

1,25-Dihydroxyvitamin D₃ affects gastric cancer progression by repressing BMP3 promoter methylation

This article was published in the following Dove Medical Press journal:
OncoTargets and Therapy

Ye Zhao^{1,2,*}
Liang-Liang Cai^{3,*}
Hui-Ling Wang¹
Xiao-Juan Shi¹
Hui-Ming Ye⁴
Ping Song⁵
Bao-Qi Huang⁵
Chi-Meng Tzeng¹

¹School of Pharmaceutical Sciences, Nanjing Tech University, Nanjing 211800, People's Republic of China; ²Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture, Nanjing Tech University, Nanjing 211800, People's Republic of China; ³Translational Medicine Research Center, School of Pharmaceutical Sciences, Xiamen University, Xiamen 361005, People's Republic of China; ⁴Department of Clinical Laboratory, Zhongshan Hospital Xiamen University, Xiamen 361004, People's Republic of China; ⁵College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211800, People's Republic of China

*These authors contributed equally to this work

Background: Vitamin D₃ has been known to have an anticancer effect, but the mechanisms underlying this is poorly explored. The present study aimed to investigate the antitumor role of vitamin D₃ on gastric cancer and mechanisms.

Methods: The Roche Elecsys platform was applied in retrospective studies to detect the role of 25-hydroxylvitamin D₃ in adenocarcinoma and colony formation assay was conducted to verify the effect of 1, 25-dihydroxyvitamin D₃ on the proliferation of gastric cancer cells. After the identification of hypermethylation of BMP3 CpG islands by bisulfite genomic sequencing (BGS), we further investigated the relationship of BMP3 expression and gastric carcinogenesis by Western blot analysis and gel electrophoresis mobility shift assay (EMSA).

Results: Here we show that low concentration of 1, 25-dihydroxyvitamin D₃ links to cancerization and significantly inhibits proliferation of undifferentiated gastric cancer cell lines SGC-7901 and BGC-823. BMP3 promoter hypermethylation was highly correlated with gastric tumor. Moreover, BMP3 expression was regulated by its promoter methylation in gastric cells. The further exploration of the relationship between 1, 25-dihydroxyvitamin D₃ and BMP3 by EMSA results that 1, 25-dihydroxyvitamin D₃ stimulates BMP3 expression by the inhibition of BMP3 promoter methylation in gastric tumor cells.

Conclusion: In combination with the data from clinical research, bioinformatics analysis and experimental verification, we propose that 1, 25-hydroxylvitamin D₃ affects gastric cancer progression by repressing BMP3 promoter methylation.

Keywords: BMP3, 1,25-dihydroxyvitamin D₃, gastric cancer, promoter methylation

Introduction

Vitamin D₃, also known as cholecalciferol, is not really a vitamin but the precursor to the potent steroid hormone calcitriol (also named as 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]), which has extensive roles through many tissues of the body. Derived from diet or sunlight, vitamin D₃ is converted into 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver, which is subsequently hydroxylated to synthesize 1,25(OH)₂D₃ by the cytochrome P450 enzyme in the kidney.¹ 25(OH)D₃ is the circulating form of vitamin D₃ that can be measured in human plasma and clinically used to establish and monitor the vitamin D₃ status of a patient.² The actions of the vitamin D hormone 1,25(OH)₂D₃ are mediated by the nuclear vitamin D receptor (VDR), a member of the steroid–thyroid–retinoid receptor superfamily of ligand-activated transcription factors that function to control gene expression. 1,25(OH)₂D₃ interacts by binding to and activating the VDR, thereby causing its dimerization with the retinoid X receptor (RXR), and binding the complex to vitamin D response element (VDRE) to promote

Correspondence: Chi-Meng Tzeng
School of Pharmaceutical Sciences,
Nanjing Tech University, No 30
Puzhu Road, Nanjing 211816,
People's Republic of China
Tel +86 25 5813 9396
Email tzengchimeng@njtech.edu.cn

the absorption or releasing of calcium in the intestine, bone, and parathyroid gland via induction of the expression of epithelial calcium channel and calcium-binding protein.^{3,4} $1,25(\text{OH})_2\text{D}_3$ is found to regulate multiple genomic actions directly and indirectly due to the existence of VDR in many normal and cancer tissues.⁵ Epidemiological and animal studies have shown that low levels of vitamin D are associated with an increased risk of cancer and $1,25(\text{OH})_2\text{D}_3$ has the potential to affect cancer involving in proliferation, apoptosis, differentiation, inflammation, invasion, angiogenesis, and metastasis.^{6,7}

Vitamin D_3 has been recognized to have multifarious antitumor effects. When binding to VDR, $1,25(\text{OH})_2\text{D}_3$ could transcriptionally activate the expression of number of target genes, including *p21*,⁸ *Myc*⁹ and *Ras*,¹⁰ which are involved in different signaling pathways during tumorigenesis. In addition, $1,25(\text{OH})_2\text{D}_3$ has been shown to synergistically or additively enhance the antitumor activities of some anti-tumor drugs, such as paclitaxel¹¹ and gefitinib.¹² In gastric cancer, several preclinical studies have proved the antitumor effects of vitamin D_3 , as well as its level being a significant independent prognostic factor in gastric cancer patients.¹³ In addition, the study of VDR expression in human malignant gastric tissue shows that the antitumor role of vitamin D_3 is largely mediated by VDR, and the VDR was expressed higher in well and moderately differentiated tissues than in tissues with poor differentiation.¹⁴ High levels of VDR in human gastric cancer tissues and cancer cell lines implicated that vitamin D_3 may display more potent pharmacological action against malignant cells. Several studies have shown that high vitamin D_3 intake and high vitamin D_3 exposure index were associated with an increased risk of gastric cancer.¹⁵ However, the precise mechanisms involved in anticancer effect of vitamin D_3 on gastric cancer are still largely unexplored as today and need to be further investigated. In this study, we aimed to investigate the antitumor effect of vitamin D_3 on gastric cancer and underlying mechanisms.

In recent years, epigenetic regulation of gene expression has emerged as a potentially important factor in tissue disorders. Aberrant DNA methylation in the promoter region of predominantly tumor suppressor genes occurs in the early stages of tumor development in precancerous lesions.¹⁶ Thus, measuring DNA methylation in a genome-wide manner would be valuable for studying mechanisms of epigenetic control involved in tumor development and progression. Previously, BMP3 promoter region methylation has been found in gastric cancer.¹⁷ The gastric carcinoma genome showed significantly higher proportions of hypomethylation in the promoter region of BMP3, which was validated in the CpG islands.¹⁸ However,

few reports are available on the role of BMP3 in gastric carcinoma development. In our study, we investigated the role of BMP3 in mediating the anticancer action of vitamin D_3 and its transcriptional activation in gastric cancer cells, further illustrating the uniqueness of vitamin D_3 in gastric cancer.

