

EZH2 regulates sFRP4 expression without affecting the methylation of sFRP4 promoter DNA in colorectal cancer cell lines

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Abstract. Abnormal activation of the Wnt signaling pathway is found in 90% of colorectal cancers (CRCs). Secreted frizzled-related protein 4 (sFRP4) serves as an antagonist of the canonical Wnt signaling pathway. Epigenetic alterations, including changes in DNA methylation and histone methylation, may influence the expression of sFRP4. Polycomb group (PcG) proteins are epigenetic transcriptional repressors that selectively repress gene expression by forming polycomb repressive complexes (PRCs). Enhancer of zeste homolog 2 (EZH2), the core component of PRC2, is a histone-lysine N-methyltransferase that interacts with DNA methyltransferases. In the present study, the promoter DNA methylation status of sFRP4 in CRC cell lines was analyzed and the underlying mechanisms of action governing this modification was investigated. Firstly, the DNA methylation status of the sFRP4 promoter in CRC cell lines was assessed using methylation-specific PCR. Subsequently, the mRNA and protein levels of sFRP4 were measured using real-time qPCR and western blot analysis, respectively, to determine whether the DNA methylation status of the sFRP4 promoter is correlated with its transcriptional levels. To screen for important epigenetic modifiers that may regulate the promoter DNA methylation status of sFRP4, the expression levels of PcG proteins were examined by gene array analysis. ChIP-qPCR was performed

to test whether the selected PcG proteins directly bind the promoter region of sFRP4. Finally, the downregulated PcG proteins EZH2, chromobox 7 (CBX7) and jumonji and AT-rich interaction domain containing 2 (JARID2) were identified and their association with sFRP4 expression levels and Wnt/ β -catenin signaling pathway activity were investigated. The present study revealed that sFRP4 was hypermethylated in the promoter region and downregulated during the progression of the CRC cell lines from Dukes A to Dukes C. Expression levels of PcG proteins EZH2, CBX7 and JARID2 were upregulated and positively associated with the aberrantly activated Wnt signaling pathway in the CRC cell lines. EZH2, CBX7 and JARID2 were all enriched in the sFRP4 promoter region in CRC cells. EZH2 downregulation did not affect the promoter DNA methylation status of sFRP4 but increased its expression levels and decreased CRC cell proliferation. DNA methylation controls the expression of sFRP4. EZH2 regulates sFRP4 expression without affecting the DNA hypermethylation of the sFRP4 promoter and influences CRC cell proliferation and Wnt/ β -catenin signaling pathway activities.

Introduction

Colorectal carcinoma is one of the major malignancies that has a serious impact on human morbidity and mortality (1). The Wnt signaling pathway regulates a number of biological processes, ranging from cell fate decision, cell differentiation, embryonic development and carcinogenesis (2). Abnormal activation of the Wnt signaling pathway has been identified in numerous types of solid tumors, particularly in colorectal cancer (CRC) (3), and dysregulation of the Wnt signaling pathway has been found in 90% of CRC case (4,5). Activation of the Wnt signaling pathway by both genetic and epigenetic mechanisms is important for both the initiation and progression of CRC. The Wnt/ β -catenin signaling pathway results in diverse downstream intracellular events, such as driving the transcription of target genes, including c-myc, cyclin D and surviving (2). The targeted inhibition of this pathway at its early stages is a reasonable and promising strategy for the development of CRC therapies (2).

Secreted frizzled-related proteins (sFRPs) are a family of secreted proteins (sFRP1-5) and are inhibitors of the Wnt signaling pathway. Several members, including sFRP1, sFRP2 and sFRP4, possess a conserved frizzled (Fzd) receptors

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Abbreviations: CBX7, chromobox 7; CCC-HIE-2, human embryo intestinal mucosa cell; CHIP, chromatin immunoprecipitation; CRC, colorectal cancer; DNMT, DNA methyltransferase; DAC, Decitabine; EZH2, enhancer of zeste homolog 2; CBX7, The Chromobox protein homolog 7; BMI1, B-lymphoma Mo-MLV insertion region 1; Fzd, frizzled receptor; JARID2, jumonji and AT-rich interaction domain containing 2; PcG, polycomb group; sFRP, secreted frizzled-related protein; HDAC, Histone deacetylase

Key words: colorectal cancer, sFRP4, epigenetics, polycomb group protein, EZH2

type cysteine-rich domain that binds Wnt and typically antagonizes Wnt signaling, presumably by preventing Wnt/Fzd interactions (6,7). Thus, sFRPs, excluding sFRP3, function as antagonists of Wnt signaling by competing with Wnt proteins via binding their receptor, Frizzled. Moreover, sFRPs attenuate Wnt signaling even in the presence of downstream gene mutations (8,9). Therefore, silencing sFRP genes may be essential for the aberrant activation of the Wnt pathway in colorectal tumorigenesis.

sFRP4, a protein with 346 amino acids, is the largest member of the sFRP family and is expressed in various tissue types, including endometrial stroma pancreas, stomach, colon, lung, skeletal muscle, testis, ovary, kidney, heart, brain, mammary gland, cervix, eye, bone, prostate and liver (10). Previous studies have suggested that sFRP4 may serve a role as a tumor suppressor and function as a modulator of cell proliferation in ovarian, prostate and breast cancer (10,11). A previous study found that sFRP4 is downregulated in 26.4% of colorectal carcinoma samples ($P=0.023$) and in 9.1% of colorectal adenoma tissue ($P=0.438$) (12). The downregulation of sFRP4 is more frequent in carcinomas than in adenomas, and sFRP4 was hypermethylated in 36.1% (26/72) of colorectal carcinomas; 24.2% (8/33) of colorectal adenomas and 2.6% (1/38) of adjacent normal mucosae (10,12). The expression levels and the underlying mechanisms of action behind the epigenetic regulation of sFRP4 in CRC remain to be elucidated.

