

ZNF280B promotes the growth of gastric cancer *in vitro* and *in vivo*

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Abstract. Zinc finger protein 280B (ZNF280B) mediates pro-survival and pro-growth functions in prostate cancer. However, in gastric cancer, its clinical significance remains poorly characterized. In the present study, the expression levels of ZNF280B in 60 patients with gastric cancer were examined using immunohistochemistry. The association between ZNF280B expression and clinicopathological features was assessed. Positive ZNF280B staining was demonstrated for 38 (63.3%) samples out of 60 gastric cancer cases in immunohistochemical analysis. ZNF280B expression was significantly associated with tumor size ($P=0.017$) and TNM stage ($P=0.001$). Furthermore, the proliferation index in the positive ZNF280B expression group was significantly higher (38.8 ± 6.2) compared with that of the negative ZNF280B expression group (16.9 ± 8.9 ; $P<0.01$). These results suggest that ZNF280B expression may be associated with the proliferation of gastric cancer cells. The role of ZNF280B in the growth of gastric cancer cells (MGC-803) was also investigated *in vitro* and *in vivo* by enhancing the expression of ZNF280B. A colony formation assay indicated that the number of colonies in the MGC-803 cells with enhanced ZNF280B (146 ± 5.8) was significantly higher than that of the MGC-803 control group (97 ± 5.1) and the negative control

group (101 ± 6.5 ; $P<0.05$). An MTT assay demonstrated that ZNF280B significantly promoted the proliferation of MGC-803 cells at days 3 and 4 ($P<0.05$). It was observed that the overexpression of ZNF280B may promote the growth of gastric cancer *in vivo* in xenograft studies. These findings indicate that ZNF280B may be a novel therapeutic target for gastric cancer.

Introduction

In recent years, gastric cancer incidence has gradually decreased in certain countries; however, it remains one of the main causes of cancer-associated mortality (1). Therefore, to improve patient prognosis, it is urgent that more biomarkers for the diagnosis and treatment of gastric cancer are identified. The development of gastric cancer is a complex process that includes the inactivation of tumor suppressor genes and the activation of oncogenes. p53, a well-established tumor suppressor, has a critical role in the anti-proliferative functions of cells. p53 mutations that lead to a protein loss-of-function are the most common genetic changes in cancer (2,3). Hence, it is important to find novel target genes that regulate p53 expression directly or indirectly.

Zinc finger (ZNF) proteins recognize various motifs in RNAs and proteins. By interacting with DNA and proteins, ZNF proteins can provide links between these molecule types (4). An increasing number of studies has reported that ZNF proteins serve critical roles in human cancers (5-15). For example, ZNF703 acts as an oncogene in invasive gastric carcinoma, and its expression levels are correlated with gastric carcinoma progression (16). The downregulation of ZNF306 expression reduces tumorigenicity in colorectal cancer (17). It is notable that certain ZNF proteins participate in tumorigenesis by influencing p53 activity. Through preventing Mdm2-mediated p53 degradation, ZNF668 has been considered an anti-oncogene in breast cancer (18). Similarly, ZNF307 inhibits p53 and p21 activity by enhancing EP300 and Mdm2 expression (19). In prostate cancer cells, ZNF280B induces p53 nuclear export, leading to subsequent proteasomal degradation (20). Therefore, ZNF280B serves pro-growth and pro-survival functions in prostate cancer cells.

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In the present study, the expression of ZNF280B in gastric cancer and its association with clinicopathological parameters were explored. Furthermore, the biological role of ZNF280B in growth of gastric cancer cells is investigated *in vitro* and *in vivo*.

Materials and methods

Patients and samples. A total of 60 gastric cancer specimens obtained from the Department of Pathology at The First Affiliated Hospital of Henan University of Science and Technology (Lyuoyang, China) from resections performed between July 2000 and March 2002 were examined. The study included 36 male and 24 female patients, with a mean age of 58.5 years (range, 45-72 years). Two experienced pathologists participated in the study and were blinded to the clinical information. Clinicopathological parameters for the patients are included in Table I, and the tissue samples were classified using a risk score TNM staging system (21). Informed consent was obtained on the collection of samples from each patient. The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital and the College of Clinical Medicine of Henan University of Science and Technology.