Materials and methods

Patients and samples

Patients were recruited from mainland China and Taiwan. All the methods were approved by the research medical ethics committee of First Affiliated Hospital of Xiamen University (Institutional Review Board Number: KYX-2015-001). All patients signed the informed consent form (ICF) and fully understood the details of the trial (XMZSHCT201301005). This study was conducted in accordance with the Declaration of Helsinki. Serum samples of rickets patients, cancer patients, and healthy normal controls were obtained, and the level of $25(\text{OH})\text{D}_3$ was tested in clinics. Primary gastric carcinoma specimens and normal controls were collected for histological examination and DNA methylation analysis.

Chemicals

$1,25(\text{OH})_2\text{D}_3$ (D1530) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Stock solution of $1,25(\text{OH})_2\text{D}_3$ was dissolved in pure ethanol at a concentration of 10 mM and kept at -80°C in the dark. The primary antibodies for BMP3 (ab134724) and VDR (ab3508) were purchased from Abcam. Anti-GAPDH (CB1001) was purchased from EMD Millipore.

Cell culture and drug treatments

Human gastric epithelial cell line GES-1 and human gastric cancer cell lines BGC-823, SGC-7901, MGC80-3, AGS-1, and MKN-28 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS in 5% CO_2 incubators at 37°C . All the culture media were purchased from Sigma-Aldrich Co., and FBS was purchased from Thermo Fisher Scientific (catalog no 16000-044; Waltham, MA, USA). Cells were incubated with $1,25(\text{OH})_2\text{D}_3$ or various doses (0, 0.1, 0.2, 0.3, 0.4, 0.5 mM) of 5-aza-2'-deoxycytidine (Sigma-Aldrich Co.) for the duration of the experiments.

Colony formation assay

Cells were seeded at a density of 1×10^3 cells/well in a six-well plate. After incubation for 24 hours, cells were treated with $1,25(\text{OH})_2\text{D}_3$ for 7 days. After that, the cells were stained by 0.1% crystal violet and the number of colonies was counted.

DNA extraction, DNA methylation microarray, and analysis

The method for DNA extraction and methylation analysis is described previously.¹⁹ Before DNA extraction, gastric specimens were formalin-fixed, paraffin-embedded and microscopically examined using H&E staining. Then, gastric tissues were dissolved in 200 μ L of lysis buffer from the DNA Micro Kit (catalog no. 56304; Qiagen NV, Venlo, the Netherlands) and incubated with proteinase K overnight at 56°C for two nights. DNA was extracted according to the manufacturer's protocol (QIAamp DNA Micro Kit; Qiagen NV) and stored at -80°C to prevent degradation. DNA concentration was determined at 260 nm using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, NC, USA).

Bisulfite modification of 500 ng of DNA from each sample was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). After bisulfite modification, the whole genome from each tissue was amplified, enzymatically fragmented, and hybridized to the Illumina Infinium HumanMethylation450 BeadChip kits (Illumina Inc.). Methylation primer was 5'-GGTATTTTGTAGGC-GCGTC-3' and 5'-AACGAAAAACGCCGCAACG-3' and nonmethylation primer was 5'-ATTGGGTATTTTGTAG-GTGTGTT-3' and 5'-AAAGAAAAAGCCAGCAAAG-3'. The BeadChips were imaged using Illumina BeadArray Reader platform, the Illumina's BeadScan software was used for analyzing image intensities, and the intensity signals were extracted using the GenomeStudio software (version 2011.1; Illumina). The transcription starts site (TSS) and first exon (1st Exon) sites were filtered for their most significant influence on the gene. The methylation level of each CpG site was recorded as a β value, indicating the ratio of the methylated signal intensity over the sum of the methylated and unmethylated intensities at each locus. The filtered methylation sites were then mapped to their potential regulated gene defined in the UCSC Genome Browser HG19 RefSeq database.

Western blot

Modified cells were washed with ice-cold Dulbecco's PBS, and cell proteins were extracted with lysis buffer and separated in the 12% SDS-PAGE. The protein bands were transferred onto a nitrocellulose membrane followed by blocking of the membrane with Tris-buffered saline (TBS) containing 10% skim milk. The membranes were incubated with anti-BMP3 antibody (1:500 dilutions, ab134724; Abcam) or anti-GDDPH antibody (CB1001; EMD Millipore) at 1:1,000 dilutions in TBS containing 1% skim milk. Then, the membrane was washed and incubated with secondary

antibodies, anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated IgG (Santa Cruz Biotechnology Inc.) at a 1:5,000 dilution for 2 hours at room temperature. Chemiluminescent signals were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from gastric cancer cells SGC-7901 using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Wild-type oligonucleotides of the BMP3 promoter (5'-GGGTGGCTAAGGGCA-3') were purchased from Sangon Biotech, Inc. (Shanghai, China). EMSA probes were synthesized as double strands after pair annealing and 3'-end labeled with biotin (Thermo Fisher Scientific). The EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) following the manufacturer's protocol. For supershift experiments, VDR (SC-13133; Santa Cruz Biotechnology) his tag and anti-his tag (LT0424; LifeTein, South Plainfield, NJ, USA) were added to the reaction solution 30 minutes prior to the addition of the probes.

Data analysis

Statistical analyses were performed by two-tailed unpaired *t*-tests using InStat version 162 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Data in bars represent mean \pm SD in histograms. The mean and SD values are derived from at least triplicates. Differences were considered as statistically significant at $P < 0.05$. DNA methylation data were analyzing using methods we described previously²⁰ by open source of ggplots2, clusterProfiler (Gene Ontology), and Kyoto encyclopedia of genes and genomes (KEGG).

Results

Low concentration of 25(OH)D₃ refers to cancerization

Our group has previously reported that genomic (in mRNA, SNP, and transcription factor [TF] studies) and nongenomic (in G protein-coupled receptor, phosphoinositide 3-kinase, and protein kinase C analyses) effects of 25(OH)D₃ and its related genes/pathways might moderately share the molecular mechanism of occurrence, development, or recurrence of adenocarcinoma.²¹ The Roche Elecsys platform applied in retrospective studies indicated the amount of 25(OH)D₃ through recruiting over a thousand patients ($n=1,433$) from mainland China and Taiwan. 25(OH)D₃ from rickets ($n=504$; 44.9 ± 13.0 nmol/L) and the cancer ($n=352$; 55.8 ± 16.6 nmol/L)

