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Original Article

Silencing of ZNF139-siRNA induces apoptosis in human gastric cancer cell line BGC823

Liqiao Fan, Bibo Tan, Yong Li, Qun Zhao, Yü Liu, Dong Wang, Zhidong Zhang

Department of General Surgery, The Fourth Hospital Hebei Medical University, Shijiazhuang, China

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Abstract: Background and purpose: ZNF139, a member of zinc finger protein family, is a transcription factor. Our previous studies have showed that the over-expression of ZNF139 in gastric cancer (GC) cells was related to the differentiation of GC. However, the function of ZNF139 in GC cells' apoptosis is still unclear. In present study, endogenous ZNF139 in GC cell line BGC823 was inhibited with siRNA, and then mechanism of ZNF139 in GC cells' apoptosis was investigated. Methods: Expression of ZNF139 in GC tissues, adjacent normal tissues, GC cell lines MKN28, SGC7901, BGC823 and that in gastric epithelial cell line GES-1 were tested. Then ZNF139-specific siRNA was transfected into BGC823 cells. Viability, cell cycle and apoptosis of GC cells were detected. Survivin, x-IAP, caspase-3, Fas, p53, Bcl-2 and Bax genes were detected with QPCR and Western blot. Results: ZNF139 expression in GC tissues was significantly higher than that in adjacent normal tissues; ZNF139 expression in GES-1 was very weak, but it expressed in various GC cell lines, with the highest expression in BGC823. After endogenous ZNF139 was inhibited with ZNF139-siRNA, FCM indicated that after transfection, GC cells in G0/G1 phase was significantly increased, but was significantly reduced in G2/M phases; also after transfection, the apoptotic rate of BGC823 cells increased significantly. 48 h after ZNF139-siRNA was transfected, the expression of Survivin, x-IAP and Bcl-2 was significantly down-regulated, while the expression of caspase-3 and Bax was significantly up-regulated. Conclusion: Our results suggest that ZNF139 functions to promote apoptosis resistance of BGC823 by regulating some apoptosis related genes.

Keywords: Gastric cancer, zinc finger protein139, cell lines, RNA interference

Introduction

Gastric cancer (GC) is the most common gastrointestinal malignancy, and it is also the leading cause of death from malignant tumors in Eastern Asia including China [1]. Recent studies indicate that GC cell is characterized by strong resistance to apoptosis, which is an important factor leading to poor prognosis. Multiple genes in GC cells play an important role in the development of resistance to apoptosis of gastric cancer cells [2-6]. It is of importance to treat GC by promoting apoptosis of GC cells. However, there is still no breakthrough in studies on the regulation of apoptosis in GC, so it is of great significance to look for new genes that can regulate apoptosis of GC cell.

ZNF139, a member in zinc finger protein family, is found to be a differentiation-related molecule identified from GC cell lines in our previous research [7]. Many of the members in Zinc finger protein family play a key role in a variety of transcriptional regulations in maintenance of normal life activities and are closely related to tumor proliferation, apoptosis, metastasis and drug resistance. Studies have confirmed ZNF217 is related with proliferation and invasion of ovarian cancer cells [8]; ZNF23 plays a role in cisplatin-induced apoptosis of hepatoma cells [9]; ZBP-89 from Krüppel family is associated with metastasis and prognosis of renal clear cell carcinoma [10]. Other studies have confirmed that the artificial transcription factors could be used in detection of drug resistance genes and pathways, which may reverse the drug resistance of the cancer [11]. Van Deken et al found that ZNF139 expression was increased in adenocarcinoma of gastroesophageal junction, which is related with tumor proliferation [12]. But the role of ZNF139 in the process of apoptosis in GC has not been reported. To understand the impact of ZNF139 on apoptosis of GC, we detected expressions of ZNF139

in GC tissues, adjacent tissues, GC cell lines and normal gastric epithelial cell line. Besides, we applied small interfering RNA transfection technique to knock down overexpressed ZNF139 in GC cell line BGC823, to observe changes in viability and apoptosis of GC cells; we further tested expression of genes involved in different apoptotic pathways, so as to explore the effect and mechanism of ZNF139 in GC apoptosis.

