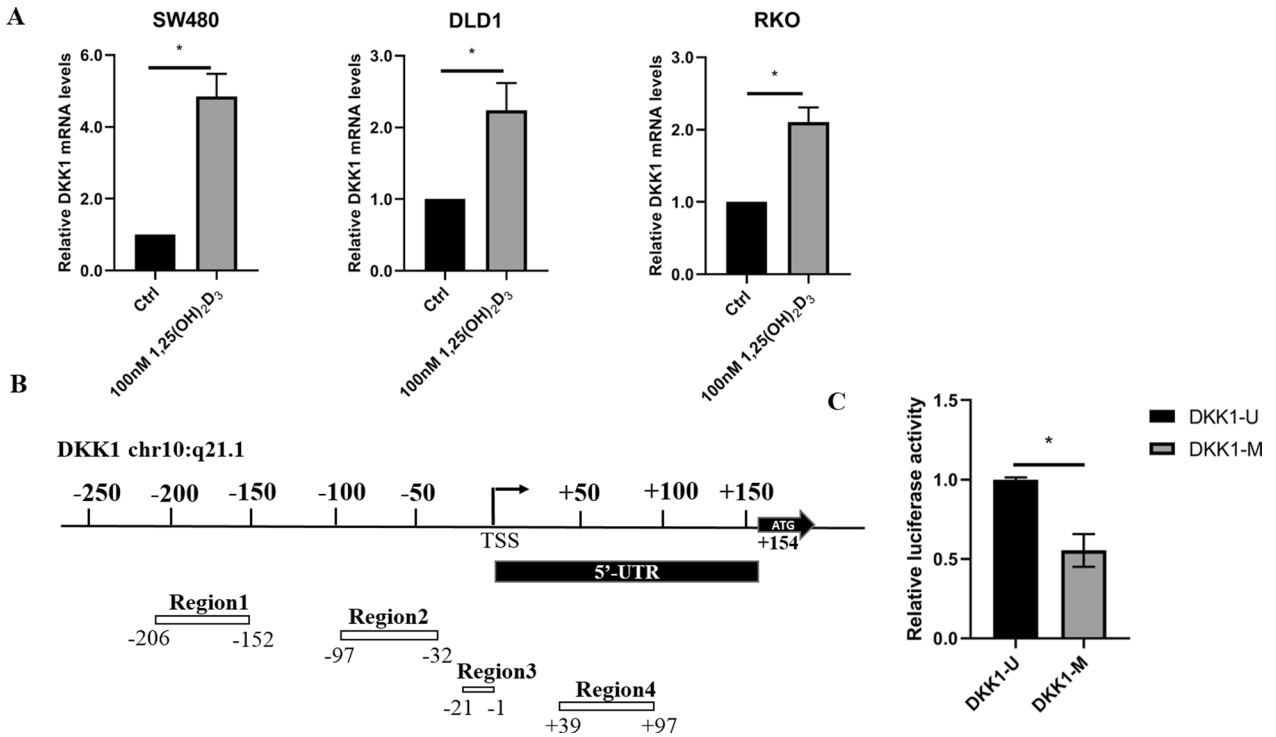

Fig. 5 $1,25(OH)_2D_3$ upregulated DKK1 expression by inducing DKK1 demethylation. **A** $1,25(OH)_2D_3$ induced DKK1 mRNA expression in SW480, DLD1 and RKO cells. **B** Pyrosequencing regions of DKK1 in this study. **C** The transcriptional activity of the differentially methylated DKK1 promoter and exon region. *$p < 0.05$

[22]. DKK1 has been shown to promote tumour metastasis in pancreatic cancer, breast cancer and non-small cell lung cancer; however, it has been shown to play an inhibitory role in ovarian cancer. The multifaceted role of DKK1 in regulating antitumour immunity has also been revealed in recent years [23]. So, further study of the role of DKK1 in cancer is imperative. In this study, we found that the serum level of DKK1 was significantly lower in CRC patients than in healthy controls and that decreased serum DKK1 levels were closely associated with nerve infiltration. In vitro, overexpression of DKK1 decreased the proliferation and migration of colorectal cancer cells. Liu et al. reported that DKK1 expression was lower in colorectal cancer tissues than in normal tissues [24], as DKK1 is a secreted protein, the serum level of DKK1 was detected in this study. Similar to the results reported in other tissues, DKK1 levels were also decreased in the serum of patients with colorectal cancer, suggesting that DKK1 has the potential to be used as a biological marker for the diagnosis of colorectal cancer.

