

# ZBTB7 evokes 5-fluorouracil resistance in colorectal cancer through the NF- $\kappa$ B signaling pathway

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**Abstract.** Zinc finger and BTB domain containing 7A (ZBTB7), a POZ/BTB and Krüppel erythroid myeloid oncogenic factor, is critical for the tumorigenicity and progression of various cancer types. ZBTB7 has been reported to promote the cell proliferation of colorectal cancers (CRC). However, the function of ZBTB7 to 5-fluorouracil (5-FU) resistance has not yet been studied. In the current study, ZBTB7 expression and function in 5-FU resistance in CRC were investigated using with multidisciplinary approaches, including western blot analysis, Transwell assay, CCK8 and a tumor xenograft model. Overexpression of ZBTB7 was increased the level of proteins associated with cell invasion and epithelial-mesenchymal transition. ZBTB7 inhibition attenuated the invasion and enhanced the apoptosis of CRC cells. IC<sub>50</sub> values and cell viability were significantly reduced in cells with short hairpin RNA (shRNA)-mediated ZBTB7 depletion compared with the control group. 5-FU administration decreased viability to a greater extent in the ZBTB7-shRNA group compared with the control, which was dose- and time-dependent. Analysis of gene expression omnibus data demonstrated that ZBTB7 mediated 5-FU resistance, potentially through nuclear factor (NF)- $\kappa$ B signaling. NF- $\kappa$ B inhibitor SN50 reversed ZBTB7-induced resistance in CRC. Collectively, the findings demonstrated that ZBTB7 mediated 5-FU resistance in CRC cells through NF- $\kappa$ B signaling. Thus, targeting ZBTB7 and NF- $\kappa$ B signaling may be an effective strategy to reverse 5-FU resistance in CRC.

## Introduction

Colorectal cancer (CRC) is a very common malignancy worldwide and is the third leading cause of cancer-associ-

ated mortality (1). The survival rate of patients with CRC remains unsatisfactory, despite effective therapeutic strategies including surgical resection and chemo-radiotherapy. 5-Fluorouracil (5-FU), antimetabolite medication, is a fluorinated analog active against a wide range of solid tumors, including pancreatic cancer (2-4), biliary tract cancer (5), breast cancer (6,7) and hepatocellular cancer (8,9). 5-FU-based therapy is one of the treatment regimens as the adjuvant or palliative chemotherapy for patients with CRC (10,11). 5-FU acts through various mechanisms, but principally as a thymidylate synthase inhibitor, eliciting cytotoxicity by interrupting the function of nucleotide synthetic enzyme thymidylate synthase. Additionally, it incorporates fluoronucleotides into RNA and DNA (12,13). A subset of patients with CRC is primarily refractory or have acquired chemoresistance to 5-FU, which has poses a major challenge for clinical practice to improve therapeutic efficiency. Thus, investigation of the mechanisms and discovery of potential treatments targets for patients with CRC with 5-FU resistance is urgently required.

Zinc finger and BTB domain containing 7A (ZBTB7), is part of the POZ and Krüppel/zinc finger and BTB (POK) protein family (14). It is involved in pleiotropic functions and cellular differentiation. ZBTB7 physically interacts with other POK family members, such as B-cell lymphoma 6 proteins (15). ZBTB7, considered to be an oncogene, is overexpressed in a variety of cancer types, and involved in the pathogenesis of cancer (16), including lung cancer (17), prostate cancer (18), liver cancer (19) and oral cancer (20). ZBTB7 drives tumorigenesis as a master regulator and is required for the regulation of other tumor suppressor genes, such as p53 (21). ZBTB7 has also been documented to be an important regulator of CRC initiation and progression (22). ZBTB7 knockdown could inhibit cell proliferation, and induce cell cycle arrest and promote apoptosis independently of p14-E3 ubiquitin-protein ligase MDM2-p53 pathway (23).

Currently, there limited knowledge of the role of ZBTB7 in CRC, and the specific mechanisms the mediate the oncogenic role of ZBTB7 in CRCs is unknown. To the best of our knowledge, no previous studied have investigated the association between of ZBTB7 expression in CRC and 5-FU resistance. In the present study, it was demonstrated that ZBTB7 knockdown sensitized the 5-FU response to in CRC, potentially via the nuclear factor (NF)- $\kappa$ B signaling pathway.

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**Key words:** zinc finger and BTB domain containing 7A, nuclear factor- $\kappa$ B, colorectal cancers, chemoresistance

## Materials and methods

**Microarray data analysis.** The genetic profiles of patients with CRC were acquired from the Gene Expression Omnibus (GEO; [ncbi.nlm.nih.gov/geo/](http://ncbi.nlm.nih.gov/geo/)) data repository (GSE39582, GSE36133, GSE17538, GSE31595, GSE33113, GSE37892, GSE38832 and GSE39084). The method used for quality control and raw data processing was previously described (24).

Differences in ZBTB7 expression among multiple cancer types were assessed using data also from GEO database (GSE39582). The subtypes with high or low expression levels of ZBTB7 mRNA were defined as: Low, 4.3352-6.1425; and high, 6.1433-7.1295. Cell line data were from GEO (GSE36133). Two subtypes of cell lines were defined (C1 and C2) according to the genetic signature, including epithelial-mesenchymal transition (EMT)-associated genetic expression, NF- $\kappa$ B signaling pathway, ABCG family and apoptosis-associated genes.