Materials and methods

Cell lines and reagents

MKN28, SGC7901, BGC823 and GSE-1 cell line were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China); RPMI 1640 culture medium and trypsin were from Gibco Company (USA); Trizol reagent and LipofectamineTM 2000 were purchased from Invitrogen (USA). Reverse transcription kit and fluorescence quantitative RT-PCR reagent were purchased from Promega Corporation, USA; PCR primers and small interfering RNA were synthesized by the Shanghai Biological Engineering Co.. Protein extraction kit was purchased from Bio-Rad company, USA; ZNF139, Survivin, x-IAP, caspase-3, Fas, P53, Bcl-2, Bax and GAPDH antibody were purchased from Santa Cruz, USA; MTT was product from Sigma, USA. Flow cytometer (Epics-XL, type II) was the product from Beckman Coulter, USA.

Clinical sample preparation

Samples of gastric adenocarcinoma and adjacent normal tissues were harvested from 25 patients (15 males and 10 females, with mean age of 62.4 ± 12.3 years), who were treated with surgery and diagnosed by pathology with advanced gastric adenocarcinoma in the Fourth Affiliated Hospital of Hebei Medical University from January 2013 to June 2013. One piece of fresh tissue (approximately 1.0 cm×0.5 cm×0.5 cm) from both cancerous tissues and adjacent normal mucosa (over 3 cm off the edge of cancerous tissues; it was pathologically confirmed after surgery that no cancer cells was present pathologically) were harvested from isolated specimens during operation and stored at -80°C.

Cell culture

Human GC cell lines (MKN28, SGC7901, BGC823) with different degrees of differentiation and gastric epithelial cell line GSE-1 were cultured in RPMI 1640 (Invitrogen) containing 10% fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin and incubated at 37°C supplemented with 5% of CO_2. 0.25% trypsin solution containing 0.02% of EDTA was used to trypsinize and split cells.

Detection for the target gene mRNA by QPCR

Total RNAs were isolated with Trizol reagent, and 2 μg was reverse transcribed to synthesize template cDNA. 2 μl reverse transcription product was subjected to PCR reactions and GAPDH served as an internal reference. According to kit instructions PCR reaction system was established with a final volume of 20 μl, 2 μl reverse transcription product, 10 μl SYBR Green Mix (Applied Biosystems, Foster City, CA), 0.5 μl of both upstream and downstream primers (10 μmol/l). PCR reaction started with 1 cycle of 95°C for 5 min, followed by 45 cycles of three steps as 94°C for denaturation for 30 S and 60°C for annealing for 30 s. Primers were designed using Primer 5.0 and detected for specificity in Blast comparison test for the experiment. The primer sequences were as follows:

ZNF139 primers: 5'-CTTCCTGAGTTCTTGGTTTCG-3' (F) and 5'-CCTTTGACCCACTGGTTTATG-3' (R);

Survivin primers: 5'-GCCAGATTTGAATCGCGGGA-3' (F) and 5'-GCAGTGGATGAAGCCAGCCT-3' (R);

x-IAP primers: 5'-CCGTGCGGTGCTTTAGTTGT-3' (F) and 5'-TTCCTCGGGTATATGGTGTCTGAT-3' (R);

caspase-3 primers: 5'-AGAGCTGGACTGCGGTATTGAG-3' (F) and 5'-GAACCATGACCCGTCCCTTG-3' (R);

Fas primers: 5'-CACTATTGCTGGAGTCATG-3' (F) and 5'-CTGAGTCACTAGTAATGTCC-3' (R);

P53 primers: 5'-GTACCGTATGAGCCACCTGAG-3' (F) and 5'-CGTCCCAGAAGATTCCCAC-3' (R);

Bcl-2 primers: 5'-TGTGTGGAGAGCGTCAACC-3' (F) and 5'-TGGATCCAGGTGTGCAGGT-3' (R);

Bax primers: 5'-TTTCTGACGGCAACTTCAAC-3' (F) and 5'-AGTCCAATGTCCAGCCCAT-3' (R);

GAPDH primers: 5'-GACCCCTTCATTGACCTCAAC-3' (F) and 5'-CGCTCCTGGAAGATGGTGAT-3' (R).