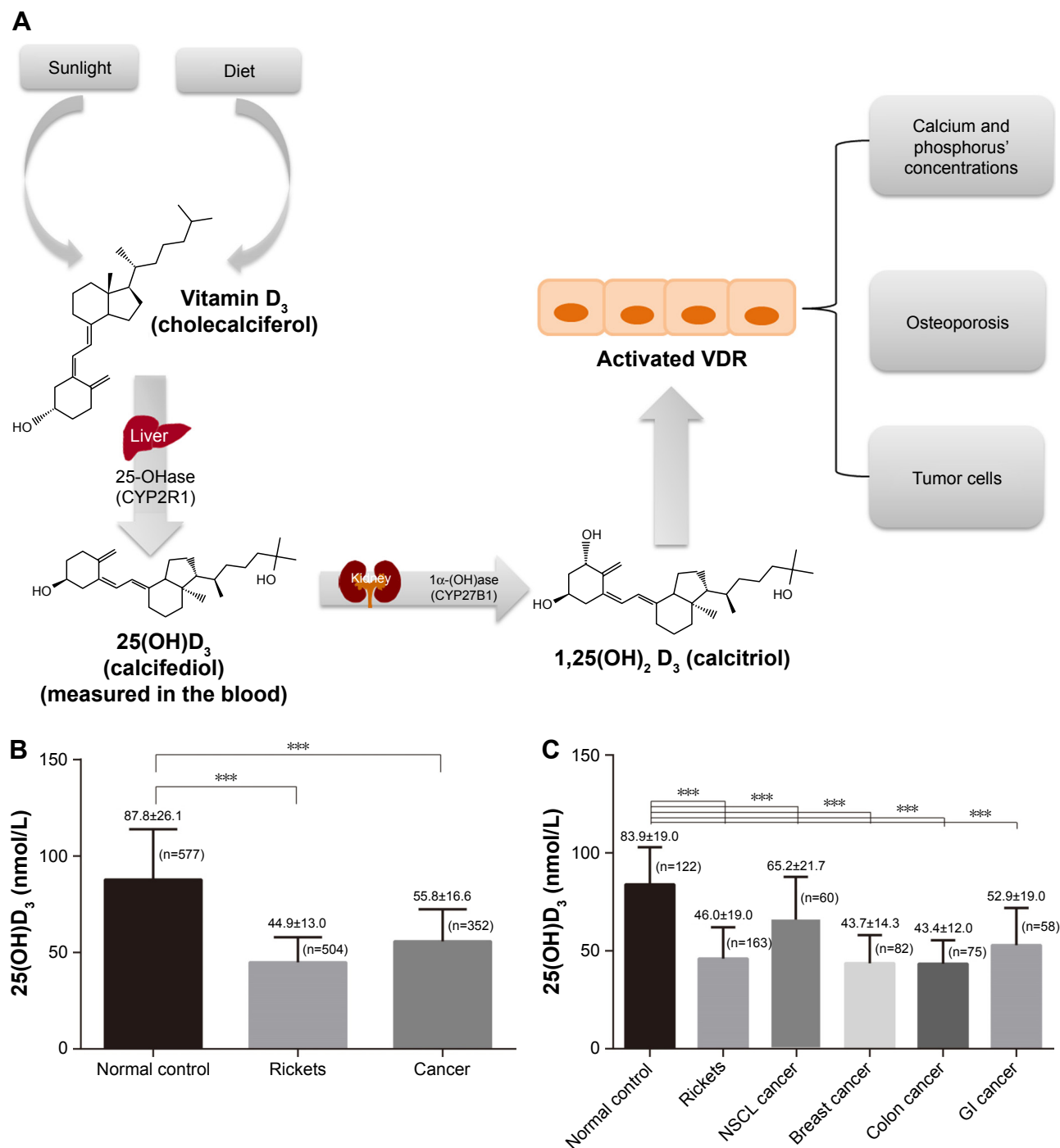


Figure 1 Concentration of 25(OH)D₃ in rickets and adenocarcinoma.

Notes: (A) Vitamin D₃ synthesis and degradation route. (B) Retrospective and (C) validated analyses of concentration of 25(OH)D₃ from rickets and adenocarcinoma in Chinese population (***) $P < 0.001$.

Abbreviations: 25(OH)D₃, 25-hydroxyvitamin D₃; 1,25(OH)₂ D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor.

patients showed much lower concentration compared to that from a healthy normal population ($n=577$; 87.8 ± 26.1 nmol/L; Figure 1B). This implies that rickets and cancerization might share the comparable pathogenesis and possess the same risk indicator for clinical diagnosis. Figure 1A shows the synthesis and degradation of vitamin D₃.

To further prove that 25(OH)D₃ could be functional as a cancer marker, we measured the concentration of 25(OH)D₃ from rickets patients ($n=163$) with 46.0 ± 16.0 nmol/L, nonadenocarcinoma healthy population ($n=122$) with 83.9 ± 19.0 nmol/L, non-small cell lung cancer patients ($n=60$) with 65.2 ± 21.7 nmol/L, breast cancer patients ($n=82$)

with 43.7 ± 14.3 nmol/L, colon cancer patients ($n=75$) with 43.4 ± 12.0 nmol/L, and gastrointestinal (GI) adenocarcinoma patients ($n=58$) with 52.9 ± 19.0 nmol/L (Figure 1C). Therefore, $25(\text{OH})\text{D}_3$ might act as a risk indicator, not only for rickets but also for adenocarcinoma.

$1,25(\text{OH})_2\text{D}_3$ inhibits the viability of gastric cancer cells

Previous studies have indicated that $1,25(\text{OH})_2\text{D}_3$ prevents cancer progression by reducing cell proliferation.²² To explore the effect of $1,25(\text{OH})_2\text{D}_3$ on the proliferation of gastric cancer cells and normal gastric cells, we conducted colony formation assay. Human gastric cancer cell lines SGC-7901 and BGC-823 were treated with $1,25(\text{OH})_2\text{D}_3$. Human gastric epithelial cell line GES-1 was used as control. Colony formation assay results (Figure 2) shows that the colony number was significantly decreased by $1,25(\text{OH})_2\text{D}_3$ in SGC-7901 and BGC-823 but not in GES-1. Thus, these results suggested that $1,25(\text{OH})_2\text{D}_3$ could exert more

selective suppressive effect in gastric cancer cells than that in normal gastric cells.

BMP3 promoter hypermethylation was highly correlated with gastric tumor

DNA methylation is crucial for regulation of gene expression and is known to be essential for the normal cellular development and maintaining of tissue characters. Tumor suppressor gene inactivation by promoter hypermethylation is thought to be important in carcinogenesis. Before further detection of mechanisms of $1,25(\text{OH})_2\text{D}_3$ on gastric tumor, we performed bisulfite genomic sequencing (BGS) for hypermethylation of BMP3 CpG island detection. Four primary gastric carcinoma specimens were collected from patients with no family history of cancer. In addition, the same number of normal tissues was also collected from patients as controls. Histology of H&E detection was performed to validate the tissues samples (Figure 3). After analyzing DNA methylation, interestingly, all four tumor