$1,25(OH)_2D_3$ can induce DKK1 expression and drive the differentiation of colon cancer cells and osteoblasts [7, 25]. A cohort study has shown that dietary vitamin D intake was strongly negatively associated with DKK1 methylation [8]. Zhu et al. reported that vitamin D deficiency is associated with global hypomethylation in African Americans. Vitamin D3 supplementation increases global DNA methylation in a dose-dependent manner in African Americans with vitamin D deficiency [26]. In breast cancer, 25(OH)D concentrations are associated with DNA methylation of candidate CpGs in several vitamin D-related genes and three immune function-related genes. Methylation of CpGs in vitamin D-related genes may interact with 25(OH)D to affect breast cancer risk [13]. In experimental studies, $1,25(OH)_2D_3$ was found to promote partial CDH1 promoter demethylation in triple-negative breast cancer, and the $1,25(OH)_2D_3$/VDR complex altered DNA methylation of the CDKN1A promoter in prostate cancer cells [27]. This study investigated whether $1,25(OH)_2D_3$ induces DKK1 expression by regulating DNA methylation in colorectal cancer. The methylation status of four regions in the DKK1 promoter and 5'UTR was analysed by pyrosequencing, and the methylation rates of the C_pG sites in regions 2 and 4 were reduced after $1,25(OH)_2D_3$ treatment. We further confirmed that CpG site methylation in regions 2 and 4 directly represses DKK1 transcription. Region 2 is located in the DKK1 promoter, and the previous studies have shown that the DKK1 promoter contains multiple TCF-binding elements (TBE,

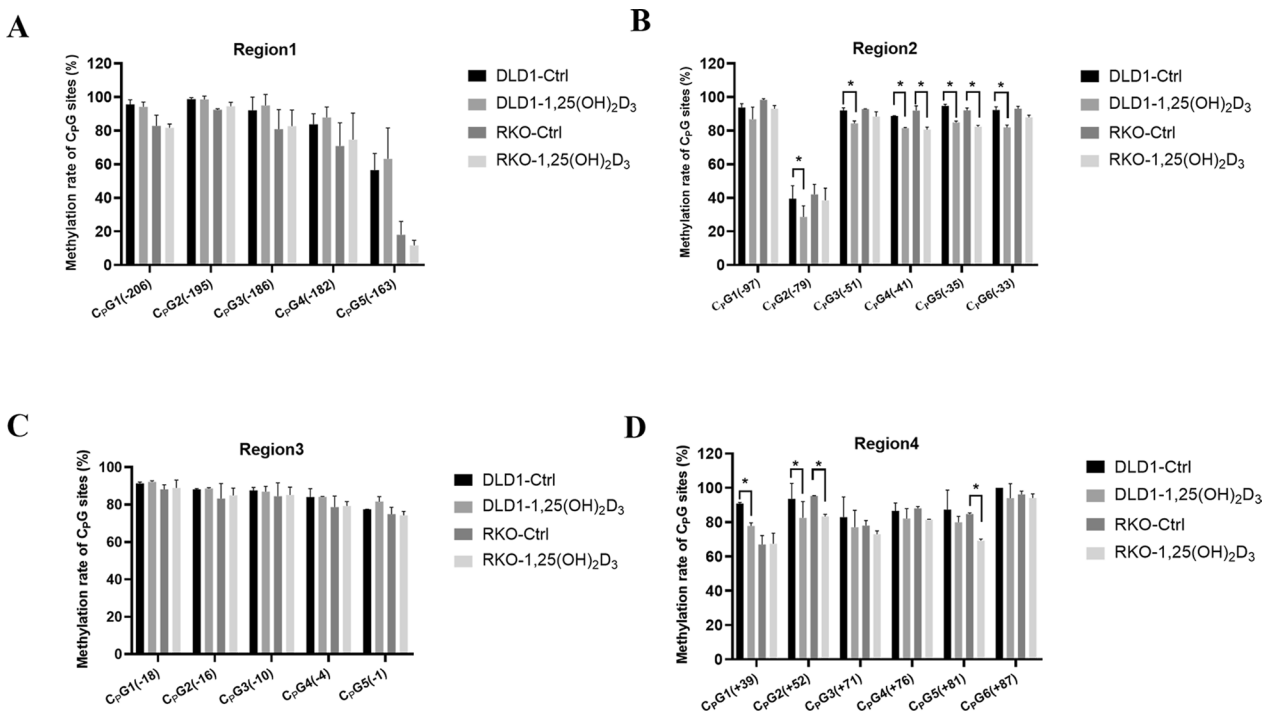

Fig. 6 The effect of $1,25(OH)_2D_3$ on the methylation of DKK1 in colorectal cancer cells. **A** Methylation rate of C_pG sites in region 1 of DKK1 in $1,25(OH)_2D_3$ treated and untreated colorectal cancer cells. **B** Methylation rate of C_pG sites in region 2 of DKK1 in $1,25(OH)_2D_3$ treated and untreated colorectal cancer cells. **C** Methylation rate of C_pG sites in region 3 of DKK1 in $1,25(OH)_2D_3$ treated and untreated colorectal cancer cells. **D**. Methylation rate of C_pG sites in region 4 of DKK1 in $1,25(OH)_2D_3$ treated and untreated colorectal cancer cells. $^*p < 0.05$