DNA methylation is one of the most well understood epigenetic regulation mechanisms. Gene silencing by promoter DNA hypermethylation is an important characteristic of colorectal tumors (13). Aberrant hypermethylation of the CpG islands in the gene promoter regions has been found to be a primary mechanism of action behind the inactivation of several tumor suppressor genes (14). DNA methylation is highly stable across cell generations and promoter DNA hypermethylation can silence gene expression without affecting the DNA sequences (15). DNA methylation is catalyzed by DNA methyltransferases, including DNMT1, DNMT3A and DNMT3B (16). DNMT3A and DNMT3B are *de novo* DNA methyltransferases and add methyl groups to the unmethylated CpG sites, while DNMT1 is a DNA methyltransferase that converts the hemi-methylated DNA into fully methylated DNA during cell mitosis (15).

Polycomb group (PcG) proteins are a group of proteins that were first discovered in fruit flies, that can remodel chromatin, such that epigenetic silencing of genes takes place. Direct interactions between PcG proteins and the DNA methylation machinery have been reported, and co-occupations were further supported by chromatin immunoprecipitation (ChIP) experiments in cancer cells (17,18). The aim of the present study was to analyze the epigenetic regulation mechanisms of action for sFRP4, to determine whether a specific PcG protein could help to establish the promoter DNA methylation status of sFRP4, and finally to investigate approaches to alter the expression levels of sFRP4.

Materials and methods

Cell culture. CRC cell lines SW1116 (Dukes A) (19), SW480 (Dukes B) and HCT116 (Dukes C) were readily available in the present laboratory (Gastroenterology Laboratory in

Zhongnan Hospital of Wuhan University, purchased from Wuhan University, Wuhan, China). The human embryo intestinal mucosa cell line CCC-HIE-2 was purchased from the China Infrastructure of Cell Line Resources. CRC cells were cultured in RPMI (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) and 1% penicillin/streptomycin (HyClone; GE Healthcare Life Sciences). CCC-HIE-2 cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 20% FBS, 0.01 mg/ml insulin (Shanghai No. 1 Biochemical Pharmaceutical Co., Ltd.), 10 ng/ml epidermal growth factor (EGF) and 1% penicillin/streptomycin. All cells were incubated at 37°C in the presence of 5% CO₂.

Methylation-specific PCR. The DNA methylation levels of the sFRP4 promoter were assessed using methylation-specific PCR in several CRC cell lines that represent different stages of CRC progression, according to the Dukes classification: SW1116 (Dukes A), SW480 (Dukes B), HCT116 (Dukes C) cells and the normal control cells CCC-HIE-2. DNA was extracted from SW1116, SW480, HCT116 and CCC-HIE-2 cells using the TIANamp Genomic DNA kit (Tiagen Biotech Co., Ltd.) according to the manufacturer's instructions. Subsequently, the DNA was treated with sodium bisulfite from the EZ Methylation-Gold™ kit (Zymo Research Corp.) according to the manufacturer's instructions. After bisulfite conversion, hot start DNA polymerase (GoldStar® Taq DNA Polymerase; CWBio) was used to amplify the converted DNA at the following thermocycling conditions: 30 cycles of 94°C for 60 sec, 37°C for 60 sec and 72°C for 2 min. A total of 10 µl PCR products were resolved and analyzed using electrophoresis on a 3% agarose gel. The primers and their annealing temperatures for the methylated and unmethylated sequences are summarized in Table I. sFRP1 is widely reported as frequently methylated and silenced in CRC (20-22); therefore, this was used as the methylation positive control in the present study.

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from SW1116, SW480, HCT116 and CCC-HIE-2 cells using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The PrimeScript RT reagent kit (Takara Bio, Inc.) was used for first-strand cDNA synthesis. For qPCR, the primers and annealing temperature used for sFRP4 are listed in Table I. GAPDH was used as an internal control. CFX Manager™ 3.0 software (Bio-Rad Laboratories, Inc.) was used to analyze the results. Each 25 µl total reaction volume contained 12.5 µl SYBR Green (PrimeScript RT reagent kit; Takara Bio, Inc.), 5 µl cDNA, 0.8 µl primer and 6.7 µl water. The thermal cycling conditions used were an initial step at 95°C for 30 sec followed by 40 cycles at 95°C for 5 sec and 60°C for 35 sec. Melting curves were presented as single curves, ensuring that the reaction products were single, specific products. Each cDNA sample was analyzed in triplicate. Each reaction was carried out in duplicate, and the 2^{-ΔΔC_q} method was employed to calculate the relative expression levels (23).

Western blot analysis. When we performing this experiment, we chose HCT116 and SW480 as represent for CRC

Table I. Primer sequences for methylation specific PCR and RT-qPCR.

sFRP4 primers	Primer sequence (5'→3')	Product length, base pairs	Annealing temperature, °C
Unmethylated	F: GGGGGTGTATGTTATTGTTTTGTATTGAT R: CACCTCCCCTAACATAAACTCAAAACA	115	54
Methylated	F: GGGTGTATGTTATCGTTTTGTATCGAC R: CCTCCCCTAACGTAACTCGAAACG	111	60
RT-qPCR	F: TGGCAACGTATCTCAGCAAA R: GGATGGGTGATGAGGACTTG	132	60
GAPDH	F: CAGCCTCAAGATCATCAGCA- R: TGTGGTCATGAGTCCTTCCA	106	60
EZH2	F: AATCAGAGTACATGCGACTGAGA R: GCTGTATCCTTCGCTGTTTCC	141	60

F, forward; R, reverse; RT-qPCR, reverse transcription-quantitative PCR; sFRP, secreted frizzle-related protein.

cell lines. The colorectal cell lines SW480 and HCT116 were lysed in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF, 1 mM Na₃VO₄ and 0.1% protease inhibitor cocktail (cat. no. P8340; Sigma-Aldrich; Merck KGaA)]. Protein concentrations of cell lysates were measured using the DC protein assay kit (Bio-Rad Laboratories, Inc.), with 10 µl (50 µg) of the protein samples for each experiment separated using 12% SDS-PAGE and transferred on to an Immobilon P PVDF membrane (EMD Millipore). The membranes were blocked with 5% milk for 2 h at room temperature. Membranes were then incubated with anti-SFRP4 (1:1,000; cat. no. A4189; ABclonal Biotech Co., Ltd.) and anti-GAPDH (1:10,000; cat. no. AC036; ABclonal Biotech Co., Ltd.) primary antibodies were added, and gently agitated for 1 h at room temperature. Subsequently, membranes were incubated with horseradish peroxidase-labeled anti-rabbit secondary antibody (1:3,000; cat. no. AS014; ABclonal Biotech Co., Ltd.) and gently agitated for 30 min at room temperature. ECL were used to visualize protein signals. Where indicated, the signals were semi-quantified using ImageJ (v1.8.0.112; National Institutes for Health).

