

# Downregulation of ZNF365 by methylation predicts poor prognosis in patients with colorectal cancer by decreasing phospho-p53 (Ser15) expression

CHAN WANG<sup>1\*</sup>, SHUIPING LIU<sup>2\*</sup>, YEYE KUANG<sup>1</sup>, XIAOTONG HU<sup>1,3</sup> and XIAO FANG<sup>4</sup>

<sup>1</sup>Biomedical Research Center and Key Laboratory of Biotherapy of Zhejiang Province, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, Zhejiang 310016; <sup>2</sup>Department of Cancer Pharmacology and Key Laboratory of Elemene Class Anti-Cancer Chinese Medicine of Zhejiang Province, Holistic Integrative Pharmacy Institutes, College of Medicine, Hangzhou Normal University, Hangzhou, Zhejiang 310012; Departments of <sup>3</sup>Pathology and <sup>4</sup>Anesthesiology and Key Laboratory of Biotherapy of Zhejiang Province, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, Zhejiang 310016, P.R. China

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**Abstract.** ZNF365 is a transcription factor that plays important roles in different types of cancer, such as colorectal cancer, breast cancer and hepatocellular carcinoma. ZNF365 can promote stalled replication fork recovery to prevent genomic instability, which is a notable feature of sporadic and hereditary types of cancers. However, the function of ZNF365 in the tumor progression of colorectal cancer (CRC) remains unclear. Thus, immunohistochemical staining was used to investigate the association between ZNF365 expression and the clinicopathological characteristics of patients with colorectal cancer. The results demonstrated that ZNF365 protein was strongly expressed in the nucleus and cytoplasm of normal colorectal mucosa. Furthermore ZNF365, which is methylated and down-regulated in most cancer cell lines and tissues, was significantly associated with lymph node metastasis ( $P=0.015$ ), depth of invasion ( $P=0.031$ ) and histopathological grading ( $P=0.042$ ). A positive correlation was observed between ZNF365 expression and phosphorylated (P)-p53 (Ser15) protein expression

( $r=0.18$ ;  $P=0.038$ ). Survival analysis indicated that patients with high ZNF365 expression had a higher survival rate than those with low ZNF365 expression ( $P=0.009$ ), suggesting that ZNF365 may be an independent prognostic factor for survival in colorectal cancer ( $P=0.046$ ). Taken together, the results of the present study demonstrated that ZNF365 was frequently inactivated by promoter methylation and independently predicted poor prognosis in patients with colorectal cancer by downregulating P-p53 (Ser15) expression.

## Introduction

Colorectal cancer (CRC) is the most common gastrointestinal malignancy, as well as the third most lethal and fourth most diagnosed type of cancer in the world, despite recent advancements in the treatment of CRC (1). CRC is caused by genetic and epigenetic alterations, including histone and chromatin structural modification, DNA methylation and microRNA (miRNA) aberrations (2). DNA methylation usually leads to the hypermethylation of gene promoters and the inactivation of tumor suppressor genes (TSGs), which plays an important role in the initiation, development and recurrence of CRC (3). Thus, identification of TSGs that undergo CpG island hypermethylation and assessment of their roles and associated molecular mechanisms in tumor progression will help to develop more effective diagnosis and individualized therapeutic strategies for patients with CRC (3,4).

The zinc finger proteins (ZNFs) are classified into eight-fold groups according to the secondary structure around the zinc-binding site and main chain conformation (5). ZNFs have several functions, including apoptosis regulation, transcriptional activation, protein folding and integration, RNA packaging, DNA recognition and lipid binding (6). ZNFs have been reported to play important roles in different types of human cancer. Some ZNFs act as oncogenes, for example, ZNF306 expression is upregulated in CRC, whereas low ZNF306 expression suppresses tumor development (7). Furthermore, ZNF307 inhibits the activity of tumor suppressor

*Correspondence to:* Dr Xiao Fang, Department of Anesthesiology and Key Laboratory of Biotherapy of Zhejiang Province, Sir Run Run Shaw Hospital, Zhejiang University, 3 Qingchun East Road, Hangzhou, Zhejiang 310016, P.R. China  
E-mail: 3195046@zju.edu.cn

Dr Xiaotong Hu, Biomedical Research Center and Key Laboratory of Biotherapy of Zhejiang Province, Sir Run Run Shaw Hospital, Zhejiang University, 3 Qingchun East Road, Hangzhou, Zhejiang 310016, P.R. China  
E-mail: hxt\_hangzhou@zju.edu.cn

\*Contributed equally

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genes, p21 and p53 by increasing the transcription of EP300 and MDM2 (8). Conversely, some ZNFs act as tumor suppressors, for example, ZNF23 inhibits the proliferation of SK-OV-3 cells by enhancing p27/kip-1 expression (9). Furthermore, ZNF668 is considered a tumor suppressor in breast cancer, which stabilizes p53 by preventing MDM2-mediated ubiquitination and degradation of p53 (10).

ZNF365 contains the N-terminus-C2H2 zinc finger motif, and was first discovered in 1998 from the human brain cDNA library (11,12). It has been reported that polymorphisms in the ZNF365 gene or its locus is associated with different types of disease. For example, polymorphisms in the Ala62Thr of ZNF365 gene are associated with susceptibility to uric acid nephrolithiasis (12,13). Furthermore, variants of ZNF365 are associated with Crohn's disease (14). A total of five single nucleotide polymorphisms (SNPs) in ADO-ZNF365-EGR2 have been demonstrated to be associated with Vogt-Koyanagi-Harada (VKH) syndrome in patients with VKH, in Thailand (15). It has been reported that genetic variations in ZNF365 affected the risk of breast cancer by influencing the proportion of dense tissue in the breast (16). Additionally, variants at the ZNF365 loci are associated with estrogen receptor subtypes of breast cancer risk in BRCA1 and BRCA2 mutation carriers (17).