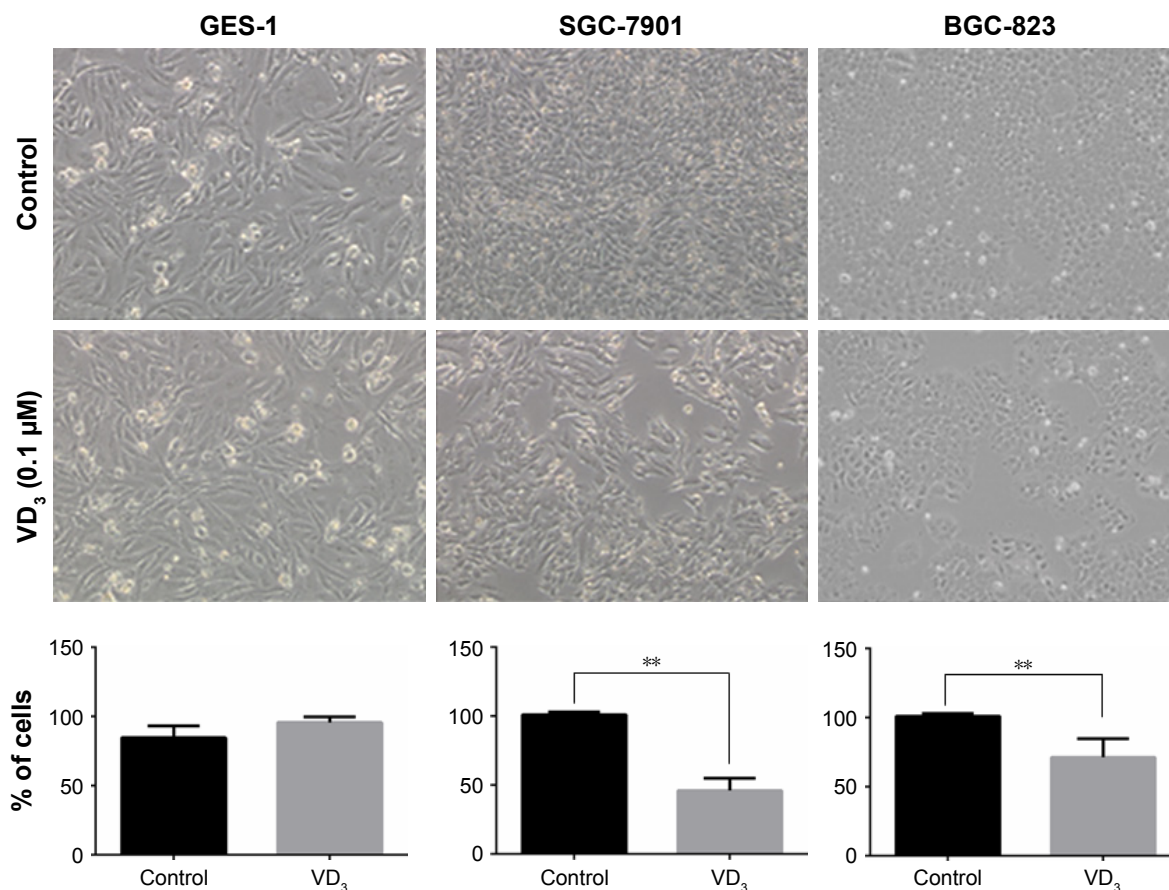


Figure 2 The antiproliferative effect of $1,25(\text{OH})_2\text{D}_3$ on gastric cells.

Notes: Cytotoxic effect of $1,25(\text{OH})_2\text{D}_3$ on GES-1, SGC-7901, and BGC-823 cells. Cells were seeded in a six-well plate at a density of 1,000 cells per well and were treated with $1,25(\text{OH})_2\text{D}_3$ for 48 hours with $1,25(\text{OH})_2\text{D}_3$ (VD₃ for short in figures) at concentrations of 0.1 μM . Colony number of each well was counted and analyzed (** $P < 0.01$).

Abbreviation: $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxyvitamin D₃.

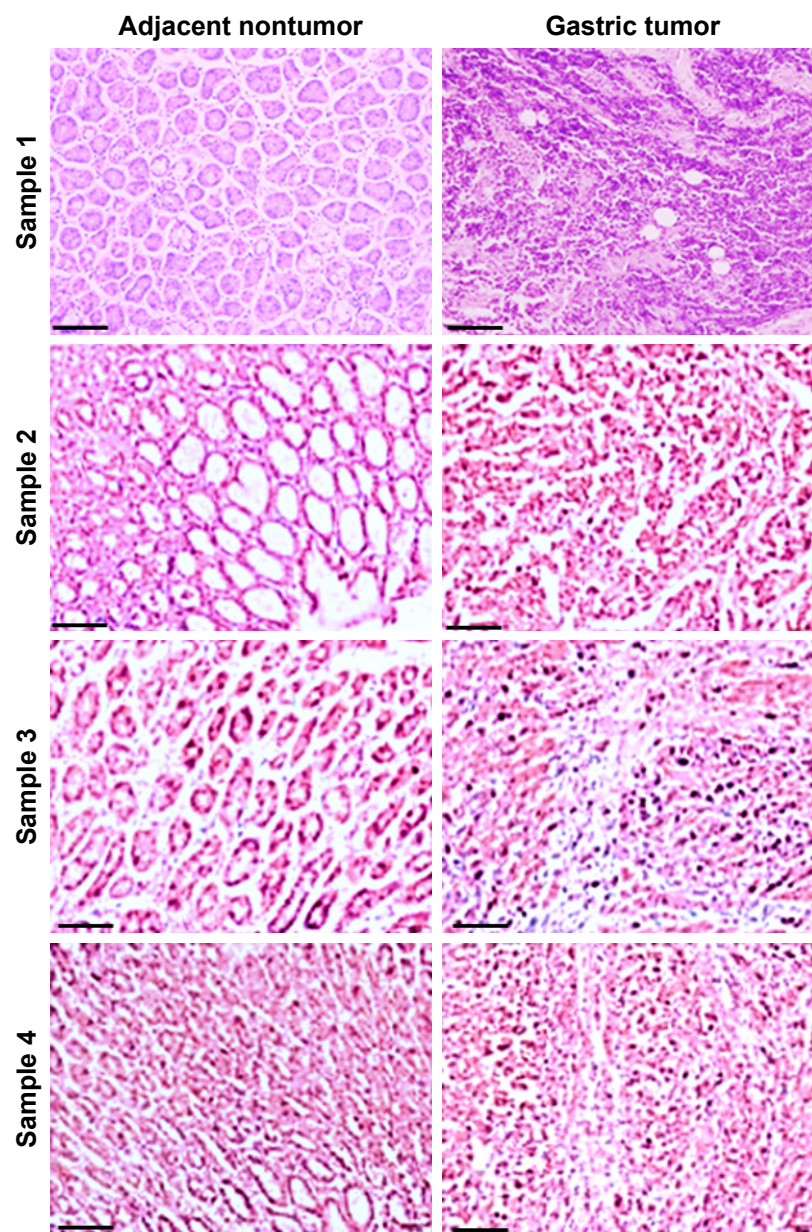


Figure 3 Histological validation of gastric specimens.