Gene array. Illumina® Whole-Genome Gene Expression Bead Chip (Illumina, Inc.), consisting of 47,322 probes, was used to evaluate genes that were differentially expressed. The whole hybridization procedure was performed following the Illumina® protocol. RNA was extracted from SW1116, SW480, HCT116 and CCC-HIE-2 cells, using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. An Illumina® TotalPrepRNA amplification kit was used to synthesize cDNA following the manufacturer's instructions, and reverse transcribed to synthesize first strand cDNA at 42°C for 2 h and stored at 4°C. Second strand cDNA was synthesized at 16°C for 2 h and stored at 4°C. cDNA products were then purified using the Illumina TotalPrep RNA Amplification kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 15 µg of each biotinylated cDNA preparation was fragmented and placed in a hybridization mixture. Samples were then hybridized onto a Sentrix BeadChip (Human WG-6 gene expression) Illumina, Inc.) at 58°C for 16 h, according to the

manufacturer's protocols. Microarray scanned images were obtained using an Illumina BeadChip Reader, using the default settings. Images were analyzed using Illumina Bead Station 500 system (Illumina, Inc.). Comparisons were made between CCC-HIE-2 samples and the SW1116, SW480 and HCT116 samples, using CCC-HIE-2 as the baseline. The fold-change values, indicating the relative change in the expression levels between CCC-HIE-2 samples and the SW1116, SW480 and HCT116 samples, were used to identify genes that were differentially expressed. To generate a heat map for the related PcG protein and Wnt signaling genes, the means of the normalized values for each protein were subjected to conditional formatting. The highest value was assigned a red color, middle values a yellow color and the lowest values a green color.

ChIP. ChIP was performed using the Magna ChIP™ A/G One-Day Chromatin immunoprecipitation kit (cat. no. 17-10085; EMD Millipore) according to the manufacturer's instructions, using 1% formaldehyde at 37°C for 10 min and neutralizing with glycine for 5 min at room temperature to achieve crosslinking of the chromatin. All colorectal cell lines (CCC-HIE-2, SW1116, SW480 and HCT116) were washed with cold 1 ml PBS + protease inhibitors (1 mM PMSF, 1 mg aprotinin and 1 mg pepstatin A). Cells were centrifuged at 4°C at 716 x g for 5 min. SDS Lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.0) was then used to disrupt the cells, which were subsequently sonicated at 150 Hz, sheared with 4 sets of 10 sec pulses on wet ice using a high intensity ultrasonic processor (Cole-Parmer). An equal amount of chromatin was immunoprecipitated at 4°C overnight. ChIP was performed using antibodies targeting chromobox 7 (CBX7; cat. no. ab21873; Abcam), enhancer of zeste homolog 2 (EZH2; cat. no. ab191250; Abcam) and jumonji and AT-rich interaction domain containing 2 (JARID2; cat. no. ab192252; Abcam). Total chromatin was used as the input. Immunoprecipitated products were collected after incubation with magnetic beads coupled with anti-mouse IgG (cat. no. ab18413; Abcam). The beads were washed using a magnetic separation rack and the bound chromatin was eluted in ChIP Elution Buffer with Proteinase K mixer, according to the manufacturer's instructions. The DNA fragments immunoprecipitated by CBX7,

Table II. Primer sequences for chromatin immunoprecipitation-quantitative PCR.

Gene	Primer sequence (5'→3')	Annealing temperature, °C	Product length, base pairs
sFRP4-1	F: ACATTGTCCCAACTGTCCTCA3 R: TTCTGCTGCCCTCTAATTCTG	60	82
sFRP4-2	F: GCAGAGGGAGCAAAGTTCAGT R: TTTTCGACACCGGATACAAGA	60	91

F, forwards; R, reverse; sFRP, secreted frizzle-related protein

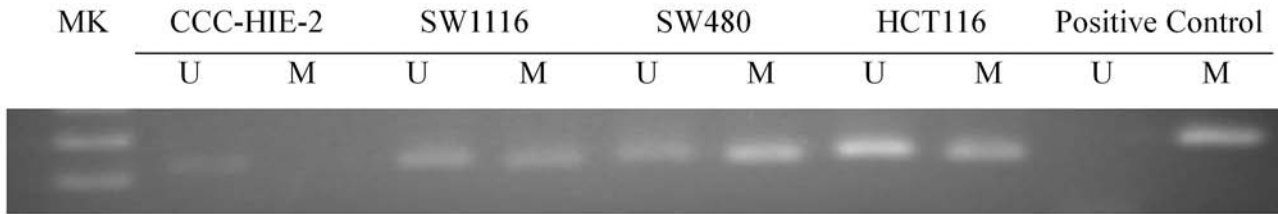


Figure 1. Methylation-specified PCR of sFRP4 in four colorectal cell lines. sFRP4 was non-methylated in CCC-HIE-2 but hypermethylated in other colorectal cancer cell lines. M, methylated DNA product amplified with methylation-specific primers; U, unmethylated DNA product amplified with non-methylation-specific primers; MK, marker; sFRP, secreted frizzle-related protein.

EZH2 and JARID2 were amplified using qPCR with the primers targeting the sFRP4 promoter, which are listed in Table II.

Small interfering (si)RNA transfections. The siRNA sequences targeting EZH2 were designed and synthesized by Guangzhou Ribobio Co., Ltd. The sequences used were as follows: siRNA (si)-EZH2-1 forward, 5'-GACUCUGAAUGCAGUUGCUTT-3' and reverse, 5'-AGCAACUGCAUUCAGAGUCTT-3'; si-EZH2-2 forward, 5'-GCAGCUUUCUGUUAACUUTT-3' and reverse, 5'-AAGUUGAACAGAAAGCUGCTT-3'; si-EZH2-3 forward, 5'-CCUGACCUCUGUCUUACUUTT-3' and reverse, 5'-AAGUAAGACAGAGGUCAGGTT-3'; and negative control (24) forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. Cells were seeded in 6-well plates (50,000 cells/ml). Transfections were performed when the cell confluence reached 50-60%. Cells were transfected with si-EZH2-1, si-EZH2-2, si-EZH2-3 or si-NC (50 nM) using GenMute™ siRNA Transfection reagent (SignaGen Laboratories, LLC), according to the manufacturer's instructions. At ~24 h after transfection, cells were harvested for subsequent analyses.