Immunohistochemistry. Staining for ZNF280B was performed using formalin-fixed, paraffin-embedded serial sections. Sections (4- μ m thick) were cut from the paraffin blocks and deparaffinized by routine techniques. The slides were microwaved in citrate buffer for 4 min for antigen retrieval. ZNF280B was detected with a rabbit polyclonal antibody (dilution, 1:150; cat. no., AP17865a; Abgent, Inc., San Diego, CA, USA). The antibody was incubated at 37°C for 3 h. The staining was detected with a biotinylated goat-anti rabbit secondary antibody, incubated at 37°C for 2 h (dilution, 1:50; cat. no., ZF-0311; OriGene Technologies, Inc., Rockville, Beijing, China), avidin-biotin complexes and diaminobenzidine (both 5 mg/ml; both Maxim Biomedical, Inc., Rockville, MD, USA), both incubated at 37°C for 5 min. Sections were then counterstained with hematoxylin at 37°C for 5 min. The expression of ZNF280B was scored according to the positive percentage and staining intensity of the stained tumor cells. The percentage was scored as 0 (0-25%), 1 (26-50%), 2 (51-75%) and 3 (>75%). The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained) and 3 (strongly stained). If the product of multiplication between staining intensity and the percentage of positive cells was ≥ 2 , it was considered immunoreaction positive (+). The goat anti-human monoclonal Ki-67 staining was achieved by a 2 h primary antibody incubation at 37°C (dilution, 1: 100; cat. no., D3B5; Gene Tech Biotechnology Co., Ltd., Shanghai, P.R. China) and a biotinylated-conjugated rabbit anti-goat immunoglobulin G secondary antibody incubation at 37°C for 2 h (dilution, 1:50; cat. no., ZF-0314; OriGene Technologies, Inc., Rockville, Beijing, China), and was scored according to the percentage of positively stained gastric cancer cells at high magnification (x200).

Cell lines and cell culture. Gastric cancer cell lines (SGC-7901, BGC-823 and MGC-803) were obtained

Table I. Association between ZNF280B expression and the clinicopathological features of gastric cancer patients.

Variable	ZNF280B expression			P-value
	n	Negative	Positive	
Sex				0.787
Male	36	14	22	
Female	24	8	16	
Age, years				0.591
≤ 60	38	15	23	
> 60	22	7	15	
Tumor size, cm				0.017 ^a
≥ 5	26	5	21	
< 5	34	17	17	
Tumor differentiation				0.573
Well/moderate	40	16	24	
Poor	20	6	14	
Tumor-node-metastasis stage				$< 0.001^a$
I/II	41	21	20	
III/IV	19	1	18	

^aP<0.05. ZNF280B, zinc finger protein 280B.

from the Department of Pathology, Guangdong Medical University (Zhanjiang, China). MGC-803 cells were stably transfected with pcDNA3.1-ZNF280B and pcDNA3.1 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were positively selected using G418 (500 μ g/ml) for 4 weeks. Surviving colonies were isolated and expanded. The cells stably transfected with pcDNA3.1-ZNF280B and pcDNA3.1 were designated as MGC-803/ZNF280B and negative control (NC) respectively. All cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μ g/ml penicillin and 100 μ g/ml streptomycin at 37°C with 5% CO₂.

Western blotting. Subsequent to washing in ice-cold PBS, cells were lysed in lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM PMSF, 5 μ l/ml leupeptin and 5 μ l/ml aprotinin]. The BCA Protein Assay Reagent kit (Pierce; Thermo Fisher Scientific, Inc.) was used to evaluate protein concentrations. Total protein (80 μ g) was boiled for 8 min prior to loading onto a 10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membrane was incubated with 5% non-fat dry milk overnight at 37°C. The membrane was incubated with the ZNF280B primary antibody (dilution, 1:200), followed by an HRP-conjugated secondary antibody (dilution, 1:2,000; cat. no., ZF-0311; Zhongshan Biology Co., Ltd., Foshan, China). Lastly, detected proteins were visualized with an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as internal/loading control

