

# ZNF703 promotes tumor cell proliferation and invasion and predicts poor prognosis in patients with colorectal cancer

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**Abstract.** Zinc finger protein 703 (ZNF703), identified as an oncogene in luminal B breast cancer, is a member of the NET/NIZ family of zinc finger transcription factors. However, the role of ZNF703 in colorectal cancer (CRC) is unknown. We investigated the expression of ZNF703 in paired tumor and corresponding normal tissues using reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot analysis. Immunohistochemistry (IHC) was applied on paraffin-embedded specimens, including 138 CRC tissues, 58 matched normal tissues and 30 paired metastatic lymph node samples. Levels of mRNA (72.72%, 16/22) and ZNF703 protein expression (65.38%, 17/26, respectively) were upregulated in CRC tissues. IHC staining revealed higher expression of ZNF703 in the CRC tissues (68/138, 49.3%) compared with that in the adjacent normal mucosal tissues (4/58, 6.9%) ( $P < 0.001$ ). Moreover, high ZNF703 expression was significantly correlated with tumor size, pathological grading, serosal invasion, lymph node metastasis and AJCC stage. CRC patients with relatively low ZNF703 expression had higher survival rates than those with high ZNF703 expression. In addition, we investigated ZNF703 expression in eight CRC cell lines (LS174T, SW480,

HT29, SW620, DLD1, SW1116, LoVo and CaCo-2) *in vitro*. The highest ZNF703 expression was detected in the LoVo cell line. RNA interference was used to assess the effects of ZNF703 knockdown in LoVo cells. Knockdown of ZNF703 expression inhibited CRC cell proliferation and migration. Collectively, these results reveal that ZNF703 may act as an oncogene in CRC and could be considered as a potential therapeutic target for metastatic CRC.

## Introduction

Colorectal cancer (CRC) is the third most frequent malignancy, and represents the fourth most common cause of cancer-related deaths in the world (1). Despite many advances in the management of early and advanced CRC, the 5-year survival rate of CRC patients remains much lower than expected (2). In the past 20 years, the encouraging improvement in patient outcome has been followed by a plethora of markers of prognosis and response to anticancer therapy, although most are not clinically viable. Nevertheless, several protein and genetic markers that predict prognosis and treatment benefit have been validated (3). The development and progression of CRC is a multistep process that occurs due to the accumulation of numerous genetic alterations, including epigenetic modifications, chromosomal abnormalities and mutations in genes that regulate proliferation, differentiation, apoptosis and angiogenesis (4).

Genes that are altered by amplification and result in concomitant overexpression in tumors are considered candidate oncogenes (5). Human ZNF703 (also known as Noca-like zinc finger protein 1, *NLZ1*) is a member of the NET gene family, which is located on chromosome 8 (8p11.23). ZNF703 protein contains six evolutionarily conserved domains, three of which have not been previously described and are specific for NET proteins (6). Several studies have revealed an amplification of the 8p11-12 chromosomal region associated with human breast cancers, particularly with the luminal B subtype (7-16). In addition, two recent studies have strongly

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**Abbreviations:** ZNF703, zinc finger protein 703; CRC, colorectal cancer; NLZ1, Noca-like zinc finger protein 1; ER<sup>+</sup>, estrogen receptor-positive

**Key words:** ZNF703, colorectal cancer, prognosis, proliferation, migration

suggested the possible role of *ZNF703* as a potential breast oncogene (15,16). For example, *ZNF703* expression is correlated with poor clinical prognosis in estrogen receptor-positive (ER<sup>+</sup>) breast cancer patients. Overexpression of *ZNF703* induces cell proliferation and interferes with transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling in breast epithelial cells (15). Furthermore, *ZNF703* is overexpressed in luminal B-type breast cancer cell lines, MDA-MB-134 and HCC1500, while its expression is low in the normal mammary epithelial cell line MCF-10A (17). In addition, relatively high expression of *ZNF703* was found in luminal breast cancers, and is associated with an intermediate tumor grade (17), and high *ZNF703* mRNA expression is correlated with poor survival in patients with ER<sup>+</sup> luminal B tumors (18). In addition, *Znf703* (Zeppl, zinc finger elbow-related proline domain protein 1) was identified as a human *ZNF703* ortholog in mice, which has been shown to regulate proliferation, migration and cell adhesion in mouse mammary epithelial cells (19). Moreover, *Znf703* was found to regulate transcription by repressing E-cadherin, Wnt and TGF- $\beta$  reporter expression, and to increase lung metastases in a mouse breast cancer model (19).

Based on these findings, clinical correlations and experimental data, it appears that *ZNF703* meets the classical definition of an oncogene in luminal B breast tumors. However, to the best of our knowledge, there are no reports on its role in the development and progression of gastrointestinal malignancies. In the present study, we examined the expression of *ZNF703* in primary CRC tissues and, using cell line models, aimed to determine its biological role in CRC.