**Unsupervised subtype identification.** Unsupervised clustering was performed using the Brunet algorithm from the R package non-negative matrix factorization (NMF), based on most variant probe sets ( $n=1,000$ ) of chemotherapy resistance associated pathway genes in Kyoto Encyclopedia of Genes and Genomes (KEGG; [genome.jp/kegg/](http://genome.jp/kegg/)) in 53 CRCs cell lines. After determining the optimal number of subtypes corresponding to high cophenetic and dispersion coefficients, the final subtype assignment was regenerated for this number of subtypes, using 200 runs.

**Gene set enrichment analysis.** Gene set enrichment analysis was performed using java GSEA Desktop Application (Broad Institute, Cambridge, MA, USA) with the hallmark gene sets ( $n=50$ ) and KEGG gene sets ( $n=186$ ) implemented in Molecular Signatures Database (v 5.1), expression data and phenotype data were formatted following user guide, samples were permuted with NMF clustering subtype or ZBTB7 expression level for 1,000 times.

**Cell lines and reagents.** Human SW620 (CCL-227), SW480 (CCL-228), LoVo (CCL-229), HCT-116 (CCL-247), and HT-29 (HTB-38) CRC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). FHC colon epithelial cell was cultured in DMEM:F12 medium supplemented with 10% fetal bovine serum. All the cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), and were cultured at 37°C in 5% CO<sub>2</sub> atmosphere. Spheroids of cells were formed using a 3D culture technique (25,26). As described previously (27), cells were seeded in 24-well plates coated with 2% SeaPlaque agarose (BioWhittaker™; Lonza Group, Ltd., Basel, Switzerland) in DMEM with 5 $\times$ 10<sup>4</sup> cells/well in 500  $\mu$ l DMEM. 5-FU was purchased from Selleck Chemicals (Houston, TX, USA). NF- $\kappa$ B inhibitor SN50 was purchased from MedChemExpress (Shanghai, China) and used at the concentration at 18  $\mu$ mol/l for the indicated times.

**Construction of overexpression vector and short hairpin RNA (shRNA) treatment.** A ZBTB7 overexpression vector and shRNA system was constructed to explore the function and

mechanism. pcDNA3.1-ZBTB7A was generated by inserting the coding region (116-1,870 bp) from SW620 cells into pcDNA3.1 vector, using *Hind*III restriction enzyme digestion and ligation (Invitrogen; Thermo Fisher Scientific, Inc.) The procedure was performed according to previous research (19). The resulting plasmid was used to express ZBTB7. Plasmid (4  $\mu$ g) was transfected into cells at 50-70% confluence using Lipofectamine 2000®. ZBTB7 primer pairs were 5'-CTTAAGCTTGCCACCATGGCCGGCGGTGG-3' and 5'-GTCAAGCTTTTAGGCGAGTCCGGCTGTGAAGTTAC-3'. The shRNA sequences used were as follows: Control shRNA, forward CGCGAATTCACCATGGCCGGCGGTGG, reverse TGGCTCGAGTTAGGCGAGTCCGCTGTGAAGTTAC; ZBTB7 shRNA, forward GATCCCCGCCCACTA CGACCTGAATTGATATCCGTTTCAGGTCGTAGTTGTGG GTTTTTTCCAAA, reverse AGCTTTTGGAAAAACCCA CAACTACGACCTGAACGGATATCAATTCAGGTCGTAG TTGTGGGCGG. The subsequent experiments were performed 48 h after the transfection.

**Transwell assay.** The chambers (BD Biosciences, San Jose, CA, USA) were precoated with Matrigel (BD Biosciences, San Jose, CA, USA). HCT116 cells (5 $\times$ 10<sup>6</sup>) and ZBTB7-shRNA cells were counted and suspended in 100  $\mu$ l FBS-free medium at the upper chamber. Medium containing 10% FBS was added in the lower chamber, and incubated for 24 h at the temperature of 37°C. Cells were fixed with 90% ethanol for 1 h at 4°C. Migrant cells on membranes were visualized with 0.1% crystal violet staining for 15 min at room temperature. Following drying, the migrant cells were counted in five  $\times$ 200 microscopic fields.

**In vitro chemosensitivity assay.** Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) to determine the IC<sub>50</sub> values of cells. Following trypsinization, the transfected cell suspensions (control shRNA, ZBTB7-shRNA and ZBTB7-OE) were transferred and dispersed into 96-well plates. The density was ~5,000 cells/well. 5-FU was added to treatment groups. After 72 h, cells were incubated with 10  $\mu$ l CCK-8 reagents for another 2 h, then detected using a microplate reader at 450 nm absorbance (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Mouse xenografts models.** Control HCT116 cells (~5 $\times$ 10<sup>6</sup>) and ZBTB7-shRNA cells were subcutaneously implanted into the posterior flank of BALB/c nude mice (male, 6 weeks old, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). A total of 12 nude mice were used with 3 mice in each group. Tumor size was measured every 3 days and recorded by using the following equation: Volume (mm<sup>3</sup>) = (length  $\times$  width<sup>2</sup>)/2. When the tumors reached 100 mm<sup>3</sup>, 5-FU was administered to the mice. Intraperitoneal injection of 5-FU was administered at a concentration of 25 mg/kg every 3 days. After ~1 month, the mice were sacrificed. The tumor tissues were extracted and the volume and weight were calculated. The maximum tumor volume was 4,200 mm<sup>3</sup>. Ethics approval for the animal experiments was provided by The Institutional Animal Care and Use Committee of Chongqing Fuling Central Hospital (Chongqing, China; ethics approval no. 2014015).

