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Original Article

Efficacy and possible mechanisms of *14-3-3zeta* inhibition of proliferation and induction of apoptosis in human gastric cancer cells

Yuan Yuan[1], Min Hu[2], Huihua Shen[1], Zhonghua Wan[1]

[1]*Department of Cardiothoracic Surgery, General Hospital of Xinjiang Military Region, Urumchi 830000, Xinjiang Uygur Autonomous Region, China;* [2]*Department of Gastroenterology, The Sixth Affiliated Hospital of Xinjiang Medical University, Urumchi 830002, Xinjiang Uygur Autonomous Region, China*

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Abstract: Background: Preliminary studies have indicated that there is abnormal expression of the *14-3-3zeta* gene in gastric cancer cells; however, the effect and mechanism of this abnormal expression are unknown. Purpose: To investigate the effect of the *14-3-3zeta* gene on proliferation and apoptosis in human gastric cancer cells. Methods: *14-3-3zeta*-siRNA was transfected into gastric cancer cells by Lipofectamine™ 2000. Cell proliferation was subsequently detected by the CCK8 assay. The cell cycle and apoptosis were detected via flow cytometry. The protein levels of p53, CylinD1, PUMA, Bax and Bcl2 were analyzed by Western blot. Results: Compared with the control groups, down-regulation of *14-3-3zeta* in gastric cancer cells resulted in a significant decrease of cell proliferation. Flow cytometry indicated a significantly increased cell proportion at the G0/G1 phase and apoptotic rate in *14-3-3zeta* siRNA cells. Furthermore, down-regulation of *14-3-3zeta* increased expression of the p53, PUMA and Bax proteins, whereas it reduced expression of the CyclinD1 and Bcl2 proteins. Conclusions: Down-regulation of *14-3-3zeta* in gastric cancer cells may inhibit proliferation and induce apoptosis via the p53 pathway by down-regulating the CyclinD1 and Bcl2 proteins and up-regulating the PUMA and Bax proteins. *14-3-3zeta* has the potential to serve as an early marker of gastric cancer.

Keywords: *14-3-3zeta*, gastric cancer, proliferation, apoptosis, p53

Introduction

Gastric cancer is a common malignant tumor; its incidence rate is high, and it causes serious harm to human health [1-3]. The diagnosis of early gastric cancer is not easy to identify, which thus delays the best timing of treatment [4]. The five year survival rate was improved by traditional surgical treatment; however, the risk of gastric cancer was not decreased [2, 4]. Therefore, it remains critical to search for a tumor specific target for the diagnosis and treatment of gastric cancer.

The occurrence and development of a tumor is a complicated process, which involves many changes in gene expression [5-7]. *14-3-3* belongs to the eukaryote acidic gene and protein family, which includes beta, gamma, zeta, eta, epsilon, sigma and tau 7 isoforms; it is an important regulatory factor involved in the regulation of growth and development, cell cycle, apoptosis and signal transduction [8-11]. Studies have shown that the *14-3-3zeta* gene is closely related to oncogenesis [12] and has an abnormal expression in lung cancer, breast cancer, colon cancer and other malignant tumors; it also induces overexpression of p53, which may decrease expression of tumor suppressor genes and activate an abnormal p53 pathway [13-17]. Our preliminary study indicates that the *14-3-3zeta* gene has an abnormal expression in gastric cancer; however, the effect and mechanism have not been clarified.

In these contexts, siRNA technology was employed in this study to interfere with expression of the *14-3-3zeta* gene of the human gastric cancer cell line SGC-7901. The proliferation and apoptosis of gastric cancer cells were sub-

Table 1. Details of primary antibodies used in this study

Antibody name	Catalogue no.	Isotype	Dilution rate	Molecular Weight (kDa)	Manufacturer
14-3-3ζ	9635	R	1:1000	28	CST
PUMA	12450	R	1:1000	23	CST
p53	2527	R	1:1000	53	CST
Bax	2772	R	1:1000	20	CST
Bcl2	15071	R	1:1000	26	CST
CyclinD1	2922	R	1:1000	36	CST
β-actin	4867	R	1:5000	45	CST

sequently assessed. Furthermore, the potential mechanisms were explored through the detection related signal pathway. This study provides a novel strategy for the diagnosis and treatment of gastric cancer.

Materials and methods

Cell culture

The human gastric cancer cell line SGC-7901 was obtained from the Institute of Cellular Biology of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (Hyclone, USA); the cells were incubated at 37°C and 95% humidity.