## Materials and methods

**Cell lines.** CRC cell lines (LS174T, SW480, HT29, SW620, DLD1, SW1116, LoVo and CaCo2) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Protein and RNA samples were extracted from subconfluent cells during the exponential phase of growth.

**Patients and samples.** Fresh frozen CRC tissue samples and their paired normal mucosal samples (10-cm distance from the tumor), as well as CRC metastatic lymph nodes, were collected at the Department of General Surgery, Nanfang Hospital, Guangzhou, China. In addition, formalin-fixed and paraffin-embedded tumor tissues (including 58 paired normal mucosal tissues) from 138 patients with a pathological diagnosis of CRC, who had undergone colonoscopy at the Department of Gastroenterology between 2008 and 2009, were also included in the present study. All patients had undergone elective surgery for CRC at Nanfang Hospital. The comprehensive set of clinicopathological data were obtained from the Tumor Tissue Bank of Nanfang Hospital. Approval for the study was obtained from the Ethics Committee of Guangzhou Southern Medical University in China, and written informed consent was obtained from all patients.

**RNA extraction, reverse-transcription and semi-quantitative RT-PCR.** Total RNA was extracted from the colorectal speci-

mens using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed in a total volume of 25  $\mu$ l with 3  $\mu$ g of total RNA using a RevertAid First Strand cDNA Synthesis kit (Promega, Madison, WI, USA). Next, 2  $\mu$ l of cDNA was used as a template to amplify the *ZNF703* fragment using the following primers (F, 5'-GATCAGGGTCCCTG AAGATGC-3' and R, 5'-CCGAGTTGAGTTTGGAGGAG-3') and the GoTaq<sup>®</sup> Green Mix kit (Promega), under the following conditions: 95°C for 2 min; 35 cycles of 95°C for 30 sec, 56°C for 30 sec, and 73°C for 30 sec; with final extension of 73°C for 5 min. The GAPDH gene was used as an internal control and amplified using the following primers: F, 5'-TATGATGATA TCAAGAGGGTAGT-3' and R, 5'-TGTATCCAAACTCATT GTCATAC-3'.

**Western blot analysis.** Tissues and cells were lysed in RIPA. Cytosolic and nuclear extract were prepared with a protocol of ProteoExtract subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). The supernatant was collected, and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

After boiling, the proteins (25  $\mu$ g) were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions and transferred to a polyvinylidene fluoride membrane (PVDF) (Millipore Corp., Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T), and then incubated for 1 h at room temperature with rabbit polyclonal antibody against human *ZNF703* (GTX107721; GeneTex, Inc., Irvine, CA, USA) (1:1,000). Mouse monoclonal anti-GAPDH (CW0100A; CWBIO, Beijing, China) or TOPO I (GTX63013; Genetex Inc., Irvine, CA, USA) was used as loading control. Next, membranes were incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (sc-45106; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse immunoglobulin G (sc-2962; Santa Cruz Biotechnology). The membranes were developed with a horseradish peroxidase chemiluminescence detection reagent (ECL Plus System; Millipore Corp.) and then exposed to Hyperfilm ECL (Millipore Corp.).

**Immunohistochemical analysis.** Formalin-fixed and paraffin-embedded tissues were cut into 4- $\mu$ m sections, deparaffinized with xylene and rehydrated through a graded series of ethanol/water. Next, the slides were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) in a water bath for 15 min at 100°C. All of the specimens were then preincubated with 10% normal bovine serum and incubated with a primary polyclonal rabbit antibody against human *ZNF703* (1:100) (GTX107721; GeneTex) overnight at 4°C. The slides were next incubated with a biotinylated goat anti-rabbit immunoglobulin G antibody, and the reaction products were visualized using diaminobenzidine (DAB; Dako, Carpinteria, CA, USA) with methyl green as a counterstain. For negative controls, the primary antibodies were omitted, but otherwise the protocol was the same.

Immunostaining in each tumor was classified into four categories depending on the intensity of staining of cancer cells as negative (0), weak (1) moderate (2) or strong (3). The percentage of stained cells was determined using the following scale: <5% (0), 5-25% (1), 26-50% (2), 51-75% (3) and >75% (4).

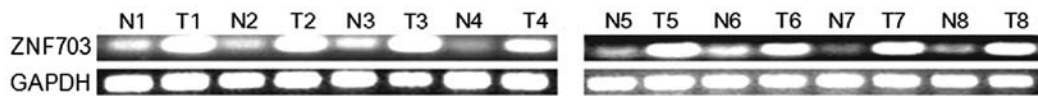


Figure 1. *ZNF703* mRNA expression in human CRC tissues and adjacent normal colorectal mucosal tissues as detected by semi-quantitative RT-PCR (GAPDH as internal control). N, normal tissue; T, tumor tissue.