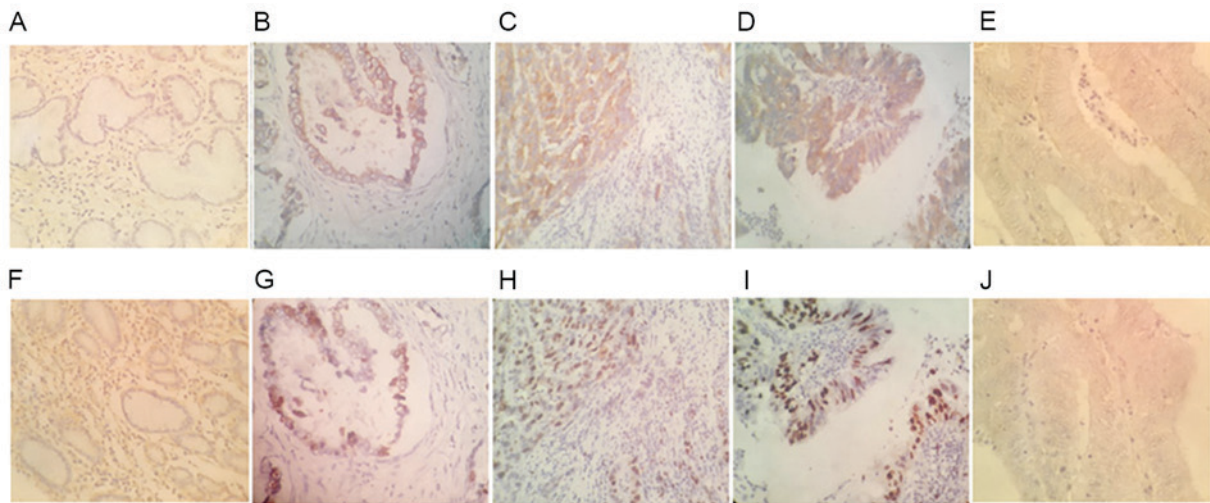


Figure 1. Immunohistochemical staining of ZNF280B and Ki-67 in gastric cancer tissues (magnification, x200). (A) Negative expression of ZNF280B in normal gastric tissues. (B-D) Positive ZNF280B staining in gastric cancer tissues, with the strongest staining intensity in D. (E) Negative ZNF280B staining in gastric cancer tissues. (F) Negative nuclear Ki-67 staining in normal gastric tissues. (G-I) Positive nuclear Ki-67 staining in gastric cancer tissues, with the strongest staining intensity in I. (J) Negative nuclear Ki-67 staining in gastric cancer tissues. ZNF280B, zinc finger protein 280B.

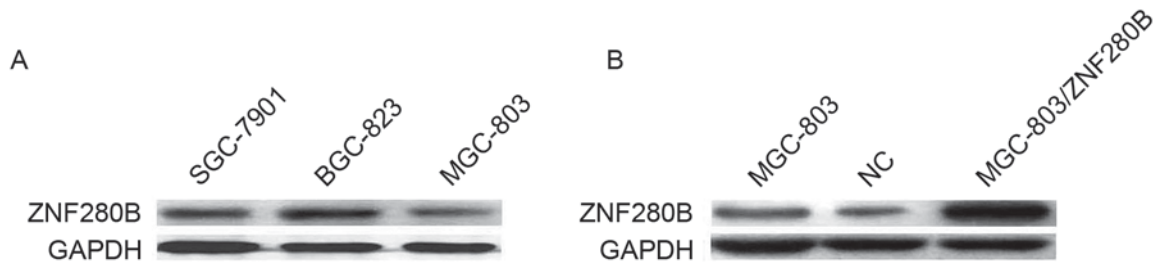


Figure 2. Expression levels of ZNF280B in gastric cancer cells. (A) Compared with SGC-7901 and BGC-823 cells, the expression of ZNF280B was low in MGC-803 cells. (B) Subsequent to transfection with a ZNF280B plasmid, the expression of ZNF280B was enhanced compared with MGC-803 and NC cells. ZNF280B, Zinc Finger protein 280B; NC, negative control.