Quantitative PCR results were calculated using the $2^{-\Delta\Delta Ct}$ method.

Detection for the target gene proteins by Western-blotting assay

The total proteins from clinical samples were extracted. After protein quantification, 60 μg protein samples in each group were electrically transferred to a PVDF membrane after gel electrophoresis with 12% polyacrylamide. They were added with 5% skim milk powder supplemented with TBST, closed at room temperature for 1 h, incubated with diluted target molecule primary antibody or internal reference β-actin primary antibody overnight at 4°C and rinsed three times with TBST. Appropriate peroxidase-labeled secondary antibody was added for incubation at room temperature for 1 h; chemiluminescence was used for coloration and bands underwent scanning for integral absorbance.

Transfection of ZNF139-siRNA into BGC823 cells

ZNF139-specific small interfering RNA (ZNF-139-siRNA) sequence was synthesized by Shanghai Biological Engineering Co., China, and the sequence was as follows: ZNF139-siRNA, 5'-ACCTCGGAAGATTCAGCAT-3'. Control siRNA (control siRNA) sequence was 5'-GACGAGTTGACTGCGATTG-3'. Before transfection, the synthesized siRNA was dissolved in solution at a concentration of 20 μmol/L. MGC803 cells were seeded in 6-well plates with the density of 4×10^5/mL for 24 h. Prior to transfection, cells were rinsed with serum-free and antibiotics-free RPMI 1640. According to the reagent instructions or by comparing control siRNA, Vav3-siRNA diluted with RPMI 1640 medium was transfected into LipofectamineTM 2000. It was held and then transfected into BGC823 cells. 24 h after transfection, the transfection efficiency was tested for subsequent experiments.

Cell vialibity test by MTT assay

BGC823 cells were seeded in 96 well plates with the density of 5×10^4 cells/mL. Cells at a confluency of 60% to 70% or compared with control siRNA were transfected with ZNF139-siRNA. Six replicate wells were made in each group. 4 hours before the end of experiment, 20 μl of MTT with a concentration of 5 mg/ml was added to each well. After 4 h, the culture medium was discarded, and 150 μl of DMSO was added in each well and shaken at room temperature for 15 min. Absorbance value (OD) at a wavelength of 490 nm were measured by a microplate reader. The above experiment was repeated 3 times. Growth inhibition rate (%)=(1-experimental group OD value/control group OD value)×100%.

Detection of cell cycle and apoptosis rate of GC by flow cytometry (FCM)

100 μl of 10^5 cells suspension were fixed with 70% ice-cold ethanol. Empty liposomes served as controls. 1 m PI was added to each group, while samples were added with 10% chicken erythrocytes as internal reference and stored at 4°C refrigerator for 30 min. Muticyle AV analysis software was employed for fitting analysis of DNA cell cycle. Apoptotic cell was measured by Annexin V-FITC/PI detection kit (Jiamei, Beijing, China), according to the instruction. Briefly, cells were harvested and resuspended in binding buffer (10^6 cells/mL). After addition of 5 μl Annexin V-FITC and 10 μl of propidium iodide (PI) with mixing, the tubes were incubated for 15 min at room temperature in the dark. Annexin V-FITC binding was detected by a FACSC alibur cytometer (Becton Dickinson, USA). The data was analyzed by the Cell Quest software. The experiment was repeated 3 times.