Notes: Histology of H&E detection was performed to validate the tissues samples of adjacent nontumor and gastric tumor. Scale bars=100 μ m.

samples showed hypermethylation of bone morphogenetic protein 3 (BMP3) gene promoter in gastric carcinoma tissue samples, which was absent in the normal tissues (Table 1). Additionally, BGS has detected eight hypermethylation of BMP3 CpG islands in a region around the translation start site of the BMP3 (Table 2). These results suggest that BMP3 genetic instability and promoter methylation are initiated during gastric carcinogenesis, which is consistent with the previous report.¹⁷ The detailed bioinformatics analyzing results (data not shown) will be shown in another published article.

BMP3 expression is regulated by its promoter methylation in gastric tumor cells

To understand the relationship between BMP3 expression and gastric carcinogenesis, we first investigated the protein expression of BMP3 in six gastric cancer cell lines and noncancer lines. Western blot analysis revealed that BMP3 protein levels were significantly downregulated in all gastric cancer cell lines compared with those in the GES-1 cell line (Figure 4A). We next determined the effects of BMP3 promoter region methylation in gastric cancer cells. The gastric

Table 1 Screening of genes related to methylation profile of human poorly differentiated gastric adenocarcinoma

Gene	Full name	Chromosome position
TRIM9	Triple domain protein 9	14q21.3
TENC1	Tensin with phosphatase activity (tensin 2)	12q13.13
PRDM2	Proteins 2 containing PR domain and zinc lipid structure	1p36
SEMA38	Brain signal protein 38	3p21.3
NEURL1	E3 ubiquitin protein ligase	10q25.1
GABRG3	γ -aminobutyric acid GABA receptor, γ -3	15q12
GLI3	GLI family of zinc lipoproteins	7p13
OPCML	Opioid-binding proteins/cell adhesion proteins	11q25
PCDH17	Pro-cadherin 17	13q21.1
ITGA8	Integrin α 8	10p13
LTF	Lactoferrin	3p21.31
TFF1	Clover factor	21q22.3
NR2F2	Nuclear receptor subfamily 2, group F, member 2	15q26
BLNK	B-cell junction protein	10q23.2–q23.33
ST5	Tumor suppressor gene 5	11p15
P2RY1	Purine receptor P2 γ , G protein-coupled 1	3q25.2
MED12L	Transcriptome subunit 12L	3q25.1
ACTA1	Skeletal muscle actin α 1	1q42.13
LMO4	LIM transcriptional regulator 4, only the LIM domain	1p22.3
BMP3	Bone morphogenetic protein-3	4q21
RASGRF1	Ras guanylate releasing factor 2	15q24
F2	Factor II (thrombin)	11p11.2
NPY4R	Neuropeptide γ receptor γ 4	10q11.2
PIK3R1	Inositol 3-kinase, regulatory subunit 1 (α)	5q13.1

cell line SGC-7901 was chosen and treated with the methylation inhibitor 5-aza-2' deoxycytidine. BMP3 expression was examined by Western blot. As shown in Figure 4B, treatment with gradient concentration of 5-aza-2' deoxycytidine restored BMP3 gene expression in gastric cancer cell SGC-7901 in a dose-dependent manner. This strongly supported promoter methylation as a mechanism for BMP3 silencing in SGC-7901. In all, BMP3 is specifically downregulated by hypermethylation in gastric tumor cells and may function as a tumor suppressor, supporting the hypothesis that the region targeted to methylation of the BMP3 promoter is important for BMP3 gene silencing.²³ The consistent conclusions were also reported in cholangiocarcinoma.²⁴

Table 2 BMP3 hypermethylation site

Target ID	$\Delta\beta$ value	Relative position	UCSC RefSeq CpG island
Cg01049530	0.34275	TSS200	Chr4:81951941-81952808
Cg01941671	0.29125	TSS200	Chr4:81951941-81952808
Cg02621694	0.22621	TSS1500	Chr4:81951941-81952808
Cg20631104	0.23562	TSS1500	Chr4:81951941-81952808
Cg20642710	0.27239	TSS200	Chr4:81951941-81952808
Cg22403273	0.34445	TSS200	Chr4:81951941-81952808
Cg26105156	0.20048	TSS200	Chr4:81951941-81952808
Cg26917673	0.30891	TSS200	Chr4:81951941-81952808

Note: $\Delta\beta$ value indicates the ratio of the methylated signal intensity over the sum of the methylated and unmethylated intensities at each locus.

Abbreviation: TSS, transcription start site.

1,25(OH)₂ D₃ stimulates BMP3 expression in gastric cells

As mentioned earlier, we have found that 25(OH)D₃ might act as a risk indicator in GI cancer. Additionally, 1,25(OH)₂ D₃ exerts selectively suppressive effects in gastric cancer cells. As BMP3 is significantly downregulated and mediated by methylation in gastric cancer cells, we wanted to investigate if 1,25(OH)₂ D₃ has a relation to BMP3 methylation. Three gastric cancer cell lines (BGC-823, SGC-7901, and MKN-28) and GES-1 were selected for 1,25(OH)₂ D₃ treatment. Interestingly, Figure 4C shows that 1,25(OH)₂ D₃ (0.1 μ M) stimulates BMP3 expression in gastric cancer cells BGC-823, SGC-7901, and MKN-28 significantly ($P < 0.05$), suggesting a strong association between BMP3 expression and 1,25(OH)₂ D₃.

Binding capacity of transcription factor VDR to the CpG methylation site of BMP3 promoter

Previous studies demonstrated that the antitumor effect of 1,25(OH)₂ D₃ was mediated by VDR, and VDR is overexpressed in human gastric cancer cells.⁸ Simultaneously, it concluded that the tissue level of VDR was the highest in the precancer stage (intestinal metaplasia) than in the cancerous stage.⁸ This important finding implies that 1,25(OH)₂ D₃ may display a more potent pharmacological action on gastric cancer cells than on normal cells. It is well recognized that VDRE is a DNA sequence that is found in the promoter region of vitamin D-regulated genes. VDR binds to VDRE and regulates the expression of some genes. To further understand better the role of 1,25(OH)₂ D₃ in BMP3 expression, gel EMSA was performed to determine whether the methylation of the CpG sites of the