(dilution, 1:400; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; catalog no., 365062).

MTT and colony formation assay. An MTT assay was used to detect the proliferation ability of MGC-803, NC and MGC-803/ZNF280B cells. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Following a 24-h incubation, 50 μ l MTT (5 mg/ml) was added to the medium. At 4 h, the medium was discarded, 150 μ l dimethyl sulfoxide was added into each well, and it was incubated with rocking for 10 min. Finally, the absorbance of each well was read at a wavelength of 492 nm. All experiments were performed in triplicate.

Subsequent to incubation for 24 h, cells from each group (MGC-803, NC and MGC-803/ZNF280B) were added to a six-well plate at 200 cells per well for the colony formation assay. Then, the tumor cells were incubated at 37°C and the medium was changed every 5 days for 2 weeks. Finally, the tumor cells were fixed by methanol, stained with trypan blue at 37°C for 20 min, and colonies were counted using a microscope at low magnification (x100) (Olympus Corporation, Tokyo, Japan). All experiments were performed in triplicate.

Xenograft studies. A total of 9 female BALB/c nude mice (4-5 weeks of age, 15-20 g) were purchased from the Shanghai

Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and kept in the Animal Center of Clinical Medicine of Henan University of Science and Technology. The temperature was maintained at 25-27°C and 40-60% relative humidity in a 12-h light/12-h dark cycle. Nude mice received sterilized water and food *ad libitum*. Mice received humane care, and all animal experiments were performed according to protocols approved by the Medical Ethics Committee of Henan University of Science and Technology. For the xenograft assay, nude mice (~8 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg) in a sterile environment. Then, 2×10^6 MGC-803, NC and MGC-803/ZNF280B cells in 50 μ l of PBS were subcutaneously injected into the mice (3 mice per group). The mice were sacrificed by cervical dislocation on day 25. Maximum tumor diameter exceeding 2 cm was deemed the humane endpoint of the study. Subsequently, the tumors were collected and the primary tumor weight was measured.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). The association between ZNF280B and clinicopathological parameters was assessed using Fisher's exact test. Multi-group comparisons were made with a one-way analysis of variance with a Student-Newman-Keuls post hoc test. $P < 0.05$ was