Transfection

The *14-3-3zeta* siRNA and control-siRNA were purchased from Santa Cruz and used as instructed by the manufacturer's protocol. Briefly, SGC-7901 cells were seeded in RPMI 1640 medium that contained 10% FCS without antibiotics. Approximately 24 h after seeding, when SGC-7901 cells were approximately 90% confluent, they were transfected with Lipofectamine™ 2000 and Opti-MEM (Life Technologies, Germany) according to the manufacturer's protocol. The transfected SGC-7901 cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Approximately 24 h after transfection, the SGC-7901 cells were used for different experiments.

RT-qPCR

Total RNA was isolated from SGC-7901 cells using Trizol solution (Invitrogen, USA) according to the manufacturer's instructions. For reverse transcription (RT)-PCR, cDNA was prepared from 1 μg of total RNA using reverse transcriptase with an oligo (dT) primer. *14-3-3zeta* mRNA was subjected to RT-PCR with a Realtime 7500 PCR apparatus (Applied Biosystems) according to the instruction manual (SYBRII Green Realtime PCR; Toyobo). The results were analyzed with Applied Biosystems 7500 system v1.4.0 software. The PCR conditions were as follows: denaturing once at 94°C (5 minutes), followed by 40 cycles at 93°C (30 seconds), 60°C (30 seconds) and 72°C (20 seconds). The primers included *14-3-3zeta* (forward, 5'-ATTGAGACGGAGCTA-3'; reverse 5'-CTEAGCCAAGTAACGGTAGTAATCT-3') and β-actin (forward, 5'-TGACGTGGACATCCGCAAAG-3'; reverse 5'-CTGGAAGGTGGACAGCGAGG-3'). The fold change was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation

To determine the effect of *14-3-3zeta* on cell proliferation, SGC-7901 cells with *14-3-3zeta* siRNA, control-siRNA and blank control were seeded on 96-well plates. A Cell Counting Kit-8 (CCK-8) was used to monitor the cell growth at 0 h, 24 h, 48 h, 72 h, 96 h and 120 h, and the number of viable cells was assessed via measurement of the absorbance at 450 nm by FIUOstar OPTIMA (BMG LABTECH, Offenburg, Germany).

Propidium iodide staining and flow cytometry

Cells in the logarithmic growth phase were used for these experiments. Cells were cultured, digested and collected at a fusion rate of 90% and fixed with 70% ethanol for 2 h at 4°C. Fixed cells were centrifuged at 800 rpm for 5 min and collected. The supernatant was discarded. Five hundred μl staining buffer solution were added to each tube that contained a cell sample to slowly and completely re-suspend the cells. Twenty μl propidium iodide staining solution were initially added to the resultant suspension, followed by 10 μl RNase A and mixed evenly. The mixture was incubated in the dark for 30 min at 37°C and tested via flow cytometry. The experiment was repeated five times.

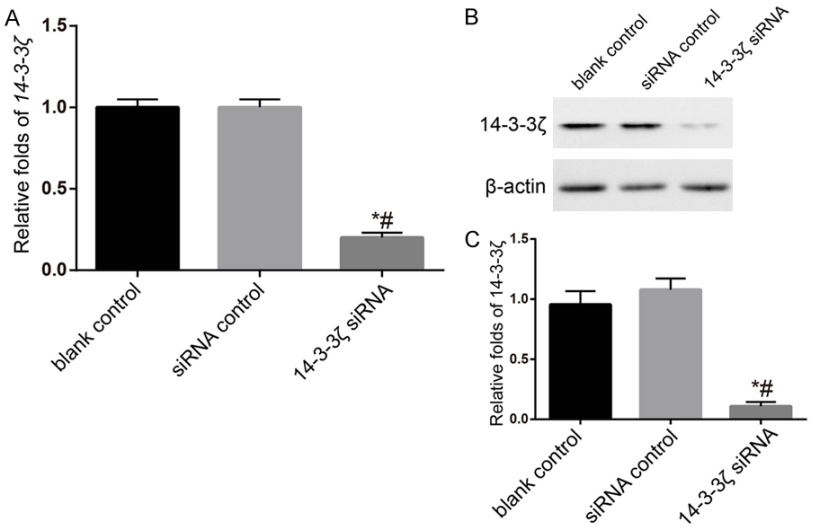

Figure 1. After transfection, the *14-3-3zeta* gene expression was tested by RT-qPCR and Western Blot. A. Relative folds of *14-3-3zeta* were tested by RT-qPCR. Data are reported as the mean ± standard error (**P*<0.01 *vs.* blank control group, #*P*<0.001 *vs.* siRNA control group). B. Representative Western blot results of 14-3-3zeta protein extracts from transfected SGC-7901 cells. β-actin was used as a loading control. C. Protein signals of 14-3-3zeta were determined via densitometry analysis of Western blotting using Image J software (NIH). Data are reported as the mean ± standard deviation (n=3, **P*<0.01 *vs.* blank control group, #*P*<0.001 *vs.* siRNA control group).