Statistical analysis

Experimental results were expressed with $\bar{x} \pm s$ and SPSS 13.0 was used for the ANOVA analysis and Dunnett test. A P value <0.05 indicated a significant difference.

Results

Expression of ZNF139 in tissues and cell lines

Results showed compared with those in adjacent tissues, ZNF139 expression was higher in GC tissues (**Figure 1A**). ZNF139 expressions in GC cell lines were higher than that in normal gastric epithelial cell line, and poorly differentiated GC cell line BGC823 showed the highest ZNF139 expression (**Figure 1B**), as shown in **Figure 1**.

Effect of ZNF139-siRNA on ZNF139 in BGC823 cells

Results showed that ZNF139 expression did not change after being transfected with con-

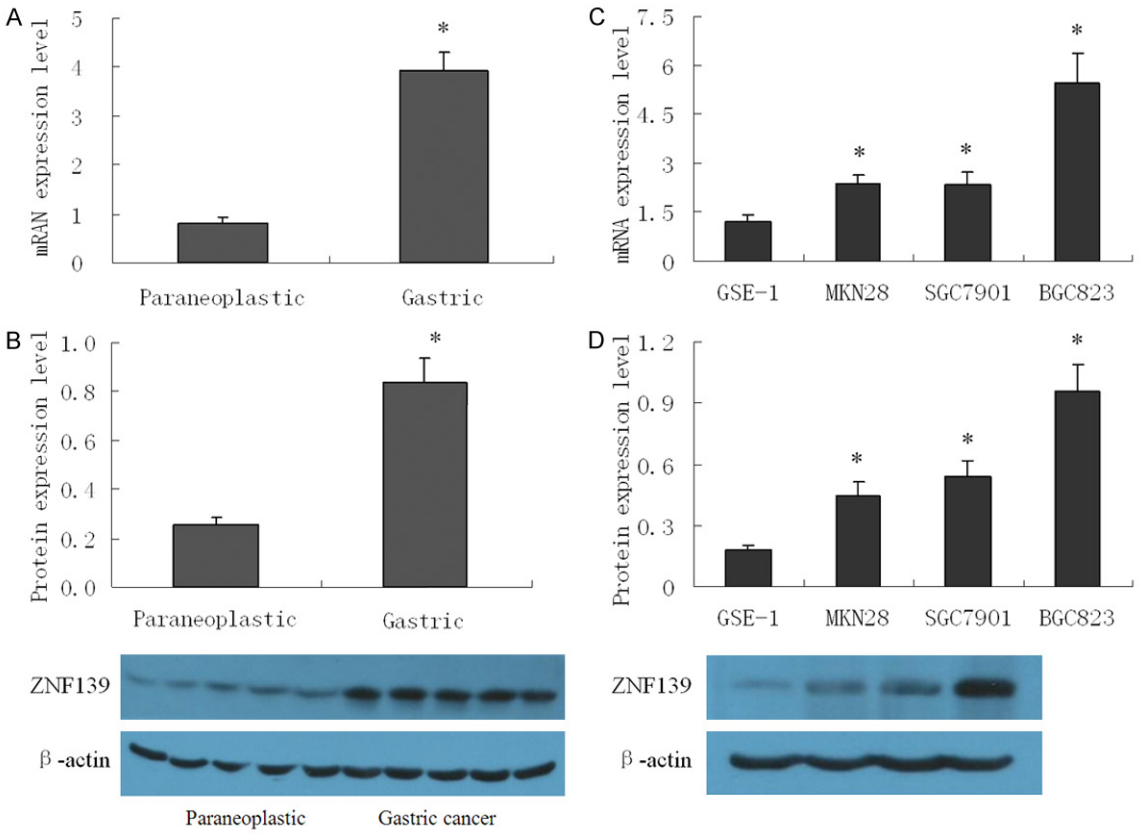

Figure 1. ZNF139 expression level in gastric cancer tissues and gastric cell lines. Clinical samples of gastric cancer tissues and paraneoplastic tissues as well as GES1, MKN28, SGC7901, BGC823 cell lines were subjected to QPCR (A) and (C) and Western-blot (B) and (D) assays to determine the expression levels of ZNF139. The relative mRNA expression levels were shown in (A) and (C), and protein levels in (B) and (D). Values were shown as mean ± SD, for tissue samples n=25, cell samples n=4 in each group. *P<0.01 versus control group (Paraneoplastic or GES-1 group).