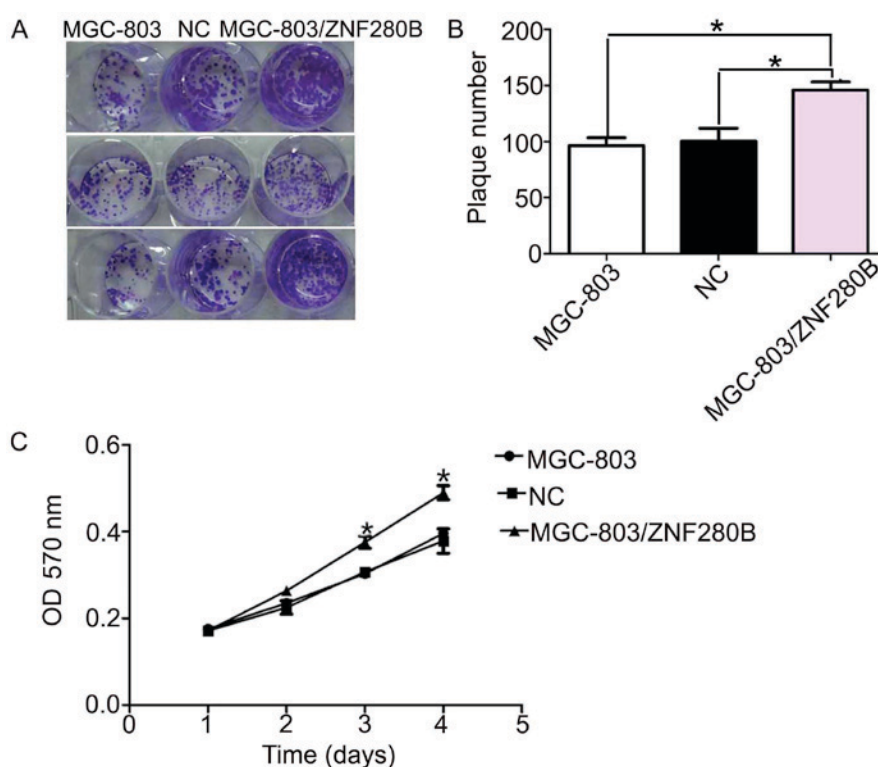


Figure 3. Effects of ZNF280B on cell proliferation and colony formation. (A) The number of MGC-803/ZNF280B cell colonies was significantly higher than MGC-803 or NC cells. (B) Quantification of part A. (C) At days 3 and 4, the MTT assay showed that proliferation of MGC-803/ZNF280B cells was significantly increased compared with that in MGC-803 and NC cells. * $P < 0.05$ vs. NC group. ZNF280B, zinc finger protein 280B; NC, negative control.

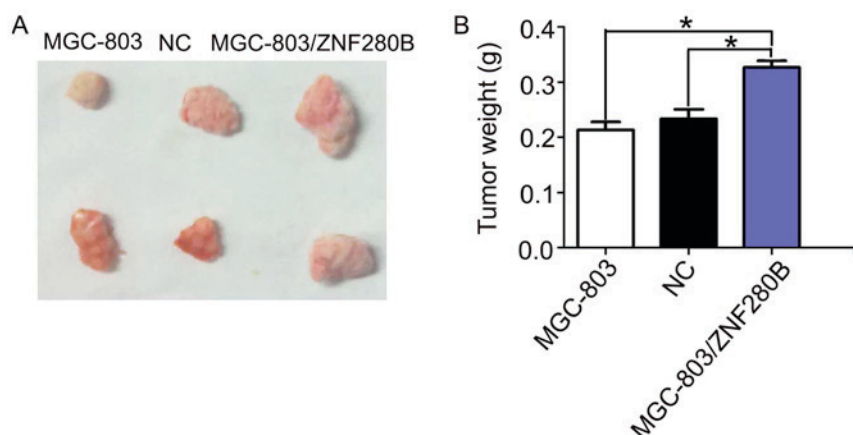


Figure 4. ZNF280B promoted the growth of tumors *in vivo*. (A) Representative images of tumors. (B) Tumor weight in the MGC-803/ZNF280B group was significantly greater than the MGC-803 and NC groups. * $P < 0.05$. ZNF280B, zinc finger protein 280B; NC, negative control.

considered to indicate a statistically significant difference. All results are expressed as the mean \pm standard deviation.

Results

ZNF280B expression is associated with the clinicopathological factors of patients with gastric cancer. To investigate the expression status of ZNF280B in gastric cancer, ZNF280B immunohistochemical staining was performed on 60 gastric cancer specimens. The results indicated that ZNF280B was distributed in the cytoplasm of gastric cancer cells (Fig. 1). Compared with the expression in normal gastric mucosal

tissues (Fig. 1A), 38 (63.3%) gastric cancer samples displayed ZNF280B-positive staining (Fig. 1B-D).

To further elucidate the clinical significance of ZNF280B in gastric cancer, the association between ZNF280B and clinicopathological characteristics was assessed. ZNF280B expression was identified in 21/26 (80.8%) cases of larger tumor size (≤ 5 cm) whereas only 17/34 (50.0%) smaller tumors exhibited positive ZNF280B immunoreactivity. Hence, ZNF280B expression was associated with tumor size ($P = 0.017$). In addition, only 20/41 (48.8%) cases in stage I and II exhibited positive ZNF280B staining, whereas ZNF280B was positively stained in 18/19 (94.7%) samples in stage III and IV

($P<0.001$). On the other hand, as demonstrated in Table I, no association existed between ZNF280B expression and other clinicopathological variables, including sex, age and differentiation degree ($P>0.05$).

ZNF280B expression is associated with the cell proliferation index (PI). To study the effect of ZNF280B on the proliferation of gastric cancer cells, the association between ZNF280B and PI was investigated. PI was determined with Ki-67 immunohistochemical staining. As demonstrated in Fig. 1, the positive ZNF280B expression group PI was significantly higher (38.8 ± 6.2) compared with that in the negative ZNF280B expression group (16.9 ± 8.9 ; $P<0.01$). These results suggest that ZNF280B expression may be associated with the rate of proliferation of gastric cancer cells.