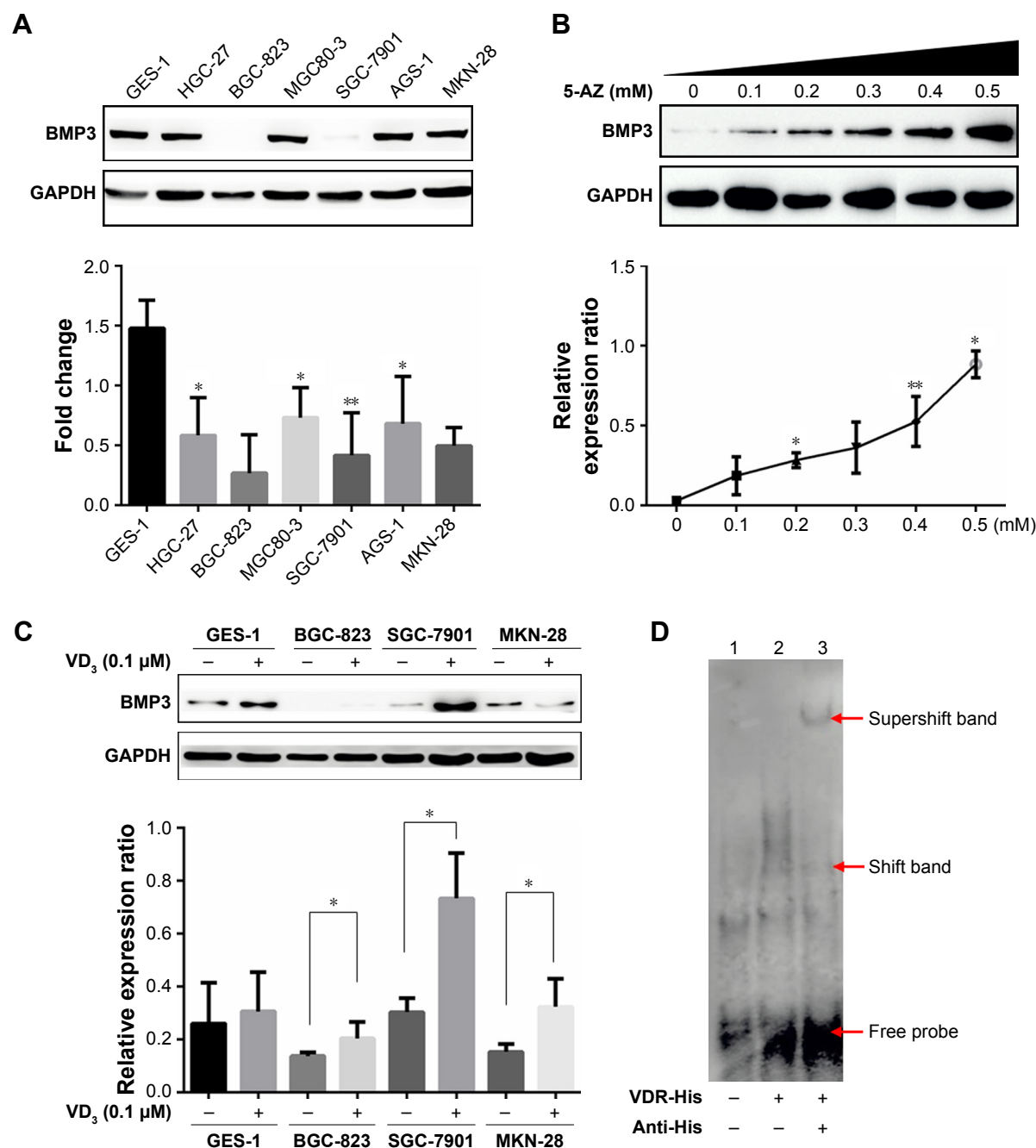


Figure 4 I,25(OH)₂ D₃ inhibits BMP3 promoter methylation required with VDR influencing.

Notes: (A) BMP3 expression in gastric noncancer and cancer cells lines was detected by Western blot. (B) BMP3 expression of gastric cancer cell line SGC-7901 was detected by Western blot after treating with gradient concentration of 5-AZ (0, 0.1, 0.2, 0.3, 0.4, 0.5 mM). (C) BMP3 expression of three gastric cancer cell lines (BGC-823, SGC-7901, and MKN-28) and normal line GES-1 was detected by Western blot with or without I,25(OH)₂ D₃ (0.1 μM) treatment. Data are represented as mean±SD (*P<0.05 and **P<0.01). (D) Gel EMSA was performed using the nuclear extract from SGC-7901 cells. A labeled wild-type probe containing the VDR consensus binding sequence (5'-GGGTGGCTAAGGGCA-3') of the BMP3 promoter was used to test VDR binding. The supershift band and bindshift band are indicated by arrows.

Abbreviations: I,25(OH)₂ D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; 5-AZ, 5-aza-2' deoxycytidine; EMSA, electrophoretic mobility shift assay.

BMP3 promoter influences the binding of transcription factor VDR to its consensus binding sequence. As shown is Figure 4D, a labeled wild-type probe containing the VDR consensus binding sequence (GGGTGGCTAAGGGCA) of the BMP3 promoter was used to test VDR binding. The incubation of nuclear extracts with a wild-type VDR

probe revealed a pattern of shifted bands representing VDR binding activity. The nuclear extracts containing BMP3 promoter region were able to bind the wild-type VDR probe (lane 3). This result suggests that the methylation sites of the BMP3 promoter have the binding site for transcription factor VDR.

Discussion

Vitamin D₃ has recently received enormous attention for a marked rise in the number of scientific and lay press articles suggesting that it might have a crucial role in the prevention of cancer, as well as in a multitude of non-skeletal and skeletal diseases.⁷ Our study also demonstrates that a low concentration of 25-hydroxyl vitamin D₃ relates to cancerization and might act as a risk indicator for adenocarcinoma. Focusing on gastric cancer, we further conclude that 1,25(OH)₂ D₃ exerts more selective suppressive effect in gastric cancer cells than in normal gastric cells. For the mechanisms of the antitumor function of 1,25(OH)₂ D₃, it has been reported that 1,25(OH)₂ D₃ plays a similar role to antiangiogenesis by the inhibition of HIF pathway.⁶ It has also been described that vitamin D₃ decreases cell viability by the inhibition of hedgehog signaling in gastric cancer cells.²² However, the target and the signals associated still need to be further explored.

Recently, epigenetic regulation of gene expression has emerged as a potentially important factor in tissue differences within a single disease since the epigenetic modifications can change important associated regulatory mechanisms for

pathogenic malignant transformation. A previous study has described that aberrant BMP3 promoter hypermethylation is highly tumor specific ($P < 0.001$) and the majority of methylated cancers (28/33, 85%) show significant downregulation of BMP3 expression.²⁵ In the present study, by BGS for hypermethylation detection of gastric tumor specimens, the methylation level in the promoter region of BMP3 gene in gastric carcinomas was found to be high, which is consistent with the findings that of BMP3 promoter methylation is associated with colon cancer development.²⁵

Bone morphogenetic proteins (BMPs) are an integral component of the transforming growth factor- β (TGF- β) superfamily with well-described functions in bone formation and are responsible for regulation of cell proliferation, differentiation, migration, and programmed cell death in a variety of cell types. Inactivation of the BMP pathway genes has been implicated as important in several cancers.²⁶ Although the role of BMP3 in the pathogenesis of cancer remains unclear, recent studies have revealed that BMP3 contributes to tumor development and progression.^{17,25} It has been shown that BMP3b is epigenetically inactivated in cancer, and aberrant promoter hypermethylation has been reported as a mechanism