MTT assays. After transfection for 24 h, SW480 cells were seeded in 96-well plates at a density of 3,000 cells/well. Briefly, 10 μ l of MTT was added into cells to incubate for 4 h and replaced with 200 μ l of DMSO. The absorbance values at 490 nm of each well was recorded. Each set was repeated at least three times. The viability of SW480 cells was detected at different time points (0, 24, 48 and 72 h).

Statistical analysis. SPSS 19.0 (IBM Corp.) was used to analyze the RT-qPCR data. One-way ANOVAs with post-hoc Dunnett's tests were used to analyze the expression levels

of sFRP4 mRNA and the data from the MTT assays. The statistical method, Normalization and Differential Analysis, developed by Illumina, Inc., was used to analyze the gene array data. $P < 0.05$, $\text{diffscore} < -20$ or $\text{diffscore} > 20$ was considered to indicate a statistically significant difference. The $2^{-\Delta\Delta C_q}$ method was used to analyze ChIP-qPCR data, which were normalized to the input DNA fraction Cq value. The % Input for each ChIP fraction was calculated as: $\% \text{ Input} = 2 \times (Cq^{\text{input}} - Cq^{\text{ChIP}}) \times Fd \times 100\%$. In this instance, Fd is the input dilution factor. For example, if 100 μ l of sonicated sample was used for ChIP, and 10 μ l of sonicated sample is used as the input, $Fd = 1/10$. Fold Enrichment = $[\% (\text{ChIP}/\text{input})] / [\% (\text{negative control}/\text{input})]$. The normalized ChIP fraction Cq value was adjusted for the normalized background (mock immunoprecipitation (25) fraction Cq value. $\Delta\Delta C_q (\text{ChIP}/\text{mock IP}) = \Delta C_q (\text{normalized ChIP}) - \Delta C_q (\text{normalized mock IP})$.

Results

Promoter DNA methylation status of sFRP4 during CRC progression from Dukes A to Dukes C. As indicated in Fig. 1, methylation of the sFRP4 promoter region was detected in SW1116, SW480 and HCT116 cells, but not in the CCC-HIE-2 cells.

sFRP4 is downregulated in CRC cells. To determine whether DNA methylation of the sFRP4 promoter coincided with decreased expression levels, the mRNA and protein expression levels of sFRP4 were measured in all four cell lines using RT-qPCR and western blotting analysis (Figs. 2 and 3, respectively). Compared with the CCC-HIE-2 cells, the mRNA expression levels of sFRP4 were significantly lower in the SW1116, SW480 and HCT116 cells (Fig. 2; $P < 0.001$), while in western blot analysis, sFRP4 appeared to be downregulated (Fig. 3). These results appear to be negatively associated with the methylation status of the sFRP4 (Fig. 1). The expression

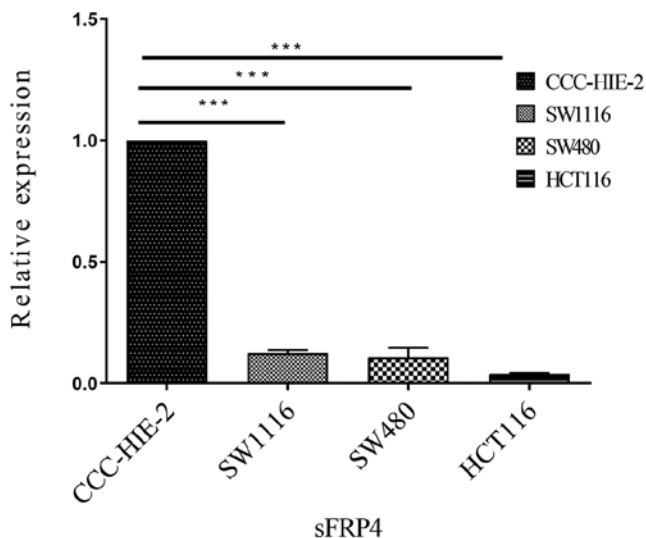


Figure 2. mRNA expression levels of sFRP4 in four colorectal cell lines. The mRNA expression levels of sFRP4 were decreased in the colorectal cancer cell lines. *** $P < 0.001$. sFRP4, secreted frizzled-related protein 4.

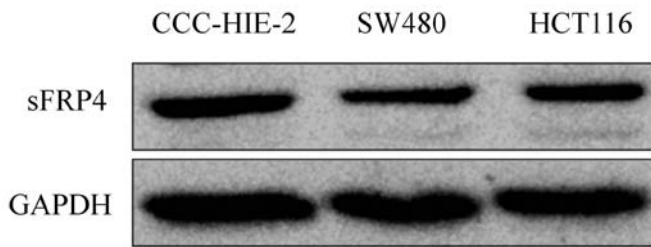


Figure 3. Protein levels of sFRP4 in colorectal cell lines. Western blotting analysis of the protein expression levels of sFRP4 in the colorectal cancer cell lines, HCT116 and SW480, and the normal colorectal cell line CCC-HIE-2. sFRP4 levels were downregulated in the CRC cell lines. sFRP, secreted frizzled-related protein.

levels of sFRP4 were downregulated by more than nine-fold in the CRC cell lines compared with the control cell line.

Expression of EZH2, CBX7 and JARID2 is upregulated and may be positively associated with the activities of Wnt/ β -catenin signaling in the CRC cell lines. sFRPs may block Wnt signaling either by interacting with Wnt proteins to prevent them from binding Fzd proteins or by forming non-functional complexes with Fzd (21). The transcriptional expression levels of components of the Wnt signaling pathway were assessed using gene array analysis of whole genome expression in SW1116, SW480, HCT116 and CCC-HIE-2 cells. The Wnt signaling pathway may be abnormally activated in all three CRC cell lines, exhibiting upregulated expression levels of the downstream genes, FZD9, Low-density lipoprotein related protein-5 (LRP5), β -catenin, T-cell factor 3 (TCF3), T-cell factor 4 (TCF4), c-myc and cycD2 (Table III, Fig. 4), and downregulated expression of glycogen synthase kinase 3 β (GSK3 β). GSK3 β is considered to serve a negative role in this pathway by promoting the degradation of β -catenin (2).