5'-CTTTG [A/T][A/T]−3') and that DKK1 is a target of β-catenin/TCF [28]. Region 2 (−97 to −32) in this study, including 2 TBEs (−89 to −95 and −70 to −76), CpG methylation of this region might suppress DKK1 expression by inhibiting TCF binding; therefore, $1,25(OH)_2D_3$ might induce DKK1 expression by reducing the methylation level of the TCF-binding region. Region 4 is located in the 5'UTR, the 5'UTR contains key elements involved in translation regulation, and changes in the 5'UTR also affect mRNA stability. Jia et al. identified diverse sequence features of the 5'UTR that control mRNA translatability [29], and the 5'-UTR methylation of DAT1 might be an epigenetic biomarker for ADHD diagnosis [30]. However, few studies have focused on the effects of 5'UTR methylation on transcription and its mechanisms, and further studies are needed.

It is noteworthy that the results of this study indicate that $1,25(OH)_2D_3$ can also induce DKK1 expression in SW480 cells that lack DNA methylation in DKK1 promoter and exon 1 region. This suggests that $1,25(OH)_2D_3$ may regulate DKK1 gene expression through other mechanisms besides demethylation. However, the inability to elucidate the precise underlying mechanism constitutes a limitation of this study. Aguilera et al. conducted relevant research and discovered that $1,25(OH)_2D_3$ can activate a 2300 bp fragment of the human DKK1 gene promoter, and exposure to $1,25(OH)_2D_3$ results in an elevation of histone 3 acetylation and trimethylation at Lys 4, which is compatible with transcriptionally active chromatin in SW480-ADH cells [7]. In addition, Aguilera et al.'s study showed that an E-cadherin blocking antibody inhibits $1,25(OH)_2D_3$-induced DKK1 gene expression, which indicates that the regulatory effect of $1,25(OH)_2D_3$ is an indirect consequence of the induction of the epithelial adhesive phenotype [7]. Furthermore, the induction of DKK1 expression by $1,25(OH)_2D_3$ may also involve epigenetic factors, as treatment with $1,25(OH)_2D_3$ has been shown to affect the expression of several Jumonji-C domain-containing histone demethylases [31]. Pereira et al. found that JMJD3, also known as KDM6B, is induced by $1,25(OH)_2D_3$ in colon cancer cells [32]. JMJD3 is the first specific demethylase targeting H3K27me3, a histone modification that typically correlates with gene repression when present at transcriptional sites. Consequently, $1,25(OH)_2D_3$ may promote gene transcription by upregulating JMJD3. Moreover, upon binding to the VDR/RXR complex, $1,25(OH)_2D_3$ recruits numerous coactivators, including histone acetyltransferases, the acetylation of histones subsequently leads to chromatin relaxation, creating an environment conducive to

gene transcription [33]. Therefore, in addition to DNA demethylation, the induction of DKK1 expression by $1,25(OH)_2D_3$ may also be associated with epithelial adhesive phenotype and epigenetic factors, including histone methylation and acetylation, these potential associations warrant further investigation.

Conclusion

In conclusion, DKK1 has been shown to act as a tumour suppressor gene in CRC, and its expression is significantly lower in CRC patients than in healthy controls. $1,25(OH)_2D_3$ could upregulate DKK1 expression by inducing demethylation of the DKK1 promoter and 5'UTR in specific colorectal cancer cell lines. Maintaining sufficient blood circulating vitamin D levels might play an important role in preventing and treating colorectal cancer.

Abbreviations

DKK1	Dickkopf-1
CRC	Colorectal cancer
BSAS	BiSulphite amplicon sequencing
MSP	Methylation-specific PCR
CCK8	Cell counting kit-8
qPCR	Quantitative real-time PCR

Supplementary Information

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Additional file 1.

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Author contributions

H.S. and X.C. designed the research and wrote the manuscript, Y.H. and Q.H. recruited the patients enrolled in this study and revised the manuscript, N.L. and Z.Z. participated in data analysis, H.S. and L.Y. performed experiments. All authors read and approved the final manuscript.

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Availability of data and materials

All the data generated in this study are included in the manuscript and supplementary files. The original datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the China-Japan Union Hospital of Jilin University and performed according to the principles of the Declaration of Helsinki. Informed consent was signed by all parents.

Consent for publication

All authors have read and revised the manuscript and consent to its publication.

Competing interests

The authors declare no competing interests.

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