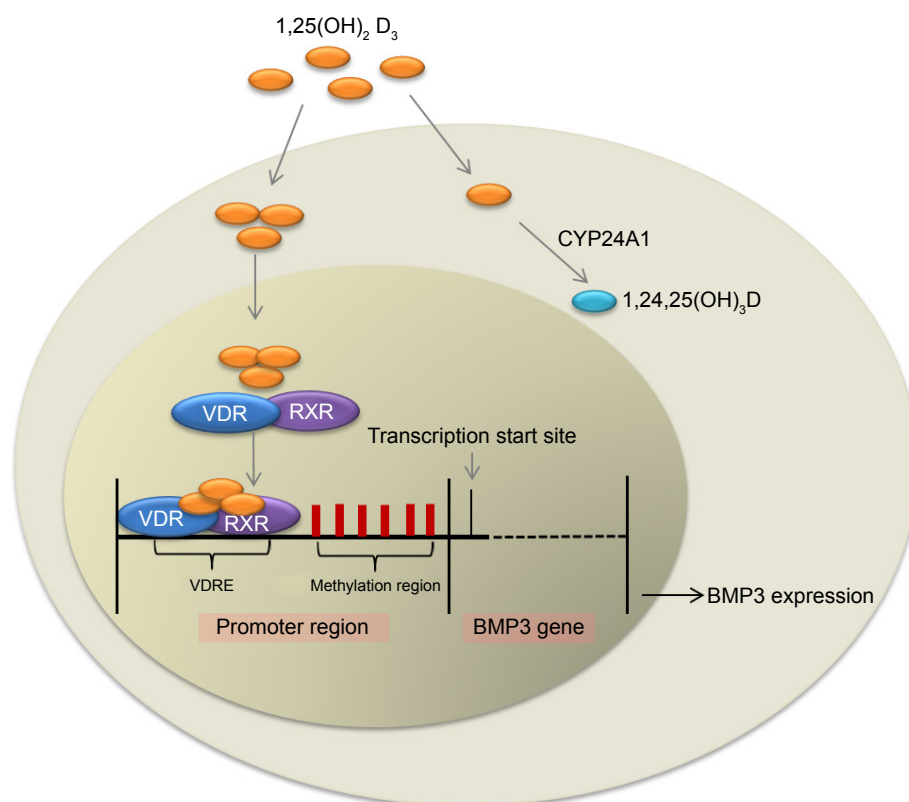


Figure 5 Regulatory effects of 1,25(OH)₂ D₃ on BMP3 promoter methylation.

Note: In gastric cells, 1,25(OH)₂ D₃ binds to VDR and forms heterodimeric complexes, which mediate the inhibition of BMP3 promoter methylation and regulate cell activations.

Abbreviations: 1,25(OH)₂ D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; 5-AZ, 5-aza-2'-deoxycytidine; VDRE, vitamin D response element; RXR, retinoid X receptor.

of downregulated expression for some family members including BMP3b in lung cancer,^{27,28} colorectal cancer,²⁵ biliary cancer,²³ pancreatic cancer,²⁹ and breast cancer.³⁰

Our study demonstrated that CpG sites in the CpG island of BMP3 promoter region were hypermethylated in gastric cancer. The sequence around the CpG site, which displays the most significant differences in methylation between cancer cases and controls, is analyzed for transcription factor-binding prediction via Transcription Element Search System (TESS) website. Several transcription factor-binding sites were predicted in the sequence (data not shown). The most frequent predicted transcription factor was VDR. VDR might be involved in the complex formation and plays a role in transcriptional regulation. We hypothesized that the BMP3 promoter region may form a DNA-binding complex mediated by the VDRE sequence. To test this hypothesis, we performed EMSAs using a polyclonal antiserum against BMP3 and VDR. We found that anti-VDR antibody could generate a supershift, suggesting that VDR participates in the formation of a BMP3 promoter complex. Activation of the VDR through direct interaction with 1,25(OH)₂ D₃ prompts the receptor's rapid binding to regulatory regions of BMP3, where it acts to nucleate the formation of large protein complexes whose functional activities are essential for directed changes in BMP3 transcription. It is also consistent with the conclusion of low-expressed VDR in gastric cancer tissues.¹⁴

The heterodimers of the VDR and the RXR have been identified as the active complex for mediating positive transcriptional effects of 1,25(OH)₂ D₃.⁴ The VDR–RXR heterodimer binds to direct repeat VDREs, located near promoters, and perform the additional functions required to modify transcriptional output.^{8,31} It is inferred in this research that VD₃-inducible heterodimeric complexes, composed of the VDR and the RXR-α, interact with the BMP3–VDREs, which mediate the inhibition of BMP3 promoter methylation (Figure 5).

Conclusion

Gastric cancer remains as one of the most fatal cancers and the second leading cause of cancer-related death worldwide.³² Thus, it is clinically significant to identify and develop novel therapeutic ways for the treatment or, even more importantly, in the prevention of gastric cancer. In combination with the data from clinical research, bioinformatics analysis, and experimental verification, we propose that 1,25(OH)₂ D₃ affects gastric cancer progression by repressing BMP3 promoter methylation. This conclusion provides a novel idea for

the 1,25(OH)₂ D₃ antitumor mechanism study and suggests that BMP3 methylation may be a new biomarker for early detection of gastric cancer.

Acknowledgment

This work was supported by grants from the National Natural Science Foundation of China (no. 31701279) and Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (no. XTE1845).

Disclosure

The authors report no conflicts of interest in this work.

References

- Meyer MB, Benkuskus NA, Kaufmann M, et al. A kidney-specific genetic control module in mice governs endocrine regulation of the cytochrome P450 gene Cyp27b1 essential for vitamin D₃ activation. *J Biol Chem*. 2017;292(42):17541–17558. doi:10.1074/jbc.M117.806901
- Gibbs DC, Fedirko V, Um C, Gross MD, Thyagarajan B, Bostick RM. Associations of circulating 25-hydroxyvitamin D₃ concentrations with incident, sporadic colorectal adenoma risk according to common vitamin D-binding protein isoforms. *Am J Epidemiol*. 2018;187(9):1923–1930. doi:10.1093/aje/kwy102
- Haussler MR, Whitfield GK, Haussler CA, et al. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res*. 1998;13(3):325–349. doi:10.1359/jbmr.1998.13.3.325
- Kim S, Shevde NK, Pike JW. 1,25-Dihydroxyvitamin D₃ stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J Bone Miner Res*. 2005;20(2):305–317. doi:10.1359/JBMR.041112
- Kong F, Li L, Wang G, Deng X, Li Z. VDR signaling inhibits cancer-associated-fibroblasts' release of exosomal miR-10a-5p and limits their supportive effects on pancreatic cancer cells. *Gut*. 2018. doi:10.1136/gutjnl-2018-316627
- Ben-Shoshan M, Amir S, Dang DT, Dang LH, Weisman Y, Majeesh NJ. 1α,25-dihydroxyvitamin D₃ (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells. *Mol Cancer Ther*. 2007;6(4):1433–1439. doi:10.1158/1535-7163.MCT-06-0677
- Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer*. 2014;14(5):342–357. doi:10.1038/nrc3691
- Li M, Li L, Zhang L, et al. 1,25-Dihydroxyvitamin D₃ suppresses gastric cancer cell growth through VDR- and mutant p53-mediated induction of p21. *Life Sci*. 2017;179:88–97. doi:10.1016/j.lfs.2017.04.021
- Salehi-Tabar R, Nguyen-Yamamoto L, Tavera-Mendoza LE, et al. Vitamin D receptor as a master regulator of the c-MYC/MXD1 network. *Proc Natl Acad Sci U S A*. 2012;109(46):18827–18832. doi:10.1073/pnas.1210037109
- DeSmet ML, Fleet JC. Constitutively active RAS signaling reduces 1,25 dihydroxyvitamin D-mediated gene transcription in intestinal epithelial cells by reducing vitamin D receptor expression. *J Steroid Biochem Mol Biol*. 2017;173:194–201. doi:10.1016/j.jsbmb.2017.01.008
- Kuittinen T, Rovio P, Staff S, et al. Paclitaxel, carboplatin and 1,25-D₃ inhibit proliferation of endometrial cancer cells in vitro. *Anticancer Res*. 2017;37(12):6575–6581. doi:10.21873/anticancer.12114
- Segovia-Mendoza M, Diaz L, Gonzalez-Gonzalez ME, et al. Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells. *J Steroid Biochem Mol Biol*. 2015;148:122–131. doi:10.1016/j.jsbmb.2014.12.006