To screen for important regulators that modulate the promoter DNA methylation of sFRP4, the expression levels of components in the PcG protein family in the three CRC cell

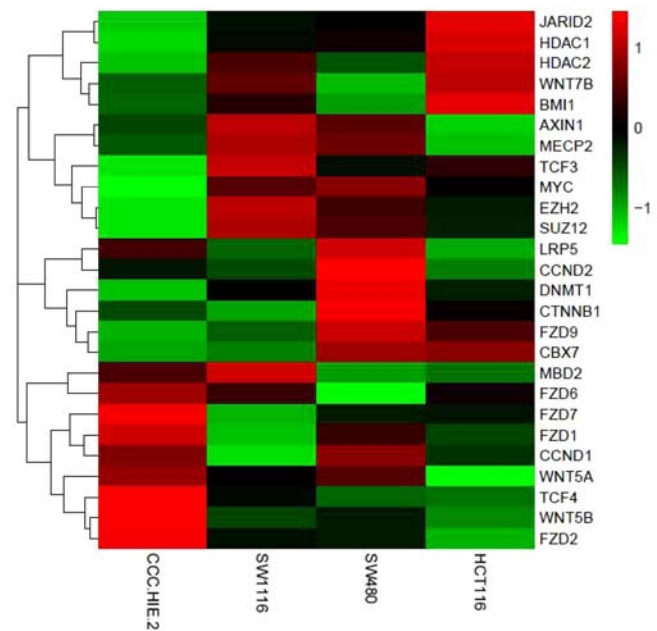


Figure 4. Heat map of differentially expressed genes in the four colorectal cell lines. PcG proteins (BMI1, EZH2, Suz12, CBX7 and JARID2) and DNMT1 were all upregulated in the cancer cell lines. Meanwhile, the mRNA expression of genes in the Wnt signaling pathway (FZD9, LRP5, β -catenin, TCF3, TCF4, c-myc and cycD2) were upregulated in CRC cell lines compared with normal colorectal cells. Red indicates high expression. Green indicates low expression. PcG, polycomb group; BMI1, B-lymphoma Mo-MLV insertion region 1; EZH2, enhancer of zeste homolog 2; Suz12, suppressor of zeste 12 homolog; CBX7, chromobox protein homolog 7; JARID2, jumoni and AT-rich interaction domain containing 2; DNMT1, DNA methyltransferase 1; cyD2, cytochrome bd-I ubiquinol oxidase subunit II; FZD9, frizzled receptors 9; LRP5, low-density lipoprotein related protein-5; TCF3, T-cell factor 3; TCF4, T-cell factor 4.

lines were analyzed. Table III lists certain PcG protein-related genes that were significantly upregulated or downregulated >1.5 -fold ($P < 0.05$; differ score >20 or differ score <20). For example, EZH2, CBX7 and JARID2 were all upregulated in cancer cells. Among these genes, EZH2 was dramatically upregulated in the SW1116 cells (59.84-fold), SW480 cells (30.27-fold) and HCT116 cells (13.69-fold). In addition, the expression levels of histone deacetylase (HDAC) 1 were higher in the SW480 cells (1.49-fold) and HCT116 cells (2.05-fold) compared with the CCC-HIE-2 control, while HDAC2 was upregulated in the SW1116 cells (2.09-fold) and HCT116 cells (2.74-fold). DNMT1 was upregulated in all CRC cell lines (1.55-fold in SW1116, 2.56-fold in SW480 and 1.41-fold in HCT116). These results suggested that histone modification and DNA methylation may serve important roles in the advanced stages of CRC and that the expression of the PcG proteins EZH2, CBX7 and JARID2 may be associated with the expression of sFRP4. RT-qPCR was showed similar results to the gene array results (Fig. S1).

CBX7, EZH2 and JARID2 are enriched in the sFRP4 promoter region in CRC cells. Since the PcG proteins have been demonstrated to be associated with the promoter DNA methylation status of targeted genes, whether the selected PcG proteins (CBX7, EZH2 and JARID2) are enriched in or bind the promoter region of sFRP4 in CRC was investigated. ChIP-qPCR using anti-CBX7, anti-EZH2 and anti-JARID2

Table III. Genes differentially expressed between colorectal cancer cell lines and the CCC-HIE-2 cell line.

Gene symbol	Fold-change in expression			mRNA Accession	Description
	SW1116	SW480	HCT116		
FZD9	1.57	8.66	4.56	NM_003508.2	Homo sapiens frizzled homolog 9
LRP5	0.52	1.51	0.45	NM_002335.1	Homo sapiens low-density lipoprotein receptor-related protein 5
β -catenin	0.72	3.65	1.60	XM_945654.1	Homo sapiens catenin (cadherin-associated protein) β 1
TCF3	2.76	1.66	1.99	NM_003200.1	Homo sapiens transcription factor 3
TCF4	4.32	1.30	5.34	NM_003199.1	Homo sapiens transcription factor 4
c-myc	9.24	15.37	4.97	NM_002467.3	Homo sapiens v-myc myelocytomatosis viral oncogene homolog
cycD2	0.33	18.18	0.02	NM_001759.2	Homo sapiens cyclin D2
JARID2	2.51	2.72	8.07	NM_004973.2	Homo sapiens jumonji and AT-rich interaction domain containing 2
CBX7	1.21	5.86	5.28	NM_175709.2	Homo sapiens chromobox 7
EZH2	59.84	30.27	13.69	NM_004456.3	Homo sapiens enhancer of zeste homolog 2
DNMT1	1.55	2.56	1.41	NM_001379.1	Homo sapiens DNA (cytosine-5-)-methyltransferase 1
HDAC1	1.39	1.49	2.05	NM_004964.2	Homo sapiens histone deacetylase 1
HDAC2	2.09	1.29	2.74	NM_001527.2	Homo sapiens histone deacetylase 2

antibodies confirmed the enrichment of these proteins at the sFRP4 promoter region (Table II). Fig. 5 demonstrated that CBX7, EZH2 and JARID2 were enriched in the sFRP4 promoter region in all four of the colorectal cell lines, while in the CRC cell lines, CBX7, EZH2 and JARID2 were enriched.