trol-siRNA. After being transfected with ZNF139-siRNA, the endogenous ZNF139 in BGC823 cells decreased differentially in a concentration-dependent manner, in which ZNF-139 expression was decreased by more than 80% in cells transfected with 80 nM of ZNF139-siRNA (**Figure 2A**, **2B**).

Effect of ZNF139-siRNA on BGC823 cell viability (MTT results)

After ZNF139-siRNA, including 20, 40, and 80 nM, was used to transfect BGC823 cells, It was showed that compared with the negative control group transfected with control-siRNA, inhibition rate of BGC823 cells transfected with ZNF139-siRNA was decreased in a dose-dependent manner (P<0.05). Results also showed that proliferation inhibition rate was not statistically significant in negative control group transfected with control-siRNA (P > 0.05) (**Figure 3**).

Effect of ZNF139-siRNA on cell cycle and apoptosis of BGC823

After 80 nM of ZNF139-siRNA were transfected into BGC823 cells, FCM results showed that BGC cells transfected with ZNF139-siRNA was significantly increased in the G0/G1 phase, while the ratio of cells in G2/M phase was significantly reduced (P<0.05), the apoptosis rate of GC cells was significantly increased after being transfected with ZNF139-siRNA-2 (P<0.05) (**Figure 4A**, **4B**).

Impact of ZNF139-siRNA-2 on expression of apoptosis-related genes (Survivin, x-IAP, caspase-3, Fas, p53, Bcl-2 and Bax)

ZNF139-siRNA and control-siRNA were transfected into BGC823 cells respectively. After 48 h, expression of Survivin, x-IAP and Bcl-2 were significantly decreased, while expression of caspase-3 and Bax were significantly increased;

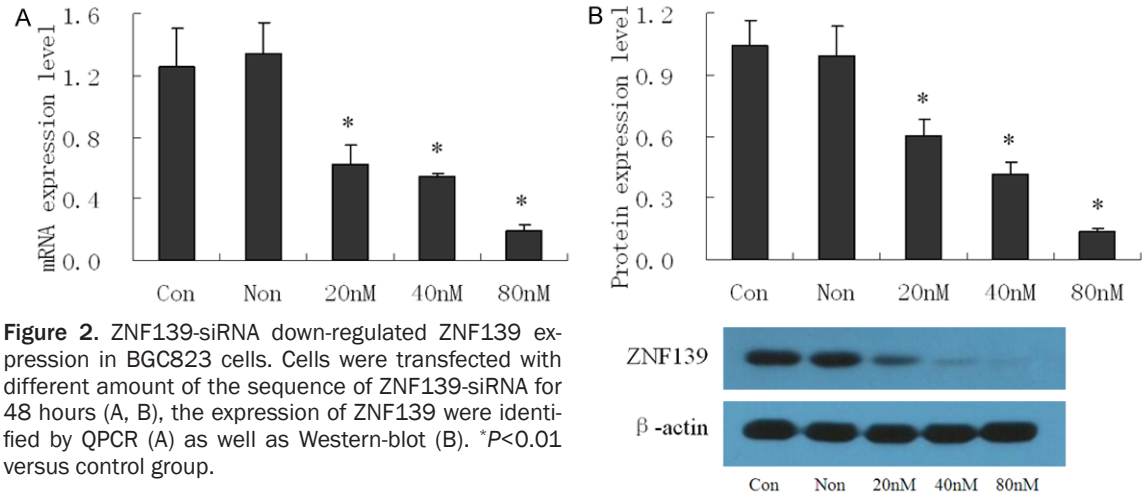

Figure 2. ZNF139-siRNA down-regulated ZNF139 expression in BGC823 cells. Cells were transfected with different amount of the sequence of ZNF139-siRNA for 48 hours (A, B), the expression of ZNF139 were identified by QPCR (A) as well as Western-blot (B). *P<0.01 versus control group.