Although ZNF365 is known to play important roles in different types of human cancer, its function in CRC remains unknown. Thus, the present study aimed to investigate the association between ZNF365 expression and tumor progression of CRC, and determine its underlying molecular mechanism.

## Materials and methods

**Cell lines and culture conditions.** The CRC cell lines (Colo320, SW620, SW480, HCT116, SW48, LOVO, HCT8, DLD1, HT29 and RKO) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) or RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) medium supplemented with 10% fetal bovine serum (cat. no. VS500T; Ausbian), in a humidified 5% CO<sub>2</sub> incubator at 37°C.

**Tissue specimens.** For immunohistochemistry (IHC) analysis, 120 patients with CRC who underwent surgery at the Sir Run Run Shaw Hospital between February 2004 and June 2006 were recruited in the present study, and 10 normal colonic mucosa biopsy samples were selected as the normal controls (4 males and 6 females; mean age, 51.12 years; age range, 32–65 years). The present study was approved by the Ethics Committee of Sir Run Run Shaw Hospital (Hangzhou, China) and all patients provided written informed consent prior to the study start (approval no. 2019ZNF365-1). Patients who had received preoperative chemotherapy, radiotherapy or immunotherapy prior to surgery were excluded. A total of 79 men and 41 women, with a mean age of 63.6 years (age range, 28–89 years) were included in the present study.

Following surgical resection, the tissue samples were fixed at room temperature in 10% formalin for 24 h, embedded in paraffin and sectioned into 4-μm-thick slices. The intensity of ZNF365 immunostaining was scored by two experienced pathologists from Sir Run Run Shaw Hospital (Hangzhou,

China) who were unaware of the clinicopathological outcomes of the patients, using the World Health Organization classification guidelines (18). A typical section for each case was selected for IHC analysis.

Differentiation status was divided into three subtypes: i) Well differentiated, including papillary adenocarcinoma and high differentiated tubular adenocarcinoma; ii) moderately differentiated, including highly to moderately differentiated tubular adenocarcinoma and iii) poorly differentiated, including poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous adenocarcinoma and undifferentiated carcinoma. According to these criteria, there were 82 well differentiated, 25 well/moderately differentiated and 13 poorly differentiated types of cancer of the 120 cases. Lymph node metastasis and depth of invasion were graded based on the 7th edition of the International Union Against Cancer tumor-node-metastasis (TNM) system (19). All patients were followed up via telephone for 36 months.

ZNF365 methylation was assessed in normal colorectal (NC) tissues and paired CRC tissues, which were obtained from 30 patients in July 2009. The patients included 11 males and 19 females with a mean age of 56.2 years (age range, 41–68 years). The corresponding NC tissues were removed from the margin of the resection with a distance >10 cm away from the tumor.

**Semi-quantitative reverse transcription (RT)-PCR.** Semi-quantitative RT-PCR was performed as previously described (20). Total RNA was extracted from fresh cells (Colo320, SW620, SW480, HCT116, SW48, LOVO, HCT8, DLD1, HT29 and RKO) using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, RNA was converted to cDNA using RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). GAPDH mRNA was used as a control. The primers used in the present study are listed in Table SI.

**Methylation analysis of ZNF365.** To determine the molecular mechanism underlying aberrant ZNF365 expression in colorectal cancer, the association between ZNF365 mRNA expression and DNA methylation was assessed using the cBioPortal online database ([www.cbioportal.org](http://www.cbioportal.org)).

**GeneMANIA analysis.** GeneMANIA is a commonly used website for constructing protein-protein interaction networks and predicting the function of favorite genes (21). The GeneMANIA database (<http://genemania.org/>) was used to assess the association between ZNF365 and p53 expression levels.

**5-Aza-2'-deoxycytidine and trichostatin A treatment.** RKO cells that do not express ZNF365 were seeded into a 10-cm dish at a density of 1×10<sup>6</sup> cells/ml and cultured overnight in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were subsequently treated with demethylating agent 5-aza (Sigma-Aldrich; Merck KGaA) at a final concentration of 10 mM for 3 days and further treated with the histone deacetylase inhibitor TSA (Sigma-Aldrich; Merck KGaA) at a final concentration of 300 nmol/l for an additional 24 h at 37°C. Cells were collected for DNA and RNA extraction.