Silencing of EZH2 rescues sFRP4 expression levels in CRC. Inhibition of EZH2 was performed to determine whether EZH2 affected the promoter DNA methylation of sFRP4. Various si-EZH2 were tested, si-EZH2-1, si-EZH2-2 and si-EZH2-3. si-EZH2-2 was selected because the EZH2 expression levels were the lowest following transfection (Fig. 6). Fig. 7 indicates that after EZH2 was inhibited in SW480 cells, the mRNA expression levels of sFRP4 were upregulated ($P < 0.01$), but the methylation status of sFRP4 was still hypermethylated (Figs. 8 and 9).

Knockdown of EZH2 influences CRC cell proliferation. To investigate the effects of EZH2 on the proliferation of CRC, knockdown of EZH2 was performed in SW480 cells for 24, 48 and 72 h and, subsequently, the cell viability was measured using the MTT assays. Fig. 10 revealed that a significant decrease in CRC cell viability was observed when EZH2 was knocked down ($P < 0.05$), with a reduction of 31.0% in the CRC cell line treated with si-EZH2 for 24 h, 32.0% at 48 h and 32.0% at 72 h.

Discussion

CRC is a leading cause of cancer-associated mortality worldwide. One of the fundamental mechanisms driving the initiation and progression of CRC is the accumulation of a variety of genetic and epigenetic changes (20). Over the past several decades, advances have been made in the understanding of cancer epigenetics, particularly for aberrant DNA methylation, microRNA and non-coding RNA dysregulation, and alterations in histone modification states (14).

sFRPs have been recognized for their potential to sequester Wnt ligands away from their receptor complexes and ultimately antagonize Wnt signaling (4,11,21). Analysis of sFRP hypermethylation may have diagnostic and prognostic value for the detection and management of CRC (24,26). To the best of our knowledge, no mutation in any sFRP gene has been reported to be associated with tumors. Previous studies have documented the loss of sFRP expression due to promoter methylation; thus, silencing sFRP expression through epigenetics may be the mechanism behind colorectal tumorigenesis (11,12,16,27).

In the present study, the methylation-specified PCR results indicated that sFRP4 was hypermethylated at the advanced stage in CRC cells. In RT-qPCR and western blot analysis, sFRP4 was downregulated in CRC cell lines compared with CCC-HIE-2 cells. In a previous study, high-dose DNMT inhibitor DAC treatment increased the expression levels of sFRP4 in CRC cells (12). These results indicated that promoter DNA hypermethylation represents a possible mechanism of sFRP4 gene silencing in CRC. By analyzing the data from the gene array, the expression of DNMT1 in CRC was found to be upregulated and it was also revealed that Wnt signaling may be aberrantly activated in CRC. DNA methylation variations have been reported to be associated with carcinogenesis (16,27), and may occur before or at the beginning of carcinogenesis. DNA methylation may silence sFRP4 expression and induce CRC initiation and progression.

DNA methylation and histone lysine methylation are dynamic chemical modifications that serve a crucial role in the establishment of gene expression patterns during development, which are considered to be tightly coordinated (28). Methylation of lysine residues on histones can initiate, target or maintain DNA methylation. Histone methylation is more commonly observed on lysine residues of histone tails H3 and H4. Common histone methylation sites associated with gene activation include H3K4, H3K48 and H3K79, while common sites for gene inactivation include H3K9 and H3K27 (28).

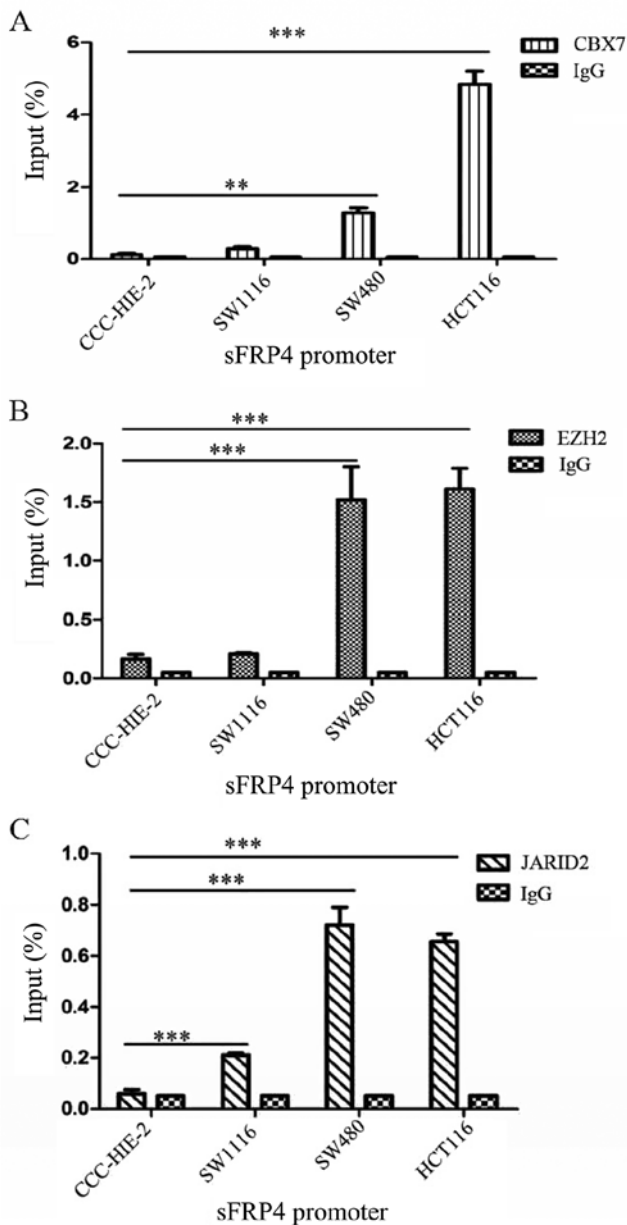


Figure 5. Chromatin immunoprecipitation-quantitative PCR of colorectal cell lines. (A) CBX7, (B) EZH2 and (C) JARID2 were found to be enriched in the promoter region of sFRP4 in colorectal cell lines, while in colorectal cancer cell lines, they were enriched. ** $P < 0.01$ and *** $P < 0.001$. CBX7, chromobox 7; EZH2, enhancer of zeste homolog 2; IgG, immunoglobulin G; Jarid 2, jumonji and AT-rich interaction domain containing 2; sFRP, secreted frizzles-related protein.