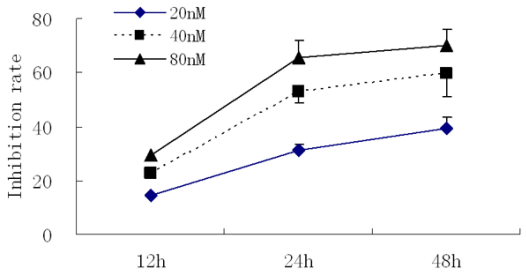

Figure 3. The effects of different amount of the sequence ZNF139-siRNA on the activity of gastric cancer cell line BGC823 with MTT assay.

expression of Fas and p53 had no obvious changes (**Figure 5**).

Discussion

GC is the most common digestive carcinoma in China, and mortality rate of GC is the highest among various malignant tumors, which seriously threatens the life of people. It is due to the insidious onset of gastric tumor cells, strong anti-apoptotic activity, poor apoptosis, and stronger resistance ability to chemotherapy drugs (such as platinum drugs), with the mechanism of apoptosis induction [13-16], that these patients typically have local or distant metastasis before diagnose, leading to ineffective comprehensive treatment, such as surgery and chemotherapy, and poor prognosis. At present, diagnose rate for early GC is less than 10% in China [17-19], the majority of patients have advanced cancer, and 50% to 80% of patients have lymph node metastasis, organ invasion and metastasis before surgery. 5-year survival of advanced GC has been fluctuating at around 40% to 50% [20, 21]. These are all associated with strong anti-apoptotic ability of GC cells. If appropriate measures could be taken to reduce the resistance to apoptosis of tumor cells and to promote apoptosis of GC cells, it would help delay the progression of cancer; improve the therapeutic effect and the prognosis. It is of importance in the field of cancer research to study resistance mechanisms of apoptosis of GC cells and to look for a new gene which can regulate apoptosis of GC cells effectively. However, there is still no breakthrough in relevant research, and the key genes that promote apoptosis of GC cells have not yet been found.

In our previous study, ZNF139 was found as one of differentiated-related proteins with proteomics identification [7]. Studies by van Deken et al [12] showed expression of ZNF139 in adenocarcinoma of gastroesophageal junction. ZNF139, a member of zinc finger protein family, has six C2H2 type zinc finger domains as well as a SCAN and KRAB (kruppel-associated box) domains. Zinc finger is a DNA binding domain, and SCAN and KRAB mediated protein-protein interaction with the cofactors, which play an important role in transcriptional regulation of the expression of downstream genes by binding the promoter region of target genes [22]. Although the relationship between ZNF139 and apoptosis of GC cells has not still been reported, our previous studies have confirmed that ZNF139 is related to GC cell proliferation, apop-