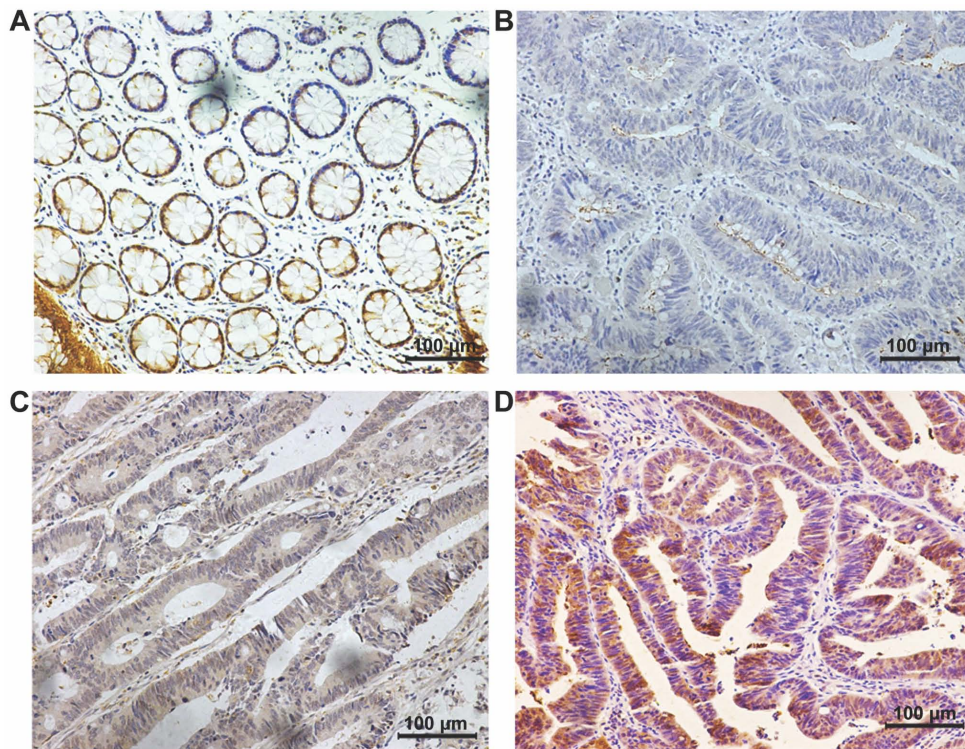


Figure 1. Representative images of immunohistochemical staining of ZNF365 in colorectal cancer tissues and normal colorectal mucosa. (A) ZNF365 expression in normal colorectal mucosa. (B) ZNF365 weak staining, (C) ZNF365 moderate staining and (D) ZNF365 strong staining in colorectal cancer tissues (magnification, x200).

**Bisulfite treatment and promoter methylation analysis.** Methylation-specific PCR (MSP), bisulfite modification of DNA and bisulfite genomic sequencing (BGS) were performed as previously described (22). The primer sequences used for MSP and BGS are listed in Table SI.

**IHC staining.** IHC staining was performed using the ChemMate™ EnVision™ detection kit (Dako; Agilent Technologies, Inc.) according to the manufacturer's instructions. Briefly, the CRC sections and the normal colonic mucosa biopsy samples were dewaxed and hydrated with 100% dimethylbenzene (cat. no. 1330-20-7; Shanghai Macklin Biochemical Co., Ltd.), and rehydrated in a descending ethanol series. Tissue sections were washed with deionized water and phosphate buffered saline (PBS). The antigen retrieval process was performed with 0.01 M citrate buffer (pH 6.0; cat. no. C1013; Beijing Solarbio Science & Technology Co., Ltd.). After cooling to room temperature, the tissue sections were blocked with 3% hydrogen peroxidase-methanol solution for 30 min to inhibit endogenous peroxidase activity, and subsequently incubated in preimmunized goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) for 30 min, both at room temperature. Tissue sections were incubated with rabbit polyclonal IgG primary antibody directed against ZNF365 (1:50; cat. no. HPA052446; Atlas Antibodies) overnight at 4°C. After warming to room temperature, the tissue sections were washed five times with PBS and subsequently incubated with ChemMateEnVision/HRP, Rabbit/Mouse reagent (Dako; Agilent Technologies, Inc.) at room temperature for 30 min. The sections were stained with ChemMate DAB+chromogen (Dako; Agilent Technologies, Inc.) and

counterstained with hematoxylin at room temperature for 1 min each. Tissue sections were dehydrated in an ascending ethanol series and dimethylbenzene, and were observed using a light microscope (magnification, x200).

**Evaluation of staining.** A total of two independent pathologists from Sir Run Run Shaw Hospital (Hangzhou, China) blindly assessed the slides three times to determine the percentage of positive cells, staining intensity and subcellular localization. ZNF365 expression was scored using the World Health Organization classification system. The percentage of positive cells was scored as follows: 0, 0-10; 1, 11-25; 2, 26-50; and 3, 51-100%. The intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate and 3, strong. The final immunoreactivity score (IRS) was equal to the sum of both scores, which ranged from 0-9. The specimens were divided into two groups according to the IRS value, high expression group (IRS 6-9) and low expression group (IRS 0-5) in order to assess the association between ZNF365 expression and clinicopathological characteristics.

**Statistical analysis.** Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). The Kaplan-Meier method was used to assess the survival curve and statistical differences were determined using the log-rank test. Pearson's  $\chi^2$  test and Fisher's exact test were used to assess the association between ZNF365 expression with clinicopathological characteristics. Cox's proportional hazards regression model was used to perform univariate (depth of invasion, tumor location, distant metastasis, sex, age, differentiation, lymph node metastasis, TNM stage and ZNF365 expression) and

Table I. Association between ZNF365 expression and clinicopathological characteristics of patients with colorectal cancer (n=120).