The final score was obtained by multiplying these two values. This resulted in an overall *ZNF703* immunohistochemical score of 0, 1, 2, 3, 4, 6, 9 or 12. *ZNF703* expression was considered low when scores were  $\leq 4$ , and high when scores were  $\geq 6$ .

**Small interfering RNA (siRNA)-mediated *ZNF703* silencing.** Expression of human *ZNF703* was knocked down with siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) duplexes 5'-CCACACACUUUGGGCCUAAAdTdT-3' (forward) and 5'-dTdTCCACACACUUUGGGCCUAA-3' (reverse) targeting the 3'UTR of endogenous *ZNF703*. The negative control siRNA 5'-UUCUCCGAACGUGUCACGUTT-3' (forward) and 5'-ACGUGACACGUUCGGAGAATT-3' (reverse) targeting an unknown mRNA sequence was used as a control. Exponential growth phase cells were plated in 6-well plates at a density of  $2 \times 10^5$  cells/ml, cultured for 48 h and transfected with 1  $\mu$ g of siRNA in reduced serum medium (OPTI-MEM-I; Invitrogen) according to the manufacturer's protocol at 30-50% confluency.

**In vitro cell growth assay.** Forty-eight hours after siRNA transfection, cells were prepared at a concentration of  $1 \times 10^4$  cells/ml. Aliquots (100  $\mu$ l) were dispensed into 96-well plates, and cells were incubated for 1, 2, 3, 4, 5 or 6 days. Following incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 ml of MTT (5 mg/ml; Promega) for 4 h. Next, the supernatants were removed, and 150  $\mu$ l of dimethylsulfoxide (Sigma, St. Louis, MO, USA) was added to each well. Fifteen minutes later, the absorbance value [optical density (OD)] of each well was measured at 490 nm with a microplate reader. All experiments were repeated three times.

**Wound closure assay.** Cells transfected with siRNA-*ZNF703* and negative control were seeded on 6-well culture plates. When cell confluency reached  $\sim 80\%$  at 48 h post-transfection, cells were scratched with a sterile 1-ml pipette tip and rinsed with medium to remove any free-floating cells and debris. Complete culture medium was then added, and plates were incubated at  $37^\circ\text{C}$ . Wound closure was observed at 0, 24 and 48 h, and representative scrape lines were photographed. Duplicate wells were examined for each condition, and each experiment was repeated three times.

**Transwell migration assay.** Cells in serum-free medium ( $2 \times 10^5$  cells/200  $\mu$ l) were added to the upper chamber of 8- $\mu$ m pore size Transwell chambers (Corning Star, Cambridge, MA, USA). The bottom chambers contained 10% FCS as a chemoattractant. Cells were allowed to migrate through the porous membrane for 24 h at  $37^\circ\text{C}$ . Non-migrating cells that remained on the upper surface of the filter were removed with cotton swabs, and the remaining cells on the lower surface of

the filter were fixed with 100% methanol, stained with crystal violet and counted under a bright field microscope in eight different fields (Nikon E400,  $\times 100$ ). Each experiment was independently performed three times.

**Statistical analysis.** Statistical software SPSS 16.0 (Statistical Package for the Social Sciences; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. The association between *ZNF703* expression and CRC clinicopathological features was analyzed using a  $\chi^2$  test. The Kaplan-Meier method was used to analyze cumulative survival rate, and differences among groups were estimated using the log-rank test.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

***ZNF703* mRNA expression in the CRC tissues.** We analyzed *ZNF703* mRNA expression in CRC tissues and adjacent normal colorectal mucosal tissues by semi-quantitative RT-PCR. *ZNF703* mRNA expression was elevated in the majority of CRC tissues compared with their normal pairs (16/22, 72.72%); representative RT-PCR results of *ZNF703* mRNA expression are presented in Fig. 1.

***ZNF703* protein expression in CRC tissues.** We also examined expression of *ZNF703* protein in the CRC tissues and adjacent normal colorectal mucosal tissues by western blot analysis. Despite the interindividual variations, the expression of *ZNF703* protein was significantly upregulated in the CRC tissues (17/26, 65.38%) (Fig. 2A). The change in *ZNF703* protein expression pattern was similar to that observed at the mRNA level.

In order to further investigate *ZNF703* protein expression and subcellular localization, we performed IHC analysis in paraffin-embedded CRC tissues, paired adjacent normal colorectal mucosal tissues and metastatic lymph nodes from the same CRC patient. Normal colorectal mucosa typically expressed low *ZNF703* protein (54/58, 93.10%). Contrary to this, expression of *ZNF703* in CRC tissues was detected in a large proportion of the samples (68/138, 49.27%) and was mainly localized in the nucleus (58/138, 42.02%) and cytoplasm. In addition, the majority of metastatic lymph nodes were positive for *ZNF703* (21/23, 91.30%). Representative results of the *ZNF703* IHC analyses are shown in Fig. 2B.