**Western blotting.** The cells were lysed in radioimmuno-precipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) and cell lysates were collected. Then bicinchoninic acid method was used to detect the protein concentration. Equal amounts of protein (20  $\mu\text{g}$ ) was separated by 10% SDS-PAGE (Thermo Fisher Scientific, Inc.) and then the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked at room temperature with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) [BSA; in TBS-Tween (TBST)] for 1 h and incubated at 4°C with the indicated antibodies overnight. TBST was used to wash the PVDF membranes three times and incubated with secondary antibodies, anti-mouse IgG (cat. no. 7076; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.; both diluted at 1:1,000) for 1 h at room temperature. TBST was used to wash again three times. Finally, proteins were observed with a chemiluminescence kit (Thermo Fisher Scientific, Inc.) by using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK). The following primary antibodies were used: GAPDH (1:3,000; Cell Signaling Technology, Inc.; cat. no. 5174), ZBTB7 (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-33683), E-cadherin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 14472), E-selectin (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-18852), integrin  $\beta 1$  (1:1,000; Cell Signaling Technology, Inc.; cat. no. 34971), integrin  $\alpha V$  (1:500; Santa Cruz Biotechnology, Inc.; cat. no. 4711) and fibronectin (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-69681).

**Immunofluorescence assay.** HCT116 control or HCT116-shRNA cells were cultured overnight with or without 5-FU (50  $\mu\text{M}$ ) to reach 80-90% confluence. Then, the cells were cultured in complete medium for another 48 h, and fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized in PBS containing 0.2 % Triton X-100 for 5 min at room temperature, washed three times in TBST and blocked with 5% BSA for 30 min at room temperature. The cells were incubated with antibody against cleaved caspase-3 (1:400; Cell Signaling Technology, Inc.; cat. no. 9661), ZBTB7 (1:100; Santa Cruz Biotechnology, Inc.; cat. no. sc-33683) overnight at 4°C. After washing, the cells were labeled with 5  $\mu\text{g}/\text{ml}$  Alexa Fluor 488-conjugated secondary antibody at 4°C for 30 min (cat. no. A-10631; Thermo Fisher Scientific, Inc.), followed by examination under a fluorescence microscope (Nikon Corporation, Tokyo, Japan). DAPI was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was incubated at room temperature for 10 min at a concentration of 1  $\mu\text{g}/\text{ml}$ . Five randomly selected fields at a magnification of x200 were counted in each slide. The experiment was repeated for three times.

**High-performance liquid chromatography (HPLC) analysis of bases released from the DNA of FU-treated cells.** HPLC assay was performed to measure the intracellular 5-FU level in the cells. The methods for HPLC assay were performed according to a standard procedure (28). Exponentially growing cells ( $\times 10^7$ ) were treated with FU-2- $^{14}\text{C}$  (53 mCi  $\text{mmol}^{-1}$ ; Sigma-Aldrich;

Merck KGaA), treated with non-radiolabeled FU and labeled with uracil-2- $^{14}\text{C}$  (52 mCi  $\text{mmol}^{-1}$ ; Sigma-Aldrich; Merck KGaA), or treated with non-radiolabeled FU. The concentration of FU (and uracil) used was 10  $\mu\text{mol}/\text{l}$  and cells were exposed to FU for 48 h (less than two cell divisions). Genomic DNA was isolated from the cells (Wizard Genomic DNA purification kit; Promega Corporation, Madison, WI, USA), and was precipitated with ethanol and the supernatant analyzed by high-performance liquid chromatography (HPLC). Fractions were collected at 0.5-min intervals, and released DNA bases detected by scintillation counting or UV absorbance at 254 nm; reference compounds were detected by UV absorbance.

**Statistical analysis.** The statistics analyses were performed using SPSS 20.0 software package (IBM Corp., Armonk, NY, USA). At least three independent experiments were performed to calculate the results presented as the mean  $\pm$  standard deviation. For comparison between two groups, two-tailed Student's t-test was performed. Multiple group comparisons were performed with one-way analysis of variance. Least significant difference was used as a post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**ZBTB7 was upregulated in CRC and promoted CRC progression.** In order to explore genetic initiation involved in CRC, ZBTB7 expression was analyzed in CRC cell lines and tissue. ZBTB7 was upregulated in CRC cells lines compared with a normal colon epithelial cell line, and HCT116 cell had a relatively high ZBTB7 level (Fig. 1A). ZBTB7 function was evaluated in HCT116 cell lines. Transwell assay demonstrated that ZBTB7 shRNA reduced the invasion of HCT116 cells (162.7 $\pm$ 34.37) compared with the control group (530.3 $\pm$ 60.73;  $P < 0.05$ ; Fig. 1B). Furthermore, three-dimensional cultures of cells were performed to mimic the complex *in vivo* tumor microenvironment. Immunofluorescence showed control RNA cells and ZBTB7 shRNA cells (Fig. 1C). The results showed the same trends (Fig. 1D).

**Silencing ZBTB7 increases the 5-FU sensitivity in CRC.** To explore the functional role of ZBTB7 in 5-FU resistance,  $\text{IC}_{50}$  values were determined in ZBTB7-shRNA HCT116 cells and control groups cells by detecting cell viability in both cell lines at different concentration of 5-FU and over time. Compared with the control group, the  $\text{IC}_{50}$  values were significantly reduced in the ZBTB7-shRNA group. As the 5-FU concentration and time increased, the cell viability was decreased, with ZBTB7-shRNA group decreased more than the control ( $P < 0.05$ , Fig. 2A-C). By contrast, overexpression of ZBTB7 in SW480 cells (relatively low ZBTB7 level compared with other CRC cell lines; Fig. 1A) exhibited the opposite effects ( $P < 0.05$ ; Fig. 2D-F). Cleaved caspase-3 is an apoptotic protein marker, and immunofluorescence demonstrated that 5-FU-induced apoptosis was increased in ZBTB7-shRNA cells compared with the control group ( $P < 0.05$ ; Fig. 3A). Furthermore, a xenograft mouse model demonstrated that the size of tumors was reduced in the ZBTB7-shRNA group following treatment