Characteristic	Number of patients, n (%)	ZNF365 expression		$\chi^2$	P-value
		Low, n (%)	High, n (%)		
Total	120	54 (45.000)	66 (55.000)		
Sex					
Male	79 (65.833)	33 (41.772)	46 (58.228)	0.973	0.324
Female	41 (34.167)	21 (51.220)	20 (48.780)		
Age, years					
$\geq 63$	62 (51.667)	28 (45.162)	34 (54.838)	0.001	0.971
<63	58 (48.333)	26 (44.828)	32 (55.172)		
Tumor location					
Rectum	77 (64.167)	32 (41.558)	45 (58.442)	1.028	0.311
Colon	43 (35.833)	22 (51.163)	21 (48.837)		
Histopathological grading					
G1 (Well)	82 (68.333)	35 (42.683)	47 (57.317)	6.349	0.042 <sup>a</sup>
G2 (Moderate)	25 (20.833)	16 (64.000)	9 (36.000)		
G3 (Poor)	13 (10.833)	3 (23.077)	10 (76.923)		
Depth of invasion					
pT1/T2	34 (28.333)	10 (29.412)	24 (70.588)	4.658	0.031 <sup>a</sup>
pT3/T4	86 (71.667)	44 (51.163)	42 (48.837)		
Lymph node status					
N0	68 (56.667)	24 (35.294)	44 (64.706)	5.973	0.015 <sup>a</sup>
N1/2	52 (43.333)	30 (57.692)	22 (42.308)		
Distant metastasis					
M0	101 (84.167)	43 (42.574)	58 (57.426)	1.517	0.218
M1	19 (15.833)	11 (57.895)	8 (42.105)		
TNM stage					
I/II	63 (52.500)	24 (38.095)	39 (61.905)	2.555	0.110
III/IV	57 (47.500)	30 (52.632)	27 (47.368)		

<sup>a</sup>P<0.05. pT, pathological tumor stage; TNM, tumor-node-metastasis.

multivariate analyses. Relative risk of mortality is presented as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CI). P<0.05 was considered to indicate a statistically significant difference.

## Results

**ZNF365 expression in CRC tissues.** ZNF365 expression was assessed in 120 cases of CRC and 10 normal colonic mucosa biopsy samples were used as the normal controls. Representative immunostaining images of ZNF365 in CRC tissues are presented in Fig. 1. The results demonstrated that ZNF365 was expressed in the cytoplasm and nucleus of cancer tissues. Patients were divided into two groups according to the IRS value, high expression group (IRS 6-9) and low expression group (IRS 0-5). A total of 54 cases of CRC tissues (45%) were classified into the low ZNF365 expression group, while the remaining 66 cases (55%) were classified into the high ZNF365 expression group (Table I).

**Association between ZNF365 expression and clinicopathological characteristics of patients with CRC.** The association between ZNF365 expression and clinicopathological characteristics of patients with CRC are presented in Table I. The results demonstrated that ZNF365 was significantly associated with lymph node metastasis (P=0.015), depth of invasion (P=0.031) and histopathological grading (P=0.042). However, there were no significant associations between ZNF365 expression and age (P=0.971), sex (P=0.324), distant metastasis (P=0.218), tumor location (P=0.311) or TNM stages (P=0.11).

**Downregulation of ZNF365 is associated with poor survival of patients with CRC.** All patients were followed up for overall survival following surgery, to further determine the role ZNF365 plays in the progression of CRC. The prognosis of patients with high and low ZNF365 expression was assessed (Fig. 2). Kaplan-Meier survival analysis demonstrated that the 3-year survival rate was higher in patients with high ZNF365



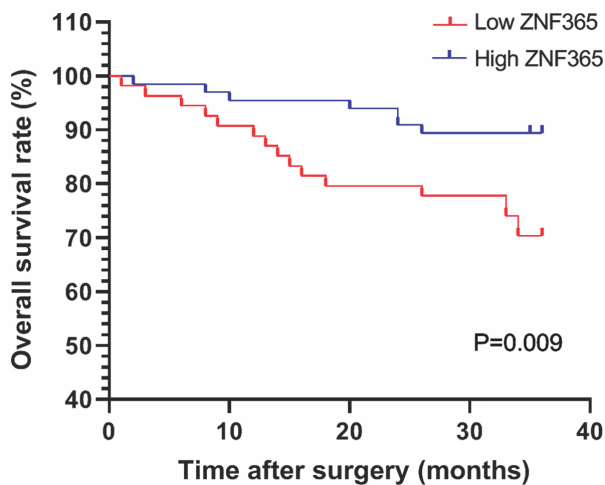


Figure 2. Kaplan-Meier survival curve of patients with colorectal cancer according to ZNF365 expression. The 3-year overall survival rate of patients with low ZNF365 expression was significantly lower than that of patients with high ZNF365 expression.

expression than those with low ZNF365 expression (Fig. 2;  $P=0.009$ ). Univariate analysis demonstrated that in addition to ZNF365 expression ( $P=0.013$ ), lymph node metastasis ( $P<0.001$ ), tumor histopathological grading ( $P=0.011$ ), distant metastasis ( $P<0.001$ ), depth of invasion ( $P=0.044$ ) and TNM stages ( $P<0.001$ ) were also significantly associated with 3-year overall survival rates (Table II).

Multivariate analysis demonstrated that tumor location (HR, 2.818; 95% CI, 1.173-6.770;  $P=0.021$ ), histopathological grading (HR, 1.907; 95% CI, 1.07-3.389;  $P=0.028$ ), TNM stage (HR, 4.801; 95% CI, 1.912-12.053;  $P=0.001$ ) and ZNF365 expression (HR, 0.386; 95% CI, 0.152-0.980;  $P=0.045$ ) were all statistically significant prognostic factors in CRC (Table III). Collectively, these results indicate that ZNF365 may be a valuable prognostic factor in CRC.

**Correlation between ZNF365 expression and p53 expression in CRC patients.** p53 plays a significant role in the development of tumors (23). The GeneMANIA database was used to assess the association between ZNF365 and p53 expression levels. The results demonstrated that ZNF365 can interact with p53 via RPRM and MAP4 (Fig. 3).