***ZNF703* expression and its relation to CRC progression and clinicopathological parameters.** To elucidate the role of *ZNF703* in the progression of CRC, we examined the correlation between *ZNF703* expression and clinicopathological features of the CRC patients including age, gender, tumor size, tumor location, pathological differentiation, serosal invasion, lymph node metastasis and AJCC stage (Table I). Expression of *ZNF703* was closely

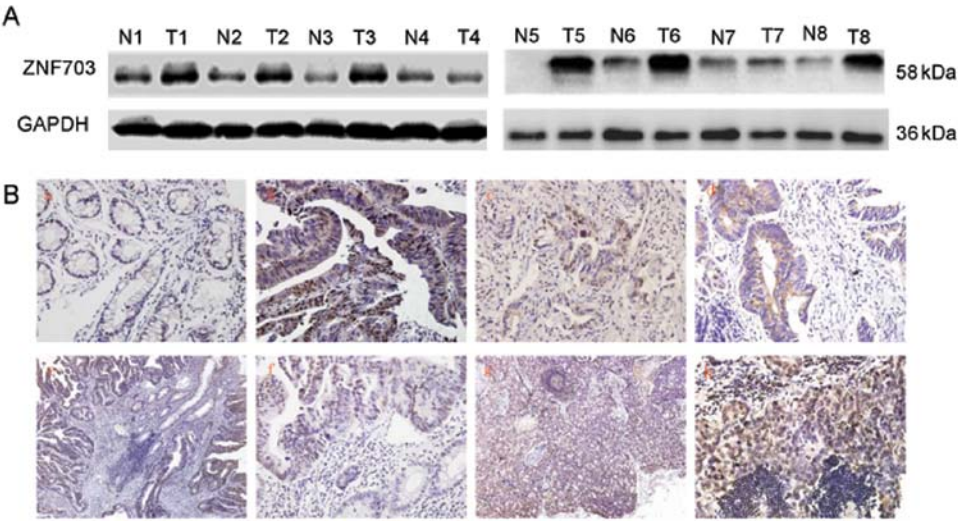


Figure 2. ZNF703 protein expression in human normal colorectal mucosa, CRC tissues and metastatic lymph nodes. (A) Human colorectal carcinoma tissues (T1-T8) and adjacent normal colorectal mucosal tissues (N1-N8) were analyzed by western blot analysis. (B) Immunohistochemical analysis of ZNF703 protein expression: (a) Negative expression in normal colorectal mucosa (original magnification, x400); (b) strong positive nuclear and weak cytoplasmic expression in CRC (original magnification, x400); (c) moderate positive nuclear and weak cytoplasmic expression (original magnification, x400); (d) moderate positive cytoplasmic expression in CRC cells (original magnification, x400); (e and f) moderate-strong positive nuclear expression in CRC cells but negative expression in normal colorectal mucosa of the same sample (original magnification, x100 and x400); (g and h) positive expression in CRC metastasis but negative expression in normal lymph nodes of the same sample (original magnification, x100 and x400).

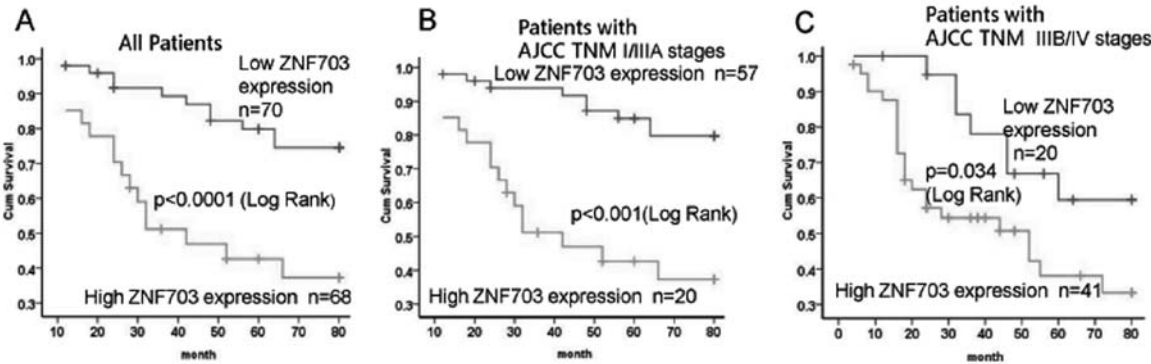


Figure 3. Kaplan-Meier survival analysis of CRC patients in relation to ZNF703 protein expression. The log-rank test was used to calculate P-values: (A) survival of all patients ( $P<0.001$ , Log Rank); (B) patients in stage AJCC I-III of CRC ( $P<0.001$ , Log Rank); and (C) patients in stage AJCC IIIB-IV of CRC ( $P=0.034$ , Log Rank).