13. Ren C, Qiu MZ, Wang DS, et al. Prognostic effects of 25-hydroxyvitamin D levels in gastric cancer. *J Transl Med*. 2012;10:16. doi:10.1186/1479-5876-10-233
14. Wen Y, Da M, Zhang Y, Peng L, Yao J, Duan Y. Alterations in vitamin D signaling pathway in gastric cancer progression: a study of vitamin D receptor expression in human normal, premalignant, and malignant gastric tissue. *Int J Clin Exp Pathol*. 2015;8(10):13176–13184.
15. Khayatizadeh S, Feizi A, Saneei P, Esmailzadeh A. Vitamin D intake, serum vitamin D levels, and risk of gastric cancer: a systematic review and meta-analysis. *J Res Medl Sci*. 2015;20(8):790–796. doi:10.4103/1735-1995.168404
16. Diez-Perez R, Campo-Trapero J, Cano-Sanchez J, et al. Methylation in oral cancer and pre-cancerous lesions (review). *Oncol Rep*. 2011;25(5):1203–1209. doi:10.3892/or.2011.1205
17. Chen XR, Wang JW, Li X, Zhang H, Ye ZY. Role of BMP3 in progression of gastric carcinoma in Chinese people. *World J Gastroenterol*. 2010;16(11):1409–1413.
18. Liu Z, Zhang J, Gao Y, et al. Large-scale characterization of DNA methylation changes in human gastric carcinomas with and without metastasis. *Clin Cancer Res*. 2014;20(17):4598–4612. doi:10.1158/1078-0432.CCR-13-3380
19. Li Z, Chen S, Yang Y, Zhuang X, Tzeng CM. Novel biomarker ZCCHC13 revealed by integrating DNA methylation and mRNA expression data in non-obstructive azoospermia. *Cell Death Discovery*. 2018;4:36. doi:10.1038/s41420-018-0033-x
20. Cai LL, Liu GY, Tzeng CM. Genome-wide DNA methylation profiling and its involved molecular pathways from one individual with thyroid malignant/benign tumor and hyperplasia: a case report. *Medicine*. 2016;95(35):e4695. doi:10.1097/MD.0000000000004864
21. Cai LL, Ye HM, Lv XN, et al. 25-(OH)VitD3, as a risk indicator in diagnosis of adenocarcinoma. *Curr Drug Targets*. 2013;14(11):1367–1376.
22. Hargrove L, Francis T, Francis H. Vitamin D and GI cancers: shedding some light on dark diseases. *Ann Transl Med*. 2014;2(1):9.
23. Kisiel JB, Li J, Zou H, et al. Methylated Bone Morphogenetic Protein 3 (BMP3) gene: evaluation of tumor suppressor function and biomarker potential in biliary cancer. *J Mol Biomark Diagn*. 2013;4(145):1000145.
24. Shire A, Zou H, Aderca I, et al. BMP3 is hypermethylated and may function as a tumor suppressor in cholangiocarcinoma. *Cancer Res*. 2008;68(9):4282.
25. Loh K, Chia JA, Greco S, et al. Bone morphogenetic protein 3 inactivation is an early and frequent event in colorectal cancer development. *Genes Chromosomes Cancer*. 2008;47(6):449–460. doi:10.1002/gcc.20552
26. Zhang L, Ye Y, Long X, Xiao P, Ren X, Yu J. BMP signaling and its paradoxical effects in tumorigenesis and dissemination. *Oncotarget*. 2016;7(47):78206–78218. doi:10.18632/oncotarget.12151
27. Kraunz KS, Nelson HH, Liu M, Wiencke JK, Kelsey KT. Interaction between the bone morphogenetic proteins and Ras/MAP-kinase signalling pathways in lung cancer. *Br J Cancer*. 2005;93(8):949–952. doi:10.1038/sj.bjc.6602790
28. Dai Z, Popkie AP, Zhu WG, et al. Bone morphogenetic protein 3B silencing in non-small-cell lung cancer. *Oncogene*. 2004;23(20):3521–3529. doi:10.1038/sj.onc.1207441
29. Kisiel JB, Yab TC, Taylor WR, et al. Stool DNA testing for the detection of pancreatic cancer: assessment of methylation marker candidates. *Cancer*. 2012;118(10):2623–2631. doi:10.1002/cncr.26558
30. Ronneberg JA, Fleischer T, Solvang HK, et al. Methylation profiling with a panel of cancer related genes: association with estrogen receptor, TP53 mutation status and expression subtypes in sporadic breast cancer. *Mol Oncol*. 2011;5(1):61–76. doi:10.1016/j.molonc.2010.11.004
31. Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D3. *Rheum Dis Clin North Am*. 2012;38(1):13–27. doi:10.1016/j.rdc.2012.03.004
32. Peng P, Wu W, Zhao J, et al. Decreased expression of Calpain-9 predicts unfavorable prognosis in patients with gastric cancer. *Sci Rep*. 2016;6:29604. doi:10.1038/srep29604

OncoTargets and Therapy

Publish your work in this journal

OncoTargets and Therapy is an international, peer-reviewed, open access journal focusing on the pathological basis of all cancers, potential targets for therapy and treatment protocols employed to improve the management of cancer patients. The journal also focuses on the impact of management programs and new therapeutic agents and protocols on

Submit your manuscript here: <http://www.dovepress.com/oncotargets-and-therapy-journal>

patient perspectives such as quality of life, adherence and satisfaction. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Dovepress