PcG proteins are transcriptional repressors that regulate several crucial developmental and physiological processes in the cell (29). More recently, these proteins have been revealed to serve important roles in human carcinogenesis and cancer development and progression (29,30). In particular, the PcG proteins and other epigenetic regulators, participate in regulation of gene transcription (29,31). PcG proteins form two major protein complexes, polycomb repressive complex 1 and 2. EZH2, the core component of PRC2, is an epigenetic regulatory protein associated with tumor aggressiveness and poor survival outcomes in several types of human cancer (32). EZH2 catalyzes the trimethylation on Lys27 of histone H3 (H3K27me3), which

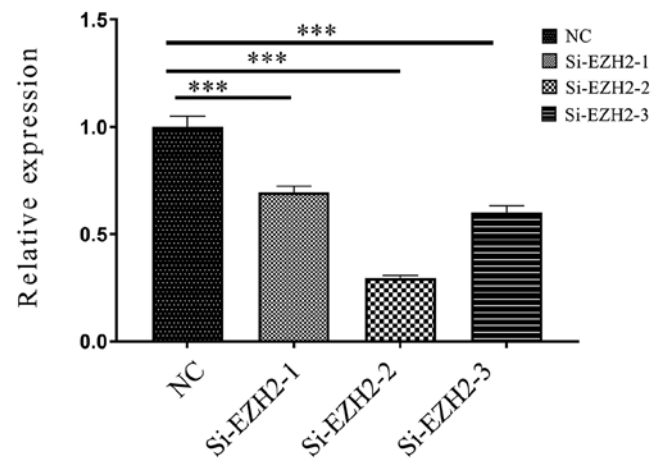


Figure 6. Silencing of EZH2 in SW480 colorectal cell lines. Knockdown of EZH2 was achieved with various siRNAs, the mRNA expression levels of EZH2 in cells treated with si-EZH2-2 were the lowest. *** $P < 0.001$. EZH2, enhancer of zeste homolog 2; NC, negative control; si, small interfering RNA.

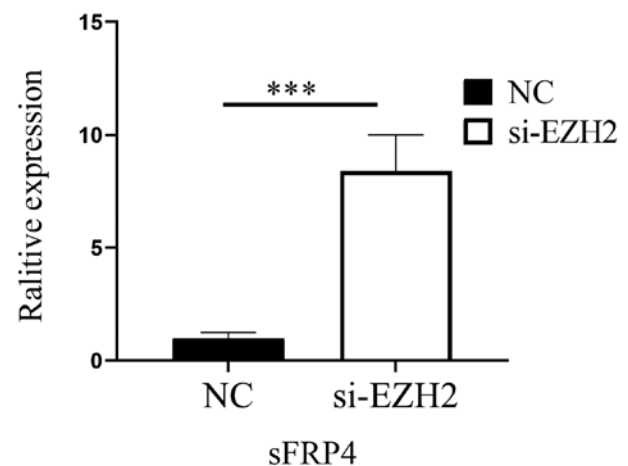


Figure 7. Expression of sFRP4 after EZH2 downregulation in the SW480 colorectal cancer cell line. After downregulation of EZH2 using siRNA, the expression of sFRP4 at the mRNA level was increased in SW480 cells. *** $P < 0.001$. EZH2, enhancer of zeste homolog 2; NC, negative control; sFRP4, secreted frizzles-related protein; si, small interfering.

recruits transcriptionally repressive complexes involved in chromatin compaction and results in stable gene silencing (33). EZH2 has also been reported to interact with both DNMT1 and DNMT3 which, in turn, methylate the target DNA where EZH2 was enriched (34). Although the mechanistic contributions of EZH2 to cancer progression are not yet determined, functional links between EZH2-mediated histone methylation and DNA methylation (35) suggest a partnership with the gene silencing machinery implicated in tumor suppressor loss.

In the present study, EZH2 was enriched in the sFRP4 gene promoter region, and knockdown of EZH2 did not influence the promoter DNA methylation levels of sFRP4. These results suggested that EZH2 regulated sFRP4 expression without affecting the promoter DNA methylation levels in CRC cells. Moreover, in SW480 CRC cells, si-EZH2 treatment restored sFRP4 expression levels and decreased CRC cell viability. These results provided evidence that EZH2 serves an important role in enhancing the proliferation of human CRC cells.

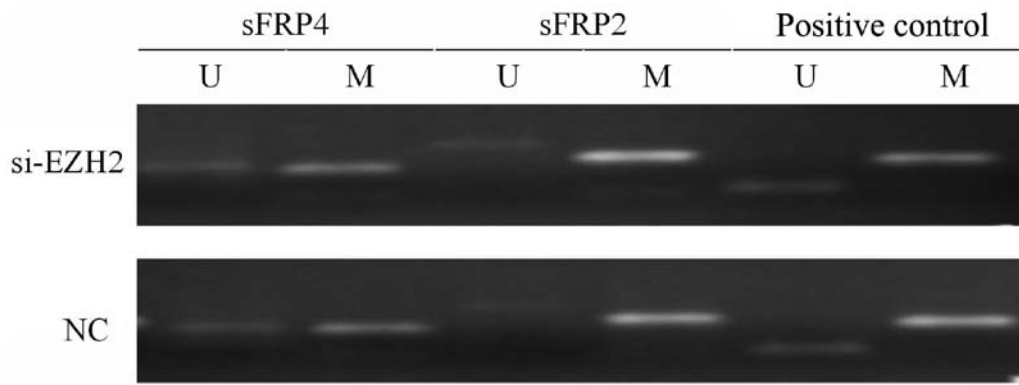


Figure 8. Methylation specific PCR of sFRP4 in SW480 cells. Inhibition of EZH2 in the SW480 colorectal cancer cells did not affect the hypermethylation status of sFRP4, and the promoter DNA methylation level was unchanged upon downregulation of EZH2. EZH2, enhancer of zeste homolog 2; M, methylation; U, unmethylation; MK, marker; NC, negative control; sFRP, secreted frizzles-related protein; si, small interfering.