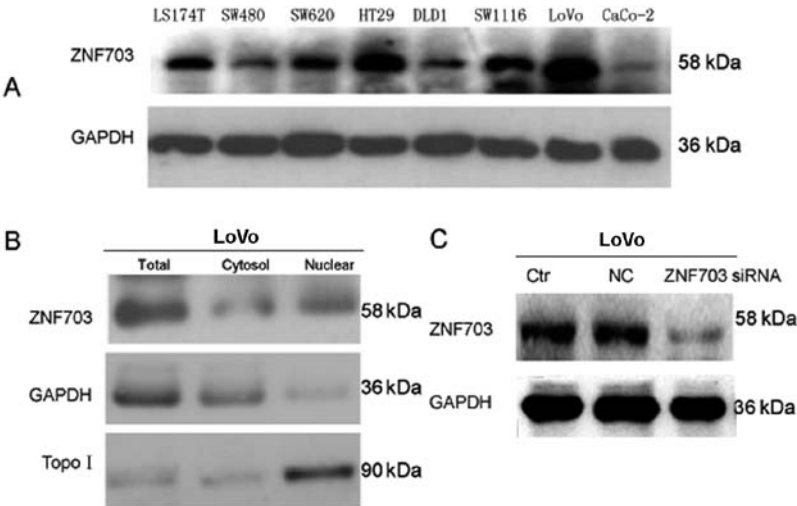


Figure 4. ZNF703 protein expression in cell lines analyzed by western blot analysis. (A) ZNF703 expression in the CRC cell lines (LS174T, SW480, HT29, SW620, DLD1, SW1116, LoVo and CaCo-2). (B) ZNF703 was expressed in the cytoplasm, and nucleus of LoVo cells. (C) ZNF703 expression was inhibited in LoVo cells by transfection with si-ZNF703 siRNA but not by transfection with the negative control siRNA.

Table I. ZNF703 expression and clinicopathological parameters of the 138 CRC patients.

Parameters	Total n	ZNF703 expression		$\chi^2$	P-value
		Score ≤4 n (%)	Score >6 n (%)		
Age (years)	70	68		2.585	0.108
≤60	84	38 (45.2)	46 (54.8)		
>60	54	32 (59.3)	22 (40.7)		
Gender					
Male	88	48 (54.5)	40 (45.5)	1.419	0.234
Female	50	22 (44.0)	28 (56.0)		
Tumor location					
Colonic and ileocecal	77	41 (53.2)	36 (46.8)	0.443	0.5006
Rectal	61	29 (47.5)	32 (52.5)		
Tumor size (cm)					
>3	75	31 (41.3)	44 (58.7)	5.797	0.016
≤3	63	39 (61.9)	24 (38.1)		
Pathological grading					
Well	76	46 (60.5)	30 (39.5)	6.502	0.011
Moderate/poor	62	24 (38.7)	38 (61.3)		
Serosal invasion					
Present	80	34 (42.5)	46 (57.7)	5.151	0.023
Absent	58	36 (62.1)	22 (37.9)		
Lymph node metastasis					
Present	62	23 (37.1)	39 (62.9)	8.365	0.004
Absent	76	47 (61.8)	29 (38.2)		
AJCC TNM stages					
I-IIIa	78	50 (64.1)	27 (35.9)	14.074	0.000
IIIB-IV	60	20 (33.3)	41 (66.7)		
Tissues					
Carcinoma	138	70 (50.7)	68 (49.3)	31.558	0.000
Normal colorectal mucosa	58	54 (93.1)	4 (6.9)		

associated with tumor size ( $P=0.016$ ), pathological grading ( $P=0.011$ ), serosal invasion ( $P=0.023$ ), lymph node metastasis ( $P=0.004$ ) and AJCC stage ( $P<0.001$ ). In addition, we examined the association between ZNF703 expression and cancer-specific survival using a Kaplan-Meier curve and log-rank test. The difference in patient survival between ZNF703-negative and weakly positive tumors was not significant. Patients in these two categories were then combined and resubjected to Kaplan-Meier analysis in comparison with patients with high ZNF703 expression. Patients with high ZNF703 expression had significantly shorter cancer-specific survival than patients with either negative or low ZNF703 expression ( $P<0.001$  for