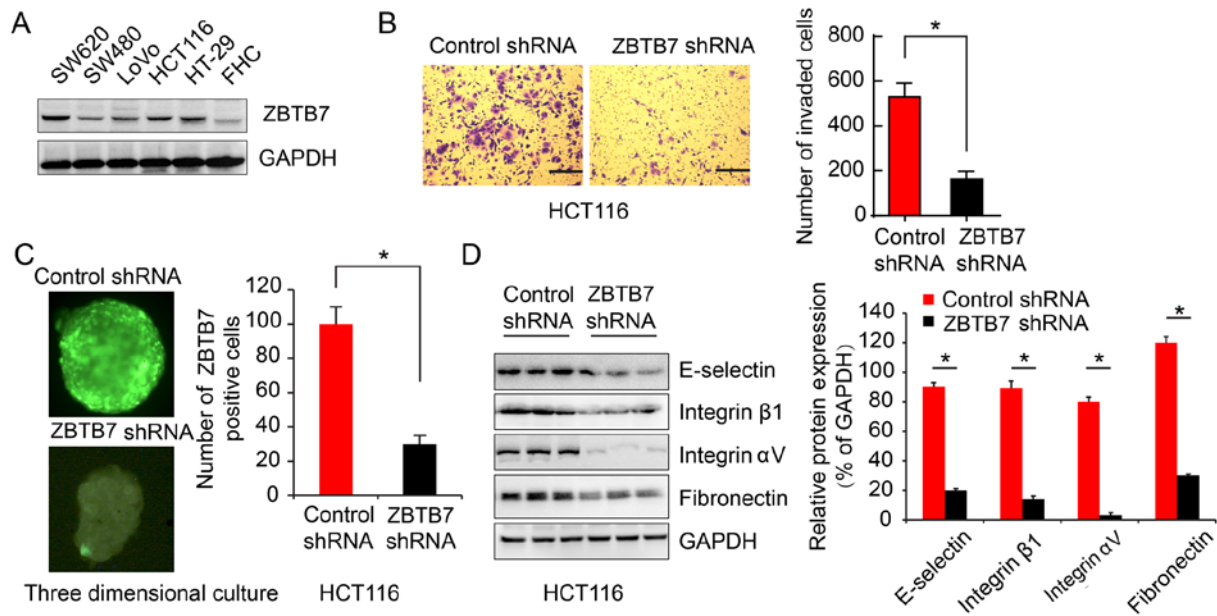


Figure 1. Expression and function of ZBTB7 in CRC. (A) Western blot analysis to measure ZBTB7 expression in CRC and normal epithelial cells lines. (B) Transwell assay of ZBTB7-shRNA and control group. ZBTB7-shRNA group showed less migrated cells than control group. (C) Representative immunofluorescence images of 3D culture of control shRNA and ZBTB7 shRNA cells. (D) Western blot analysis of relative protein expression in 3D culture. The column presents the relative protein expression  $^*P < 0.05$ . CRC, colorectal cancer; ZBTB7, zinc finger and BTB domain containing 7A; shRNA, short hairpin RNA.