ZNF280B transfection enhances ZNF280B protein expression in MGC-803 cells. The endogenous expression of ZNF280B was assessed in 3 gastric cancer cell lines, including SGC-7901, BGC-823 and MGC-803, and was revealed to be the lowest in MGC-803 cells, which were used for the subsequent experiments (Fig. 2A). As demonstrated in Fig. 2B, 48 h after MGC-803/ZNF280B transient transfection, ZNF280B protein expression was evidently increased in MGC-803 cells. These results indicate that ZNF280B expression was effectively upregulated subsequent to transfection.

ZNF280B overexpression enhances the proliferation and colony formation ability of MGC-803 cells. A colony formation assay indicated that the number of MGC-803/ZNF280B colonies (146 ± 5.8) was significantly higher than that of the MGC-803 (97 ± 5.1) and NC groups (101 ± 6.5 ; $P=0.039$ and $P=0.042$, respectively). However, there was no significant difference between the MGC-803 and NC groups ($P=0.369$) (Fig. 3A and B).

An MTT assay was performed to study the role of ZNF280B in the proliferation of MGC-803 cells. As demonstrated in Fig. 3C, ZNF280B significantly enhanced the proliferation of MGC-803 cells at days 3 and 4 ($P<0.05$). These results support the previous finding that ZNF280B expression is associated with tumor size.

Upregulated expression of ZNF280B promotes growth of gastric cancer cells in vivo. It was identified that ZNF280B acts as an oncogene in gastric cancer by promoting cell proliferation. It was further investigated whether ZNF280B would have a similar effect *in vivo*. MGC-803, MGC-803/ZNF280B and NC cells were subcutaneously injected into nude mice. It was found that upregulated ZNF280B expression significantly promoted tumor growth, compared with the MGC-803 and NC groups (Fig. 4). In addition, compared with the MGC-803 and NC groups, tumor weight was significantly heavier in the MGC-803/ZNF280B group ($P<0.05$). These results imply that upregulated ZNF280B expression promotes the growth of gastric cancer *in vivo*.

Discussion

There exist a number of publications describing the correlation between the expression of ZNF280B and the growth of

various types of cancer (5-22). For example, Gunn *et al* (22) reported that ZNF280B acts as a tumor suppressor in chronic lymphocytic leukemia. However, the biological roles of ZNF280B in carcinogenesis remain poorly characterized. In the present study, the overexpression of ZNF280B protein was confirmed by the immunohistochemical analysis of gastric cancer samples. It was identified that ZNF280B expression was associated with a larger tumor size. In addition, the present study demonstrated a positive association between ZNF280B and Ki-67, suggesting that ZNF280B is associated with the proliferation of gastric cancer cells. Furthermore, ZNF280B expression was significantly higher in stages III and IV compared with stages I and II, suggesting that it may serve a vital role in the progression of gastric cancer.

To further investigate the role of ZNF280B in tumorigenesis of gastric cancer, the effects of ZNF280B on the proliferation of gastric cancer cells were studied *in vitro* and *in vivo*. The results of an MTT assay indicated that upregulated ZNF280B expression effectively promoted the proliferation of MGC-803 cells. Furthermore, colony formation was greatly increased following the transfection with a ZNF280B plasmid.

Finally, the present study confirmed that upregulated ZNF280B expression promoted tumor growth *in vivo*. Similarly, Gao *et al* (20) identified that ZNF280B served pro-growth and pro-survival functions in prostate cancer with knockdown and overexpression experiments. Additionally, ZNF280B induces the expression of Mdm2, thereby controlling the subcellular localization and stability of p53; the pro-cancer functions of ZNF280B may be mediated by the downregulation of p53 in prostate cancer cells (20).

To the best of our knowledge, this is the first study that evaluates the association between ZNF280B and gastric cancer, and although further confirmation using large cohort samples is required, the data of the present study demonstrate that ZNF280B may promote the progression of gastric cancer. Hence, ZNF280B may be a promising target for treatment of gastric cancer.

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