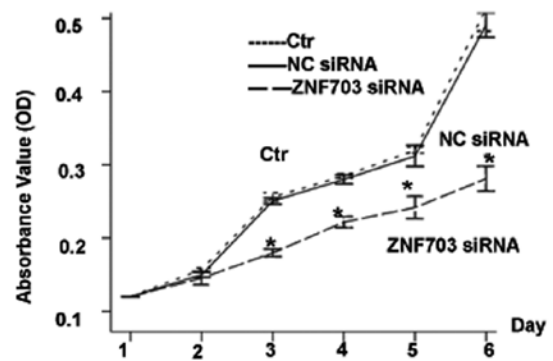


Figure 5. *In vitro* silencing of ZNF703 inhibits cell proliferation. Data are presented as mean  $\pm$  SD ( $n=3$ ,  $^*P<0.01$  by t-test, comparison of ZNF703 siRNA-transfected cells and negative control siRNA-transfected cells) of the absorbance optical density (OD) of LoVo cells. Significant differences in the OD values among days 3, 4, 5 and 6 were not ed.

all patients in Fig. 3A). This relationship was more obvious in patients with AJCC stage I-IIIa CRC ( $P<0.001$ ; Fig. 3B) than in AJCC stage IIIB-IV patients ( $P=0.034$ ; Fig. 3C). Unfortunately, the prognostic value of ZNF703 expression was not evident in patient subgroups stratified according to AJCC stage IIIB-IV (Fig. 3). Furthermore, multivariate analysis failed to confirm overexpression of ZNF703 as an independent prognostic factor for CRC.

**ZNF703 protein expression in cell lines.** Next, we analyzed ZNF703 expression and function at the cellular level. Expression of ZNF703 in CRC cell lines (LS174T, SW480, HT29, SW620, DLD1, SW1116, LoVo and CaCo-2) was examined by western blot analysis (Fig. 4A). The highest ZNF703 expression was detected in LoVo cells (Fig. 4A). Therefore, LoVo cells were selected for further experiments.

Based on the results of IHC staining in tumor cells, we decided to examine expression of ZNF703 in protein extracts of LoVo cell, cytoplasm and nuclei by western blot analysis (Fig. 4B). As expected, ZNF703 was expressed in LoVo cell, cytoplasm and nuclei.

***In vitro* silencing of ZNF703 affects the function of LoVo cells.** We investigated the role of ZNF703 in the display of aggressive phenotypes of CRC cells *in vitro*. siRNA transfection was employed to knock down ZNF703 expression in LoVo cells with high endogenous ZNF703 expression, and high proliferation and invasion capability. Western blot analysis showed 80% knockdown of the ZNF703 protein when compared to cells treated with negative control siRNA or to untreated cells (Fig. 4C).

As shown by MTT assays, ZNF703 knockdown inhibited cell proliferation when compared with the control cells ( $P<0.0001$ ) (Fig. 5). We also used a wound-healing assay to examine the impact of ZNF703 expression on migration of LoVo cells. ZNF703 inhibited the migration of LoVo cells that had been physically wounded and incubated for 24 or 48 h (Fig. 6A). To analyze invasiveness, another important feature of malignant cells, we performed Transwell invasion assays using cell culture inserts covered with extracellular matrix components; ZNF703/RNAi-transfected cells had relatively weak invasive abilities (Fig. 6C). Collectively, the wound-healing