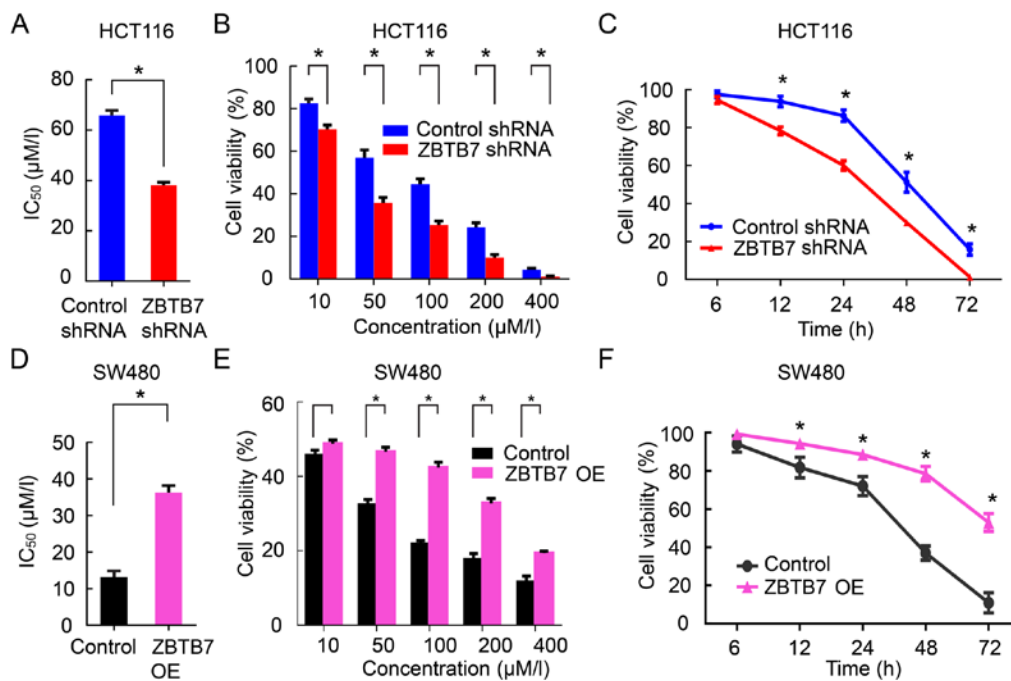


Figure 2. Silencing ZBTB7 restores the sensitivity of colorectal cancer to 5-FU. (A) CCK-8 method was measured IC<sub>50</sub> values in control or ZBTB7-shRNA groups exposed to 5-FU. (B) Cell viability in control or ZBTB7-shRNA groups exposed to different 5-FU concentration (10, 50, 100, 200 and 400 μM). (C) Cell viability in control or ZBTB7-shRNA groups exposed to 5-FU at different time-points (6, 12, 24, 48 and 72 h). (D) IC<sub>50</sub> values in control or ZBTB7-OE groups (SW480) exposed to 5-FU. (E) Cell vitality in control or ZBTB7-OE groups (SW480) exposed to different 5-FU concentration (10, 50, 100, 200 and 400 μM). (F) Cell viability in control or ZBTB7-OE groups (SW480) exposed to 5-FU at different time-points (6, 12, 24, 48, and 72 h).  $^*P < 0.05$  vs. control. 5-FU, 5-fluorouracil; shRNA, short hairpin RNA; ZBTB7, zinc finger and BTB domain containing A; OE, overexpression.

with 5-FU (Fig. 3B). The tumors volume were calculated and recorded. ZBTB7 shRNA cell-derived tumors and 5-FU were the slowest growing among all the groups ( $P < 0.05$ ; Fig. 3B).

*ZBTB7 is associated with NF- $\kappa$ B signaling pathways.* Subsequently, it was aimed to uncover the potential mechanism

of ZBTB7-driven 5-FU resistance. The original genetic profiles of patients with CRC were acquired from the GEO data repository. Differences in ZBTB7 expression among multiple cancer types were assessed using data from GEO. To explore the potential role and the mechanism of ZBTB7 in regulating chemotherapeutic resistance, data from cell lines

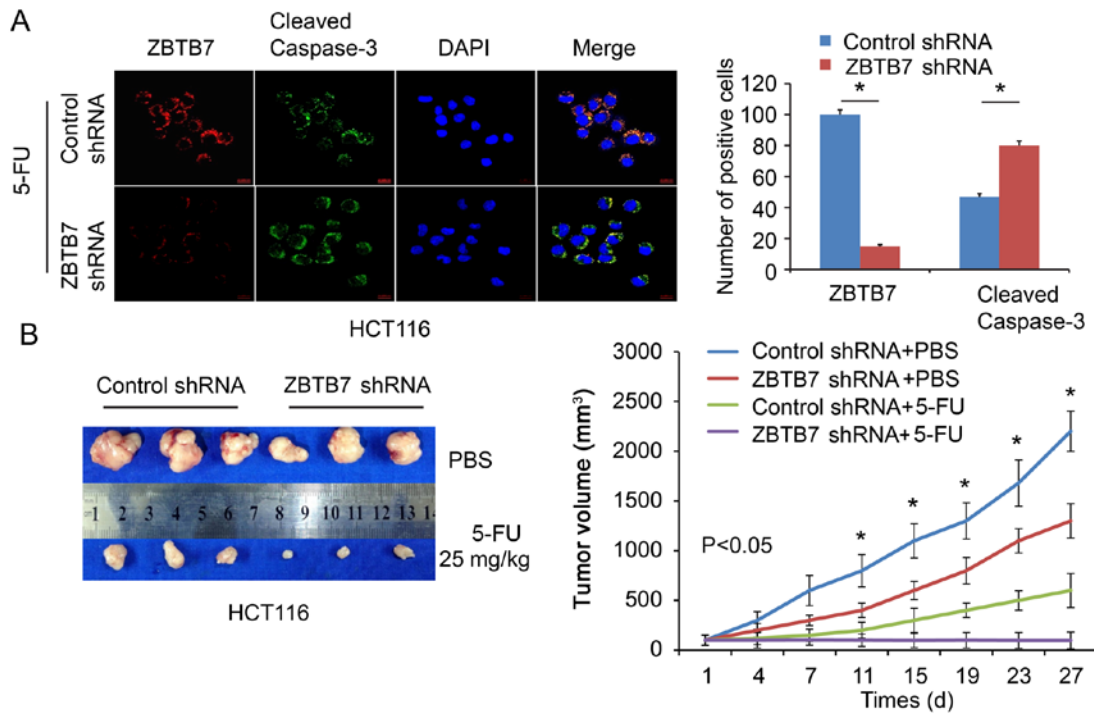


Figure 3. Effect of ZBTB7 on cell apoptosis assay and xenografts *in vivo*. (A) Immunofluorescence for cleaved caspase-3 and ZBTB7 expression following treatment with 5-FU in control and ZBTB7-shRNA groups. Control group (ZBTB7, 100±4; cleaved caspase-3, 48±2), ZBTB7-shRNA group (ZBTB7, 16±2; cleaved caspase-3, 80±4). The number of positive cells was counted for quantification. Five randomly selected fields at a magnification of x200 were counted for each slide. The experiment was repeated three times. (B) Mouse xenografts tumor volume in control and ZBTB7 shRNA groups with or without 5-FU (25 mg/kg) in 27 days. \*P<0.05 vs. control group. 5-FU, 5-fluorouracil; shRNA, short hairpin RNA; ZBTB7, zinc finger and BTB domain containing 7A.