IHC analysis was performed to confirm the correlation between ZNF365 protein expression and total p53 and P-p53 (Ser15) protein expression in 120 cases of CRC. No significant correlation was observed between ZNF365 expression and total p53 expression, while ZNF365 expression was positively correlated with P-p53 (Ser15) protein expression, with a correlation coefficient of 0.189 ( $P=0.038$ ; Table IV). Representative IHC staining images of ZNF365 and P-p53 (Ser15) in CRC tissues are presented in Fig. 4.

**ZNF365 is downregulated by methylation in most CRC cell lines and tissues.** In order to determine the molecular mechanism by which ZNF365 expression is decreased in CRC, the cBioPortal database was used to assess the association between ZNF365 expression and DNA methylation. The results demonstrated a statistically significant negative correlation between ZNF365

Table II. Univariate survival analysis of prognostic factors in colorectal cancer.

Characteristics	HR	95% CI	P-value
Sex			0.419
Male vs. Female	1.468	0.579-3.723	
Age, years			0.561
$\geq 63$ vs. $<63$	1.277	0.560-2.912	
Tumor location			0.067
Rectum vs. Colon	2.148	0.948-4.871	
Histopathological grading			0.011 <sup>a</sup>
Well vs. Moderate vs. Poor	1.914	1.163-3.15	
Depth of invasion			0.044 <sup>a</sup>
T1+T2 vs. T3+T4	2.629	1.025-6.744	
Lymph node metastasis			$<0.001^b$
N0 vs. N1/2	3.111	1.84-5.258	
Distant metastasis			$<0.001^b$
M0 vs. M1	6.326	2.792-14.496	
TNM stage			$<0.001^b$
I/II vs. III/IV	2.863	1.723-4.756	
ZNF365 expression			0.013 <sup>a</sup>
Low vs. High	0.324	0.133-0.787	

<sup>a</sup> $P<0.05$ ; <sup>b</sup> $P<0.001$ . HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.

Table III. Multivariate survival analysis of prognostic factors in colorectal cancer.

Characteristic	HR	95% CI	P-value
Sex			0.784
Male vs. Female	1.149	0.424-3.115	
Age, years			0.616
$\geq 63$ vs. $<63$	1.249	0.524-2.973	
Tumor location			0.021 <sup>a</sup>
Rectum vs. Colon	2.818	1.173-6.770	
Histopathological grading			0.028 <sup>a</sup>
Well vs. Moderate vs. Poor	1.907	1.073-3.389	
Depth of invasion			0.336
T1 vs. T2+T3+T4	0.582	0.193-1.753	
TNM stage			0.001 <sup>b</sup>
I+II vs. III+IV	4.801	1.912-12.053	
ZNF365 expression			0.045 <sup>a</sup>
Low vs. High	0.386	0.152-0.980	

<sup>a</sup> $P<0.05$ ; <sup>b</sup> $P<0.001$ . HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.

gene expression and DNA methylation (Spearman,  $-0.32$ ;  $P=1.016 \times 10^{-4}$ ; Fig. 5). Semi-quantitative RT-PCR analysis was

Table IV. Correlation between ZNF365 expression and p53 expression in patients with colorectal cancer (n=120).

p53 expression	Number of patients, n (%)	ZNF365 expression		r value	P-value
		Low, n (%)	High, n (%)		
Total p53					
Low	40 (33.333)	21 (52.500)	19 (47.500)	0.107	0.247
High	80 (66.667)	33 (41.250)	47 (58.750)		
Phospho-p53 (Ser15)					
Low	52 (43.333)	29 (55.769)	23 (44.231)	0.189	0.038
High	68 (56.667)	25 (36.765)	43 (63.235)		

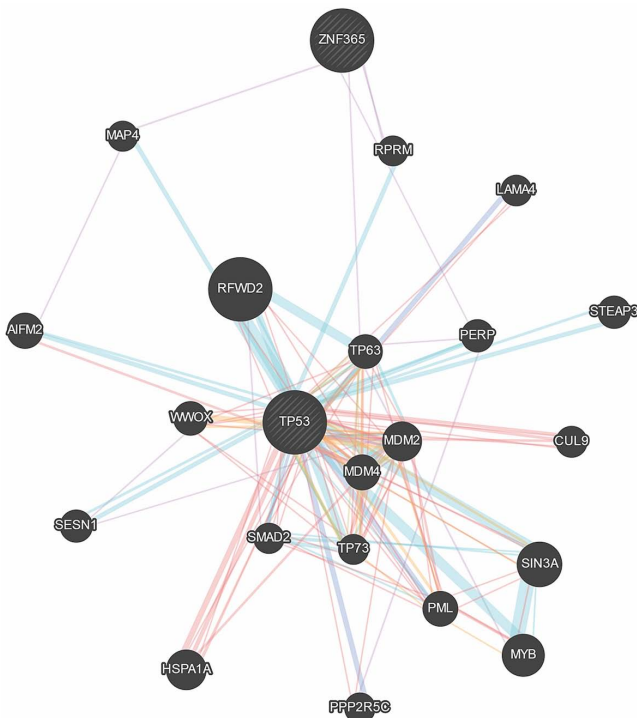


Figure 3. Interaction network between ZNF365 and p53. ZNF365 can interact with p53 through RPRM and MAP4, as predicted by the GeneMANIA database.

subsequently performed to assess ZNF365 expression in CRC cell lines (Colo320, SW620, SW480, LOVO, HCT116, SW48, HCT8, DLD1, HT29 and RKO). The results demonstrated that ZNF365 expression was downregulated or even silenced in most cell lines (Fig. 6A). In addition, MSP primers of ZNF365 were designed to determine its methylation status according to the ZNF365 CpG island (CGI) sequence (Fig. 7A). As expected, the ZNF365 promoter was methylated in the cell lines with decreased ZNF365 expression or ZNF365 silenced (Fig. 6A).