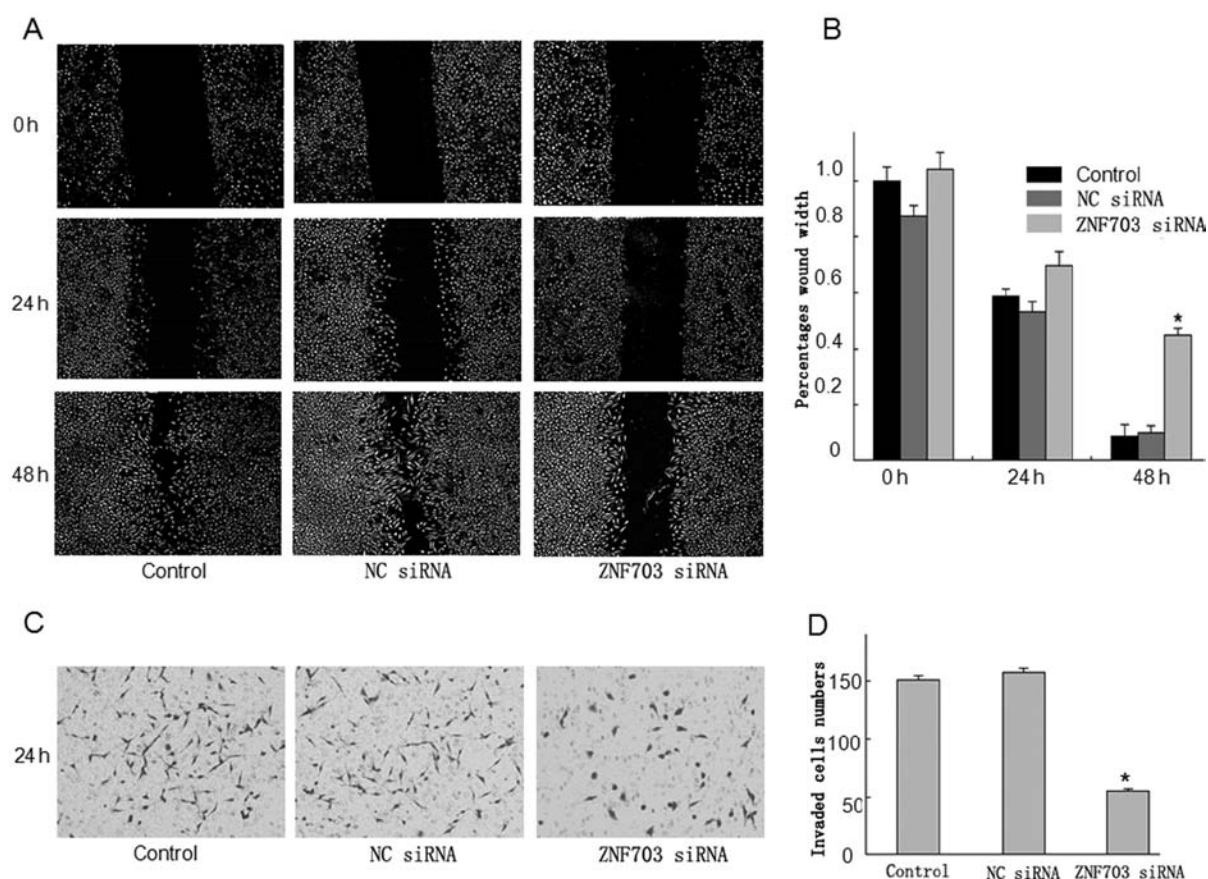


Figure 6. Inhibition of ZNF703 expression facilitates the migration and invasion of LoVo cells. Migration and invasion of cells treated with ZNF703 siRNA (or negative control siRNA) were analyzed at 48 h post-infection. (A) Wound-healing assays of cells treated with blank control, negative control siRNA or ZNF703 siRNA. Migration of cells to the wound was visualized at 24 and 48 h with an inverted Leica phase-contrast microscope (original magnification,  $\times 100$ ). (B) A histogram reveals the relative migratory distances of the cells. Data are presented as means  $\pm$  SEM ( $n=3$ ,  $^*P<0.01$  by t-test, comparison of the blank control and negative control siRNA-transfected cells). (C) Transwell assays of cells treated with blank control, negative control siRNA or ZNF703 siRNA. The ZNF703 siRNA-transfected cells showed lower invasion rates compared with blank controls and negative control siRNA-transfected cells. (D) The number of cells that invaded through the membrane was counted in eight fields under a  $\times 10$  objective lens. Data are presented as mean  $\pm$  SEM from 3 independent experiments. Bars, SD.  $^*P<0.01$  by t-test.

and invasion assays indicated that downregulation of ZNF703 expression inhibited migration and invasion of LoVo cells.

## Discussion

ZNF703 is an oncogenic transcription factor that regulates expression of numerous genes involved in multiple aspects of the cancer phenotype, including proliferation, increased self-renewal and invasion (13-17).

The aim of the present study was to examine the role of ZNF703 in CRC. According to our results, ZNF703 expression was higher in CRC tissues than in normal colorectal mucosa at both the mRNA and protein levels. IHC staining revealed that the subcellular localization of ZNF703 was mainly in the nucleus, and partly in the cytoplasm or membrane of CRC cells. In addition, elevated ZNF703 expression was correlated with serosal invasion, lymph node metastasis and AJCC stage. CRC patients with low ZNF703 expression had higher survival rates. At the cellular level, CRC cell lines had higher ZNF703 expression than the normal 297T cell line. Due to their relatively high ZNF703 expression, LoVo cells were chosen for siRNA silencing of ZNF703. Based on these knockdown experiments, ZNF703 silencing led to reduced cell prolifera-

tion and migration. Collectively, these findings indicated that ZNF703 may not be important in the differentiation of cancer cells, but may play an important role in the progression and metastasis of CRC.

Our findings in regards to the role of ZNF703 in CRC are in agreement with the results of a previous study examining the role of ZNF703 in breast cancer. According to the study of Zhang *et al* (17), ZNF703 is overexpressed in a number of breast cancer cell lines, while its expression is low in normal mammary epithelial cells. In addition, high ZNF703 expression contributes to tumor aggressiveness (17). The mechanisms leading to ZNF703 overexpression in human tumors are not well established, and it still remains to be determined how ZNF703 expression or function is regulated. In addition, major downstream effectors of oncogenic functions of ZNF703 still remain elusive.