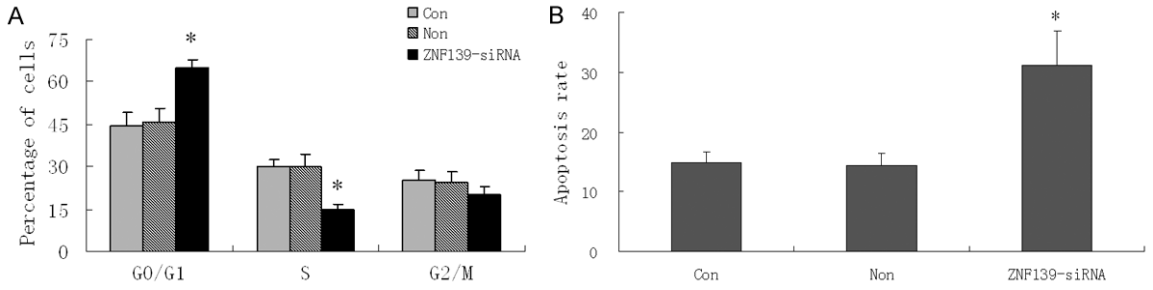

Figure 4. The effects of ZNF139-siRNA-2 on the proliferation, apoptosis of gastric cancer cell line BGC823 with FCM. Cells were transfected with ZNF139-siRNA or control NS-siRNA, and then were tested by FCM. Cell cycles were shown as (A), and apoptosis rates were shown as (B). *P<0.01 versus control group.