In order to further determine whether promoter methylation directly mediated silencing of ZNF365, its expression was compared before and after treatment in these cell lines, with 5-aza and TSA. The results demonstrated that ZNF365 expression significantly recovered following demethylation treatment in the assessed cell lines, (Fig. 6B). BGS analysis of 49 CpG sites was performed to determine the methylation profiles of

ZNF365 CGI, including those CpG sites analyzed by MSP (Fig. 7). Densely methylated CpG sites were detected in the cell lines without ZNF365 expression. Both BGS and MSP analyses demonstrated that the ZNF365 CGI was significantly demethylated following TSA and 5-aza treatment, suggesting a direct association between ZNF365 silencing and CGI methylation (Figs. 6B and 7B). Furthermore, MSP analysis was performed to detect ZNF365 methylation in 30 primary colorectal tumors (T) and paired adjacent normal tissues (N). In 63.3% (19/30) of cases, ZNF365 methylated bands in tumor tissues were stronger than the paired adjacent normal tissues (Fig. 6C), which was confirmed following BGS analysis (Fig. 7B).

## Discussion

ZNF365 serves as a transcription factor, playing key roles in transcriptional activation, protein folding, DNA recognition, lipid binding, RNA packaging and apoptosis regulation (7). The present study aimed to investigate the association between ZNF365 protein expression and the clinicopathological characteristics of patients with CRC. ZNF365, which is predominantly expressed in the cytoplasm and nucleus of CRC tissues (24), was significantly associated with histopathological grading, lymph node metastasis and depth of invasion. However, no significant associations were observed between ZNF365 expression and age, sex, tumor location, distant metastasis or TNM stage. Furthermore, the survival rate of patients with high ZNF365 expression was significantly higher than that of patients with low ZNF365 expression. ZNF365 expression was downregulated in tumor tissues, particularly in poorly differentiated and advanced CRC. The association between ZNF365 expression and CRC from a histological level was also assessed. Multivariate survival analysis demonstrated that ZNF365 protein expression may be used as a prognostic and diagnostic marker for CRC.

Previous studies have reported that genetic polymorphisms of ZNF365 are associated with immune-related diseases in Latin America and Asia (25,26). It has been demonstrated that ZNF365 is associated with atopic dermatitis (AD) in Japanese (26) and overall susceptibility to Crohn's disease in Canadian children (25). Variants of ZNF365 are also associated with susceptibility to breast cancer (16). A total of five SNPs in ADO-ZNF365-EGR2 have been reported to be associated with Vogt-Koyanagi-Harada (VKH)

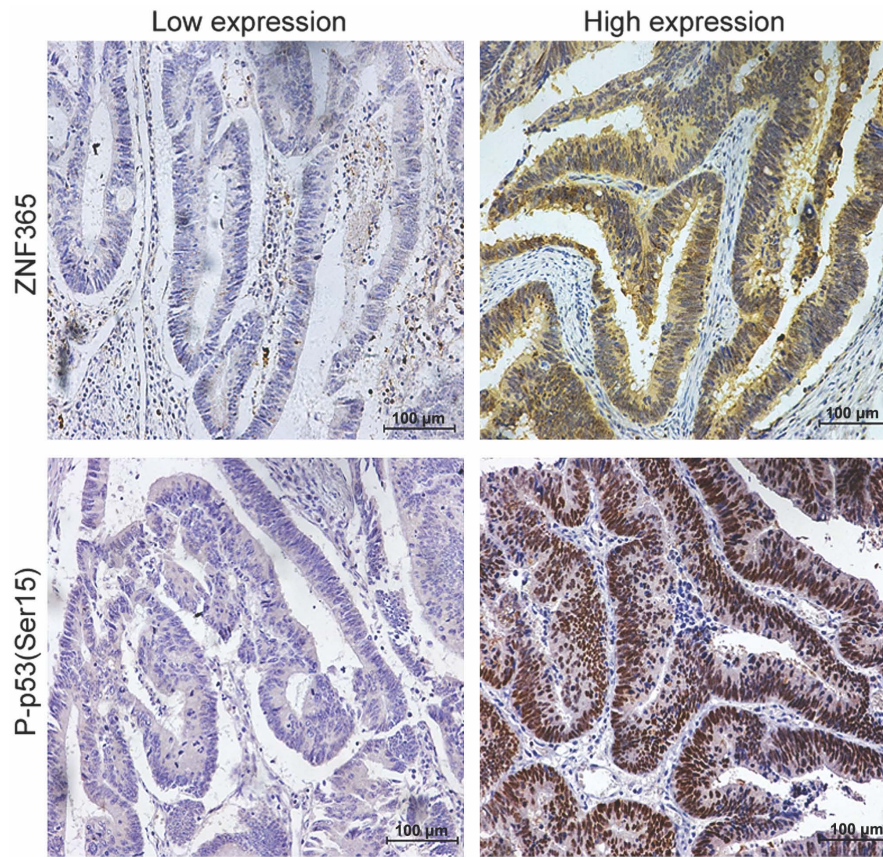


Figure 4. Images of immunohistochemical staining of ZNF365 and P-p53 (Ser15) in CRC tissues. ZNF365 protein expression was positively correlated with P-p53 (Ser15) expression in CRC (magnification, x200). P, phosphorylated; CRC, colorectal cancer.