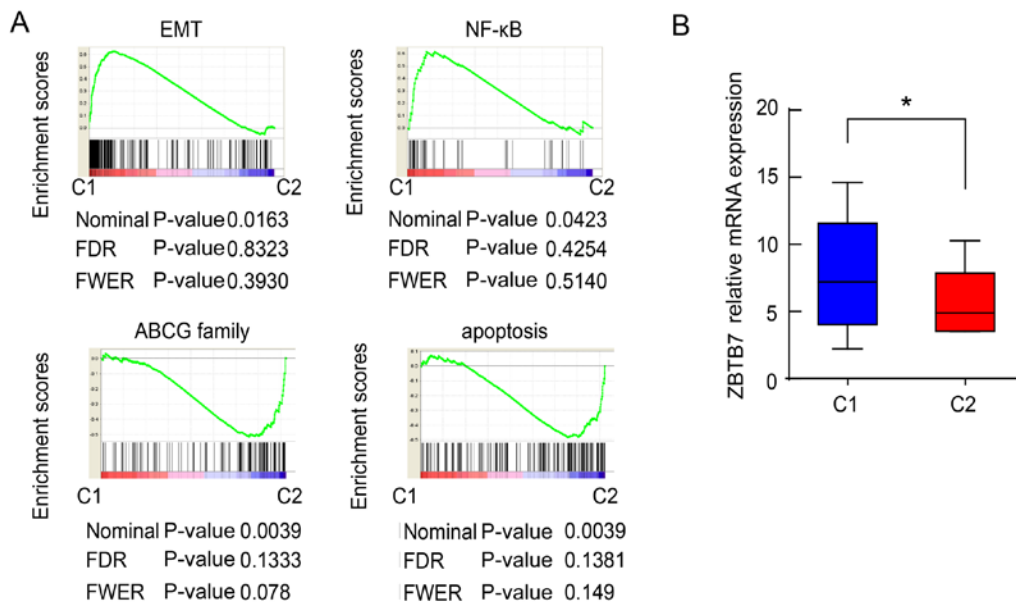


Figure 4. ZBTB7 may be associated with NF-κB signaling pathways. (A) C1 subtype was particularly enriched high in EMT and NF-κB. C2 subtype was enriched in ABCG family and apoptosis. (B) The relative ZBTB7 level in group C1 was found to be significantly higher than that in group C2. Data was obtained from Gene Expression Omnibus. \*P<0.05. EMT, epithelial-mesenchymal transition; FDR, false discovery rate; FWER, family-wise error rate; NF-κB, nuclear factor-κB; ABCG, ATP-binding cassette subfamily G.

(GSE36133) derived from naturally occurring tumors were analyzed because they recapitulate various aspects of the tissue type and genomic context of cancer. NMF, a recently established approach for consensus clustering, was performed onto 53 CRC cell lines according to their transcriptional features. This analysis revealed two subtypes of CRC cell lines

with adequate data coherence. Bioinformatics analysis split cell lines into two subtypes according to the EMT-associated genetic expression, the NF-κB signaling pathway, ABCG family and apoptosis-associated genes. One subtype (39.6% of all lines, n=21) was especially high in epithelial- mesenchymal transition and NF-κB (Fig. 4A), and was termed C1 subtype.

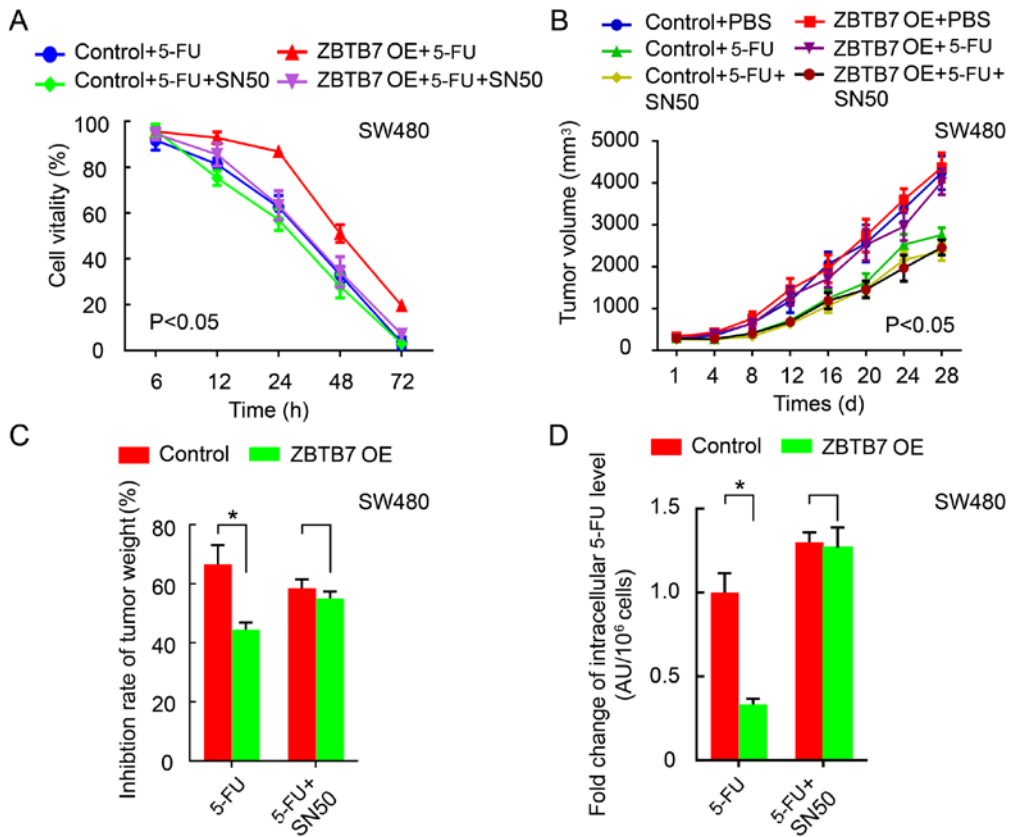


Figure 5. NF-κB inhibitor SN50 reverses ZBTB7-induced resistance in colorectal cancer cells. (A) CCK8 method was used to detect cell viability in control+5-FU with or without SN50 group, in OE+5-FU with or without SN50 group. (B) Tumor volume in control+PBS, control+5-FU, control+5-FU+SN50, in OE+PBS, OE+5-FU, OE+5-FU+SN50 group. (C) Inhibition rate of tumor weight in control+5-FU with or without SN50 group, in OE+5-FU with or without SN50 group. (D) HPLC assay was performed to measure intracellular 5-FU concentration in control+5-FU with or without SN50 group, in OE+5-FU with or without SN50 group in control and ZBTB7-OE cells with or without 5-FU and SN50. \*P<0.05. NF-κB, nuclear factor-κB; 5-FU, 5-fluorouracil; OE, overexpression.