ZNF703 plays a role in tamoxifen resistance induced by activation of the Akt/mTOR signaling pathway and down-regulation of ER $\alpha$ , providing a potential mechanism of its action in tumorigenesis (18). In addition, ZNF703 regulates transcription in mouse Eph4.9 cells, complexing with Groucho and repressing E-cadherin, Wnt and TGF- $\beta$  reporter expression (19). Possible mechanisms of its activation include

point mutation, gene amplification, gene rearrangement and insertion of strong promoter or enhancer. Epigenetic modifications including demethylation and deacetylation may also be responsible for activating ZNF703 in tumorigenesis. Nevertheless, the exact mechanisms leading to ZNF703 oncogenic activation have yet to be examined.

In conclusion, ZNF703 was upregulated in CRC patients, particularly in those with metastatic disease, implying an involvement in poor clinical outcomes. Although the molecular mechanism of ZNF703 action in carcinogenesis remains unexplored, we found that silencing ZNF703 inhibited cancer cell growth and migration *in vitro*. Hence, ZNF703 should be considered as a potential therapeutic target for metastatic colorectal disease.

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### References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 27: 2893-2917, 2010.
2. Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. *CA Cancer J Clin* 60: 277-300, 2010.
3. Tänzler M, Liebl M and Quante M: Molecular biomarkers in esophageal, gastric, and colorectal adenocarcinoma. *Pharmacol Ther* 140: 133-147, 2013.
4. Vogelstein B, Fearon ER, Hamilton SR, *et al*: Genetic alterations during colorectal-tumor development. *N Engl J Med* 319: 525-532, 1988.
5. Santarius T, Shipley J, Brewer D, Stratton MR and Cooper CS: A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer* 10: 59-64, 2010.
6. Pereira-Castro I, Costa AM, Oliveira MJ, Barbosa I, Rocha AS, Azevedo L and da Costa LT: Characterization of human NLZ1/ZNF703 identifies conserved domains essential for proper subcellular localization and transcriptional repression. *J Cell Biochem* 114: 120-133, 2013.
7. Garcia MJ, Pole JC, Chin SF, *et al*: A1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes. *Oncogene* 24: 5235-5245, 2005.
8. Gelsi-Boyer V, Orsetti B, Cervera N, *et al*: Comprehensive profiling of 8p11-12 amplification in breast cancer. *Mol Cancer Res* 3: 655-667, 2005.
9. Chin K, DeVries S, Fridlyand J, *et al*: Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell* 10: 529-541, 2006.
10. Adélaïde J, Finetti P, Bekhouche I, *et al*: Integrated profiling of basal and luminal breast cancers. *Cancer Res* 67: 11565-11575, 2007.
11. Kwek SS, Roy R, Zhou H, Climent J, Martinez-Climent JA, Fridlyand J and Ibertson DG: Co-amplified genes at 8p12 and 11q13 in breast tumors cooperate with two major pathways in oncogenesis. *Oncogene* 28: 1892-1903, 2009.
12. Melchor L, Garcia MJ, Honrado E, *et al*: Genomic analysis of the 8p11-12 amplicon in familial breast cancer. *Int J Cancer* 120: 714-717, 2007.
13. Haverty PM, Fridlyand J, Li L, *et al*: High-resolution genomic and expression analyses of copy number alterations in breast tumors. *Genes Chromosomes Cancer* 47: 530-542, 2008.
14. Sircoulomb F, Nicolas N, Ferrari A, *et al*: ZNF703 gene amplification at 8p12 specifies luminal B breast cancer. *EMBO Mol Med* 3: 153-166, 2011.
15. Holland DG, Burleigh A, Git A, *et al*: ZNF703 is a common Luminal B breast cancer oncogene that differentially regulates luminal and basal progenitors in human mammary epithelium. *EMBO Mol Med* 3: 167-180, 2011.
16. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM and Dunning MJ: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486: 346-352, 2012.
17. Zhang X, Mu X, Huang O, Xie Z, Jiang M, Geng M and Shen K: Luminal breast cancer cell lines overexpressing ZNF703 are resistant to tamoxifen through activation of Akt/mTOR signaling. *PLoS One* 8: e72053, 2013.
18. Reynisdottir I, Arason A, Einarsdottir BO, *et al*: High expression of ZNF703 independent of amplification indicates worse prognosis in patients with luminal B breast cancer. *Cancer Med* 2: 437-446, 2013.
19. Slorach EM, Chou J and Werb Z: Zeppol is a novel metastasis promoter that represses E-cadherin expression and regulates p120-catenin isoform expression and localization. *Genes Dev* 25: 471-484, 2011.