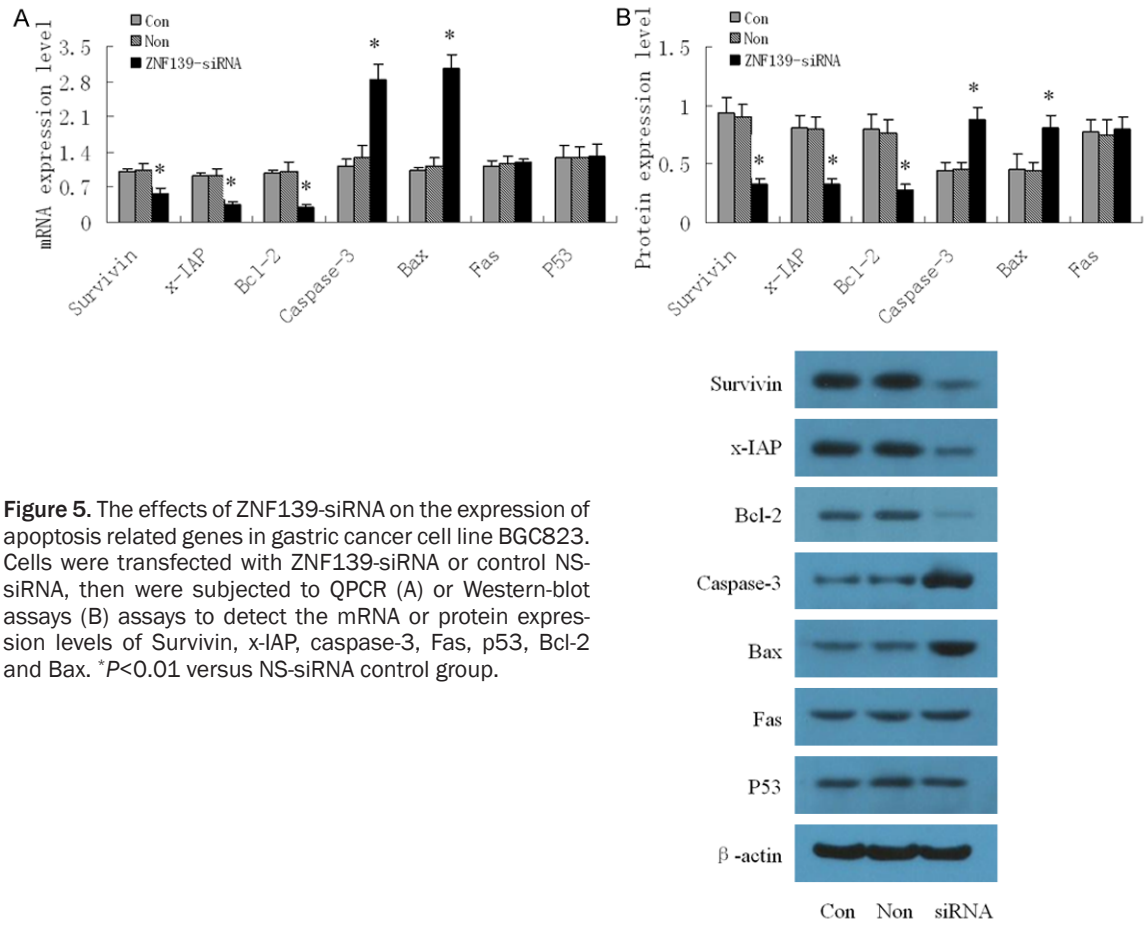

Figure 5. The effects of ZNF139-siRNA on the expression of apoptosis related genes in gastric cancer cell line BGC823. Cells were transfected with ZNF139-siRNA or control NS-siRNA, then were subjected to QPCR (A) or Western-blot assays (B) assays to detect the mRNA or protein expression levels of Survivin, x-IAP, caspase-3, Fas, p53, Bcl-2 and Bax. *P<0.01 versus NS-siRNA control group.

tosis, invasion, metastasis and drug resistance [8-11]. Then we further explored the relationship between ZNF139 and apoptosis of GC.

In the present study, ZNF139 expression in GC tissues and GC cell lines was significantly higher than that in the adjacent tissues and normal gastric epithelial cell lines, indicating that ZNF139 had a close relationship with GC. Since apoptosis of tumor cells is an extremely complex pathological process with multi-step and multi-factor involvement, various genes and pathways, such as inhibition of apoptosis pathway, the mitochondrial pathway and death receptor pathway involved in this process [2-6]. Our study confirmed ZNF139 expression was closely related to the activity of GC cells. Inhibition of ZNF139 expression could help

inhibit the activity of BGC823 cells. Flow cytometry results showed that in G0/G1 phase gastric cells transfected with ZNF139-siRNA were significantly increased, and in G2 and M phase cells was significantly decreased; after transfection with ZNF139-siRNA, apoptosis rate of GC cells increased significantly, suggesting that ZNF139 overexpression of GC cells could promote cell division and enhance apoptosis resistance.

Mechanism of tumor cell apoptosis is extremely complex, in which Bcl-2 family members of the mitochondrial pathway play an important role. Bcl-2 can enhance the apoptotic resistance of tumor cells in a variety of ways, while Bax has a strong pro-apoptotic effect. Bcl-2 and Bax are combined into Bcl-2/Bax dimers, and changes in the proportion of Bcl-2/Bax make imbalance in tumor cell apoptosis [23-25]. Fas is an important member in the death receptor pathway of all apoptosis pathways, and it also plays an important role in tumor cell apoptosis [26, 27]. P53 is the most studied tumor gene, and wild-type P53 can promote tumor cell apoptosis. Mutant p53, a key factor in tumor, has more effects on inhibition of apoptosis [28, 29]. The inhibitor of apoptosis proteins (IAPs) is an important gene family in tumor cell apoptosis pathway, and Survivin and xIAP in the family have a strong effect to inhibit apoptosis [30-33]. Caspase family is the core in apoptosis, directly inducing apoptosis, and caspase-3 is one of the main members [34, 35]. The results of our study showed that after ZNF139 was inhibited, expressions of Survivin, x-IAP and Bcl-2 were decreased, while expressions of caspase-3 and Bax were increased, and expressions of Fas and P53 did not change significantly. These indicated that ZNF139 gene was closely related to GC cell apoptosis, and its regulation mechanism may be caused by regulating genes in both mitochondrial and inhibiting apoptosis pathway.

In conclusion, our study showed the up-regulation of ZNF139 expression in GC. SiRNA-mediated ZNF139 silencing may decrease expressions of Survivin, x-IAP and Bcl-2, and increase expressions of caspase-3 and Bax. These results suggested that ZNF139 is a new molecular gene that may be involved in regulation of apoptosis in GC cells. Therefore, ZNF139 may have a regulatory effect in apoptosis of GC as a target gene in a potential therapeutic treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yong Li, Department of General Surgery, The Fourth Affiliated Hospital, Hebei Medical University, Shijiazhuang 050011, China. Tel: +8631186095678; Fax: +86-31186077634; E-mail: li_yong_hbth@126.com

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