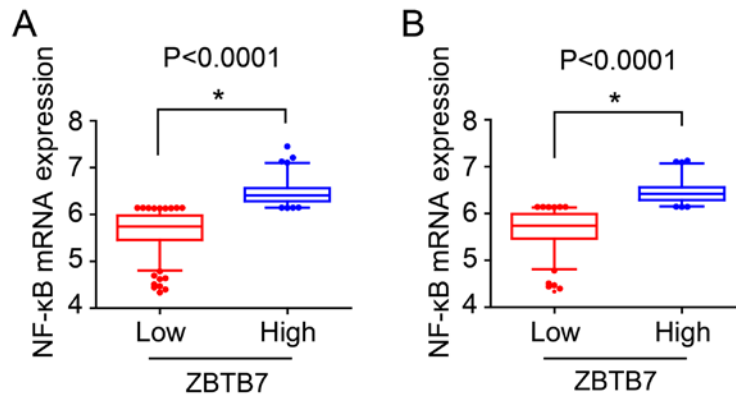


Figure 6. ZBTB7 mRNA level is associated with NF-κB mRNA. NF-κB mRNA level in (A) stage II and (B) stage III colorectal cancer patients. Differences in ZBTB7 expression were assessed using data from Gene Expression Omnibus database (GSE39582). \*P<0.05. NF-κB, nuclear factor-κB; 5-FU, 5-fluorouracil.

The other subtype (56.2% of all lines, n=27) especially high in ABCG family and apoptosis (Fig. 4B) were named C2 subtype. Notably, the relative ZBTB7 level in group C1 was significantly higher than that in group C2 (Fig. 4B). This evidence indicates that ZBTB7 may be involved in chemotherapeutic resistance of CRCs via regulation of the NF-κB pathway.

*NF-κB inhibitor SN50 reverses ZBTB7-induced resistance in CRC.* The role of NF-κB in ZBTB7-driven 5-FU resistance in

CRC was investigated. 5-FU reduced cell viability, and overexpression of ZBTB7 depleted the inhibitory effects. Treatment with NF-κB inhibitor SN50 reversed the proliferation enhancing effects of ZBTB7 (P<0.05; Fig. 5A). These results were verified *in vivo*. The tumor volume (P<0.05; Fig. 5B) and weight (P<0.05; Fig. 5C) exhibited the same trends. Finally, ZBTB7 overexpression increased the 5-FU level while SN50 significantly increased the intracellular 5-FU level (P<0.05; Fig. 5D).

Table I. Effect of ZBTB7 expression on overall survival and relapse-free survival of patients with stage II/III colorectal cancer according to Gene Expression Omnibus (GSE39582) clinical annotations.

A, Overall survival

Treatment	Mean months (standard error)			P-value <sup>b</sup>
	Total	ZBTB7 Low <sup>a</sup>	ZBTB7 High <sup>a</sup>	
5-FU	52.28 (1.84)	53.99 (1.98)	47.46 (4.07)	0.041
CC <sup>c</sup>	53.19 (1.70)	55.60 (1.84)	48.81 (3.43)	0.043

B, Relapse-free survival

Treatment	Mean months (standard error)			P-value <sup>b</sup>
	Total	ZBTB7 Low <sup>a</sup>	ZBTB7 High <sup>a</sup>	
5-FU	43.16 (2.61)	46.60 (2.80)	32.93 (5.58)	0.019
CC <sup>c</sup>	47.36 (2.66)	51.53 (2.79)	39.77 (5.16)	0.103

<sup>a</sup>ZBTB7 mRNA expression levels: low=4.3352-6.1425, high=6.1433-7.1295; <sup>b</sup>Log-rank (Mantel-Cox); <sup>c</sup>Chemotherapy regimen: Folinic acid, 5-FU and oxaliplatin; 5-FU and folinic acid; 5-FU, folinic acid and irinotecan.

*ZBTB7 mRNA level is associated with 5-FU sensitivity and prognosis.* The above results had demonstrated that higher ZBTB7 expression promoted tumorigenesis and susceptibility to 5-FU resistance through the NF- $\kappa$ B pathway. The clinical significance was investigated using GSE39582 from the GEO database. The patients received 5-FU alone or 5-FU-based adjuvant chemotherapy; the subgroup with ZBTB7-low tumors substantially benefited from adjuvant chemotherapy and exhibited a significantly increased probability of relapse-free survival and overall survival relative to those in the subgroup with ZBTB7-high tumors. (P<0.05; Fig. 6; Table I).