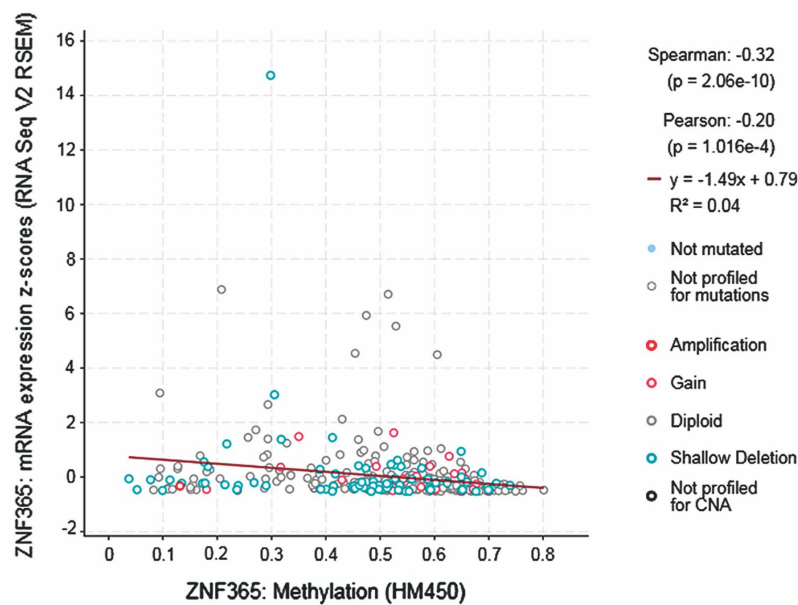


Figure 5. Correlation between ZNF365 mRNA expression and promoter methylation analyzed using the cBioPortal database. There was a significantly negative correlation between ZNF365 gene expression and DNA methylation. Seq, sequence; RSEM, RNA-Seq by Expectation Maximization; CNA, copy number alteration.

syndrome in Thai patients with VKH but not in other Asian patients (15). In addition, Zhang *et al* (24) demonstrated that ZNF365 acts as a transcriptional target of p53, which is a novel factor caused genomic instability (24). A mechanistic

study demonstrated that ZNF365 can suppress expression of a group of common fragile sites including telomeres, and thus, that polymorphisms in the ZNF365 locus are associated with an increased risk of cancer that may result from



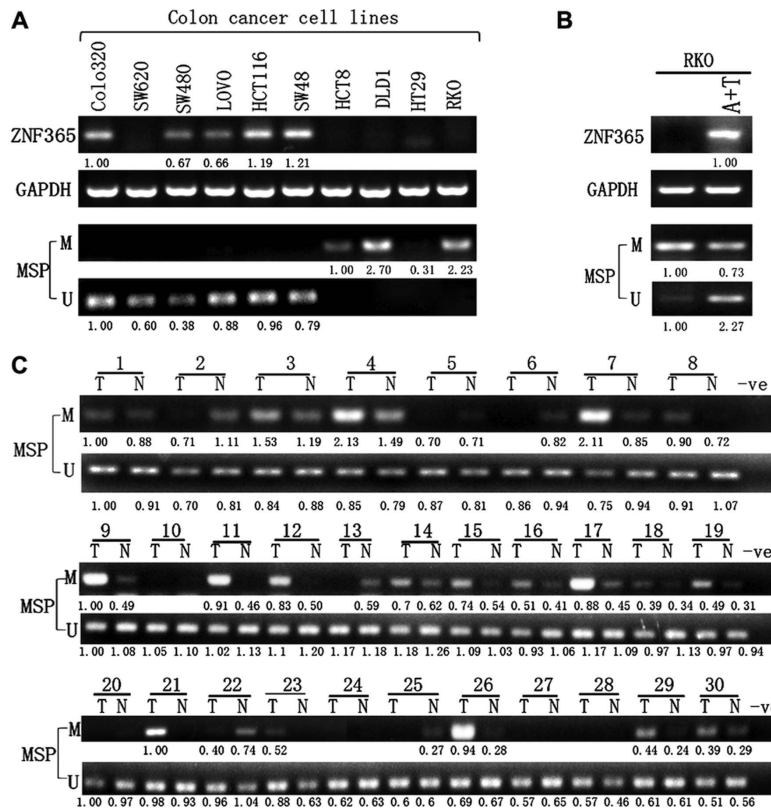


Figure 6. Expression and CGI methylation of ZNF365 in CRC cell lines and primary tumors. (A) ZNF365 expression was downregulated or silenced in several CRC cell lines due to its CGI methylation. (B) Pharmacological demethylation with 5-aza and TSA recovered ZNF365 expression in methylated and silenced cell lines. Typical results are presented. A+T, treatment with 5-aza and TSA. (C) Methylation of ZNF365 in primary T tissues and paired N tissues by MSP. CGI, CpG island; CRC, colorectal cancer; T, tumor; N, non-tumor; M, methylated; U, unmethylated; TSA, trichostatin A; MSP, methylation-specific PCR.