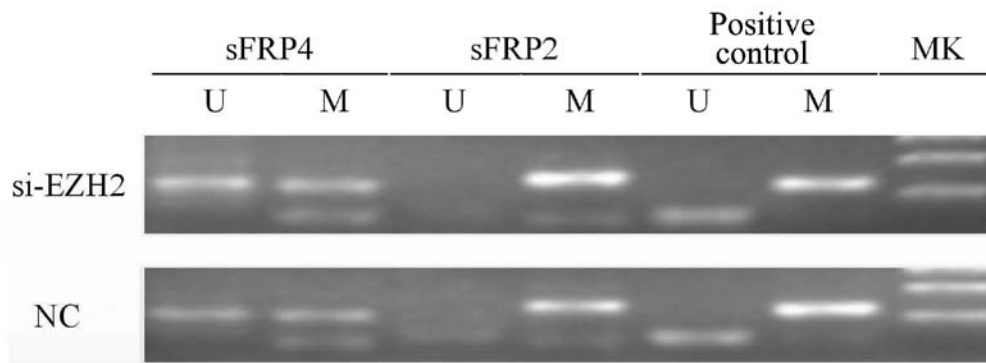


Figure 9. Methylation-specific PCR of sFRP4 in HCT116 cells. Inhibition of EZH2 in HCT116 colorectal cancer cells did not affect the hypermethylation status of sFRP4, and the promoter DNA methylation level was unchanged upon EZH2 knockdown. EZH2, enhancer of zeste homolog 2; M, methylation; U, unmethylation; MK, marker; NC, negative control; sFRP, secreted frizzles-related protein; si, small interfering.

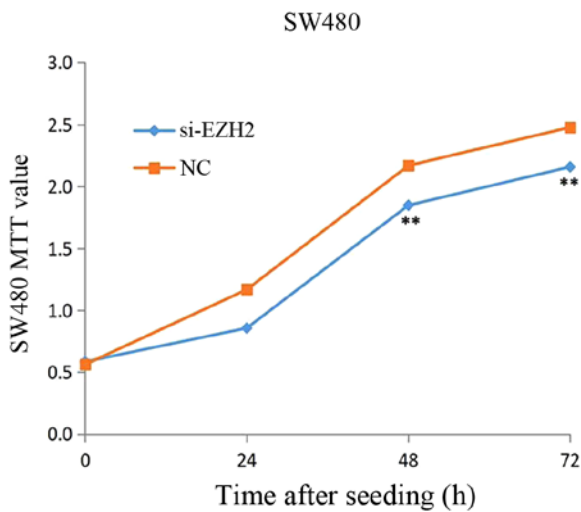


Figure 10. Downregulation of EZH2 in cell proliferation. Knockdown of EZH2 decreased proliferation in CRC cells, with a reduction of ~31.0% in si-EZH2 treated CRC cells at 24 h, 32.0% reduction at 48 h and 32.0% at 72 h. **P<0.01. CRC, colorectal cancer; EZH2, enhancer of zeste homolog 2; NC, negative control; si, small interfering.

Given that EZH2 did not affect the DNA methylation level of sFRP4, it was speculated that EZH2 may regulate sFRP4 expression via histone methylation.

Additionally, the Wnt signaling pathway has several key components, such as GSK3 β and β -catenin (2). GSK3 β is considered to serve negative roles in this pathway by promoting the degradation of β -catenin (2). The mRNA expression levels of GSK3 β were confirmed to be downregulated in CRC cell lines via a gene array. EZH2 was revealed to have a significant influence on the downstream activity in the Wnt signaling pathway. Chen *et al* (36), reported that the expression levels of β -catenin, vimentin and c-Myc were detected via western blotting after EZH2 knockdown in SW480 cells. These data reveal that knockdown of EZH2 decreased the expression of c-Myc and vimentin, which are known downstream target genes of the Wnt/ β -catenin signaling pathway. In hepatic cancer, EZH2 reduces the expression levels of nuclear β -catenin, TCF transcriptional activity and downstream proliferate targets cyclin D1 and epidermal growth factor receptor (37). In cervical carcinoma, EZH2-mediates the repression of GSK3 β and tumor suppressor p53 (TP53), which promotes Wnt/ β -catenin signaling-dependent cell expansion (38). Inhibition of GSK 3 β activity is associated with excessive EZH2 expression levels and enhanced tumor invasion in nasopharyngeal carcinoma (39).

In the present study, the enrichment of CBX7 and JARID2 at the sFRP4 promoter was also increased in all three colorectal cell lines in which the sFRP4 promoter was hypermethylated. CBX7 is a canonical component in

PRC1, which can physically interact with H3K27me3 via its N-terminal chromodomain (40). CBX7 is also known to be involved in repressive chromatin modifications and to be able to form complexes with DNA methyltransferase enzymes, but knockdown of CBX7 is unable to relieve the suppression of silenced genes in cancer cells (37,38). JARID2, a member of the Jumonji family, is a DNA binding protein that binds to the promoter region of specific myogenic genes in rhabdomyosarcoma cells in conjunction with a PRC2 protein and regulates H3K27me3 (25). CBX7, JARID2 and EZH2 all show close connections with histone methylation (25,29). The functions of CBX7 and JARID2 still need to be analyzed.

In conclusion, based on the current results, the expression levels of sFRP4 appear to be associated with its DNA methylation. CBX7, EZH2 and JARID2 were enriched in the sFRP4 promoter region when sFRP4 was hypermethylated, and there was a greater level of enrichment with more malignant CRC cell lines. The PcG protein may have participated in sFRP4 promoter DNA hypermethylation and induced sFRP4 silencing. In particular, EZH2 regulated sFRP4 expression without affecting the hypermethylation of sFRP4 in CRC cells, while there may be a potential regulation of EZH2 and sFRP4. This mechanism of action may be of interest for developing a new therapy for CRC.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JQ collected the data and designed the study. YL performed the experiments, analyzed the data and wrote the manuscript. JY, YX, ML and FW aided in experiments, analyzed the data and revised it critically for important intellectual content. JZ aided in experiments, analyzed the data and revised it critically for important intellectual content. JQ supervised the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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