**Discussion**

5-FU-based chemotherapy is the basic classical treatment for patients with CRC (29). However, chemoresistance, either primary or acquired, is as a major challenge for clinical practice. 5-FU primary chemoresistance is predominantly due to increased thymidylate synthetase mRNA or protein level. Acquired chemoresistance mechanisms include the depletion of certain enzymes, such as thymidylate synthase, which is the target of 5-FU, or certain genetic mutations (30). Up to 40% of patients with stage II and III CRC receiving 5-FU-based adjuvant chemotherapy experience recurrence or mortality within 8 years of follow-up (31). Of patients with metastatic CRC, 50% are resistant to 5-FU-based chemotherapy (32,33). It is important to investigate the 5-FU resistance mechanisms and set up therapeutic strategies to reverse the chemoresistance to improve prognosis. In the current study, ZBTB7 was overexpressed in CRC cells compared with normal colon epithelial cells. Additionally, ZBTB7 knockdown increased 5-FU sensitivity. Mechanistically, ZBTB7 potentially promoted 5-FU resistance through the NF- $\kappa$ B signaling pathway. Targeting NF- $\kappa$ B signaling using SN50 reversed ZBTB7-mediated 5-FU resistance. Therefore, a ZBTB7/

NF- $\kappa$ B axis is involved in mediating 5-FU resistance in patients with CRC.

Though ZBTB7 is aberrantly overexpressed in human cancers, little is known about the mechanism that regulates this. A previous study demonstrated that the ZBTB7 gene is at the genomic locus chromosome 19p13.3, which is frequently mutated. The t(14;19)(q32;p13.3) translocation is common in B-cell non-Hodgkin's lymphoma (34). ZBTB7 overexpression may aberrantly activate regulatory pathways, such as fibronectin-mediated h1-integrin ligation in precursor B leukemia cells (35). However, its function in 5-FU resistance is unknown. In the current study, ZBTB7 was upregulated in CRC cell lines compared with normal colon epithelial cells. A Transwell assay demonstrated that ZBTB7-shRNA cells were less invasive than the control group. E-selectin, integrin  $\beta$ 1, integrin  $\alpha$ V and fibronectin are adhesion molecules. Typically, cancer cells interactions initially require a selectin-mediated initial attachment and then the circulating cancer cells roll along the endothelium. Locally released chemokines activate the rolling cancer cells. This triggers integrin activation, making a firmer adhesion to cell adhesion molecules, initiating and driving the trans-endothelial migration and extravasation processes (36). In the current study, three-dimensional cultures were used to mimic the tumor microenvironment. The results revealed that integrin  $\beta$ 1, integrin  $\alpha$ V and fibronectin expression were reduced in ZBTB7-shRNA cells compared with the control group. These results indicated that ZBTB7 could regulate adhesion molecules expression *in vitro* and *in vivo*, leading to tumor progression. Furthermore, ZBTB7 knockdown and 5-FU treatment could increase apoptosis. EMT was previously reported to be associated with 5-FU resistance in pancreatic cancer (37) and lung cancer (38). We hypothesized that ZBTB7 may be involved in 5-FU resistance in CRC. Previous research demonstrated that ZBTB7 promoted the migration and invasion of hepatocellular carcinoma by increasing

myocyte enhancer factor 2D expression (19). Mak *et al* (39) reported that ZBTB7 promoted cell migration and invasion via phosphoinositide 3-kinase/Akt signaling. Taken together, the results suggest that ZBTB7 may regulate EMT- and apoptosis-associated proteins to promote cell invasion and decrease cell apoptosis, which may mediate cell chemoresistance.

ZBTB7 was reported to be associated with efficacy of paclitaxel and cisplatin combination chemotherapy and overall survival in patients with NSCLC (40). The function of ZBTB7 in 5-FU resistance was examined in the present study. IC<sub>50</sub> values were determined using ZBTB7-shRNA cells and control groups by measuring cell viability over different concentrations and time-points of 5-FU. The results demonstrated that the 5-FU IC<sub>50</sub> values were reduced upon ZBTB7 depletion compared with control group, in dose- and time-dependent manners. Additionally, overexpression of ZBTB7 exerted the opposite effects. The results were also validated in animal models. The volume and the weights of the tumor were lower in the ZBTB7 shRNA group following exposure to 5-FU.

GEO data was analyzed to identify genes that may be involved in the 5-FU resistance of CRC. The results demonstrated that the 5-FU resistance may be associated with the NF- $\kappa$ B signaling pathways. NF- $\kappa$ B signaling is reported to mediate chemoresistance in various ways (41,42). Kwon *et al* (43) reported that gastric cancer cell resistance to 5-FU was mediated via activation of NF- $\kappa$ B. ZBTB7, as an oncogene, participated in NF- $\kappa$ B signaling pathway. For instance, ZBTB7 reduces Bcl-2 expression through NF- $\kappa$ B in hepatocellular carcinoma (44). In the current study, NF- $\kappa$ B altered in ZBTB7-driven 5-FU resistance in CRC. 5-FU inhibited cell viability and overexpression of ZBTB7 depleted the inhibitory effects. NF- $\kappa$ B inhibitor SN50 reversed the proliferation enhancing effects of ZBTB7 *in vitro* and *in vivo*. Finally, clinical data was analyzed and indicated that patients with high expression of ZBTB7 have worse prognosis under 5-FU adjuvant therapy.

In conclusion, the finding indicated that the ZBTB7/NF- $\kappa$ B axis contributes to 5-FU resistance of patients with CRC, and may be serve as potential therapeutic targets to overcome 5-FU resistance.

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#### Availability of data and materials

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

#### Authors' contributions

ZW and XZ performed the cellular and animal studies, the statistical analysis and drafted the manuscript. WW and MZ

analyzed the GEO database. YiL, YaL, JG and GX performed the cellular studies. CW and RL participated in designing the study. QZ was involved in designing the study and drafting the manuscript and revising it critically for important intellectual content and given final approval of the version to be published. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Ethics approval for the animal experiments was provided by The Institutional Animal Care and Use Committee of Chongqing Fuling Central Hospital (ethics approval no. 2014015).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no conflicts of interests.

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