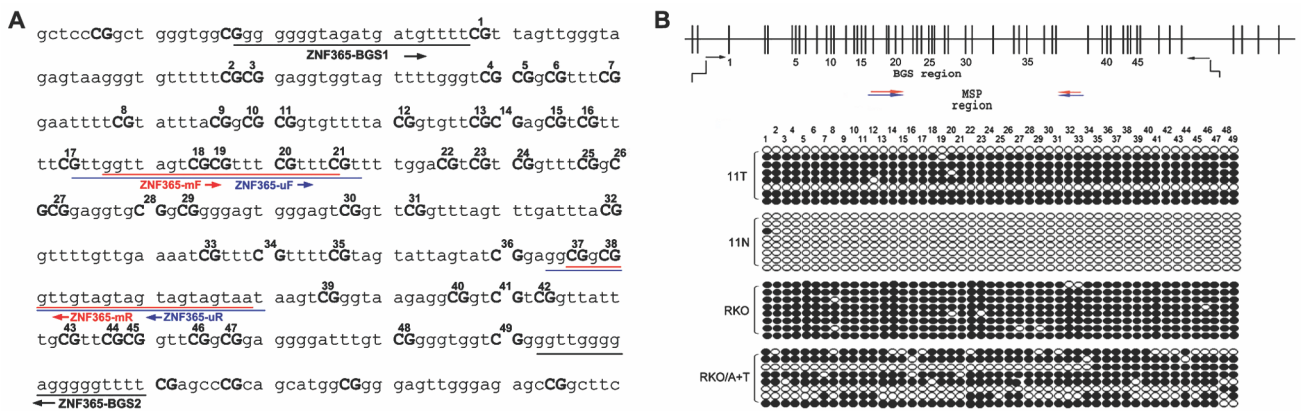


Figure 7. ZNF365 CGI methylation in colorectal cancer cell lines and primary tumors. (A) Sequence of ZNF365 CGI with locations of the 49 CpG sites analyzed and primers used. MSP and BGS regions are also presented. (B) Each vertical line indicates individual CpG sites. Products of cloned BGS-PCR were sequenced and each clone is exhibited as an individual row, representing a single allele of the CGI. Open circles represent unmethylation, while filled circle represent methylation. CGI, CpG island; BGS, bisulfite genomic sequencing.

telomere dysfunction (24). Another study revealed that when induced by DNA double strand break signals, ZNF365 can participate in the homologous recombination repair pathway and maintain genomic integrity during DNA replication by interacting with poly(ADP-ribose) polymerase 1 to tether MRE11 to the DNA end resection site (27). Telomere dysfunction triggers inaccurate DNA repair followed by genomic instability, which is a feature of almost all types of human cancer (28).

p53, activated as a transcription factor, plays an important role in preventing tumorigenesis and tumor progression (29). Following DNA damage, p53 protein rapidly accumulates through post-transcriptional mechanisms, which induces growth arrest or apoptosis (30,31). In some cells with defective DNA repair, p53 accumulation is absent or delayed (32). However, mutant p53, detected in >50% of cases of solid cancers, loses its ability to specifically bind to sequences of genes that respond to senescence, cell cycle arrest and



apoptosis, which results in the transformation of this tumor suppressor into an oncogenic factor (33-35). The results of the present study demonstrated that ZNF365 expression did not correlate with total p53 expression; however, it was positively correlated with phospho-p53 (Ser15) expression. Upon DNA damage, p53 is phosphorylated at serine double 15, which results in decreased interaction of p53 with its negative regulator, the tumor protein MDM2, both *in vitro* and *in vivo* (32). This suggests that ZNF365 acts as a suppressor in CRC tumorigenesis and progression by downregulating phospho-p53 (Ser15) expression.

Regarding the expression control mechanisms, the results of the present study demonstrated that ZNF365 expression was downregulated or even silenced in most cell lines. ZNF365 expression was also downregulated in tumor tissues, particularly in advanced cancer and poorly differentiated cancer. Downregulation of ZNF365 expression may be due to its methylation (36), which was detected in 63.3% of CRC tumors and only 36.7% of adjacent non-tumor tissues. DNA methylation is a vital component in multilevel gene control in eukaryotes (37). Increasing evidence demonstrates that methylation levels and patterns are deranged in tumor cells (38,39). DNA methylation, together with other epigenetic changes such as RNA editing, affects chromatin structure and thus regulates processes, including allele-specific expression of imprinted genes, X-chromosome inactivation, transcription and inactivation of tumor suppressor genes (40). It was also demonstrated that in the Colo320 and SW620 cell lines, unmethylated alleles coexisted with silencing, suggesting that other expression control mechanisms, such as deletions may also be involved. However, promoter methylation is the main cause of downregulation of ZNF365 (36), and the results of the present study suggest that ZNF365 methylation is a common cancer-specific event in CRC.

The present study is not without limitations. First, as this was a single retrospective study, more research is required to determine the underlying molecular mechanisms between ZNF365 and P-p53. To the best of our knowledge, the present study was the first to investigate the association between ZNF365 expression and CRC. Taken together, the results suggest that downregulation of ZNF365 by methylation may independently predict poor prognosis in patients with CRC, by decreasing P-p53 (Ser15) expression.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

XH, CW, SL, YK and XF designed the study, analyzed data and approved the version to be published. CW performed the experiments and drafted the manuscript. SL and YK revised it critically for important intellectual content. XF reviewed the manuscript and gave final approval of the version to be published. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sir Run Run Shaw Hospital (Hangzhou, China) and all patients provided written informed consent prior to the study start (approval no. 2019ZNF365-1).

## Patient consent for publication

Patients agreed to the use of their samples in scientific research and provided consent for publication of their information.

## Competing interests

The authors declare that they have no competing interests.

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