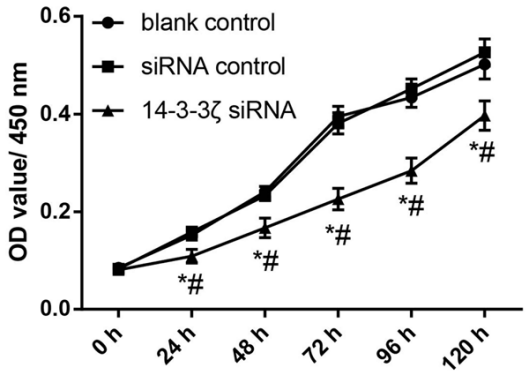

Figure 2. Proliferation of transfected SGC-7901 cells and blank control cells for 0 h, 24 h, 48 h, 72 h, 96 h and 120 h of culture. Data are reported as the mean ± standard deviation (**P*<0.01 *vs.* blank control group, #*P*<0.001 *vs.* siRNA control group).

Annexin V-FICT/PI flow cytometry double staining

After transfection, the apoptotic cell death was determined using an Annexin V-FITC/PI (propidium iodide) apoptosis detection kit according to the modified manufacturer's protocol. In brief, transfected 14-3-3zeta siRNA SGC-7901 cells, transfected control-siRNA SGC-7901 cells and SGC-7901 cells were seeded on a 12-well plate for 48 h in an incubator. After the incubation, the cells were harvested and washed with phosphate buffered saline (PBS) twice to determine apoptosis. The adherent and floating cells were subsequently combined and subjected to Annexin V and PI staining. The samples were analyzed on a FACS can cytometry instrument (Becton Dickinson, San Jose, CA), and the data were subsequently analyzed using CellQuest software to differentiate apoptotic cells from all cells following the step-by-step protocol provided by the manufacturer.

Western blotting

The transfected *14-3-3zeta* siRNA SGC-7901 cells, transfected control-siRNA SGC-7901 cells and SGC-7901 cells were seeded on a six-well plate in different groups for 48 h. The cells were subsequently washed with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA lysis buffer and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The protein concentration was determined by a BCA protein assay kit. The immuno-signal was detected with an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, MD, USA). The details regarding the primary antibody used in this study are included in **Table 1**. As a loading control, β-actin was probed and visualized. The protein signals were subsequently determined by densitometric analysis using Image J software (NIH).

Statistical analysis

All experiments were repeated at least three times, and numerical data were reported as the mean ± standard deviation (SD). The statistical significance of cell proliferation was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. The cell cycle, apoptosis and densitometric analysis of We-

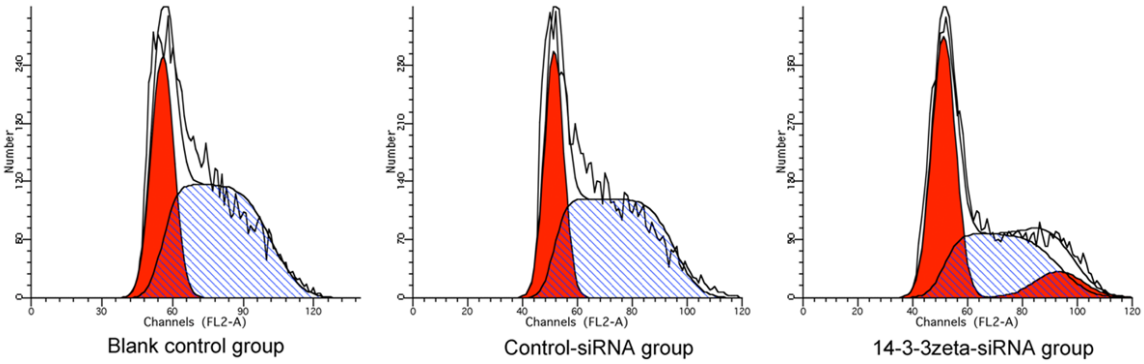

Figure 3. Representative flow cytometry results of cell cycle of transfected SGC-7901 cells and blank control cells.

Table 2. Proportion of cell cycle by FCM (n=5, $\bar{x} \pm s$)

Group name	G0/G1 (%)	G2/M (%)	S (%)
Blank control group	62.53 ± 3.11	5.23 ± 0.45	32.23 ± 2.58
Control-siRNA group	63.17 ± 2.97	5.48 ± 0.41	31.36 ± 2.69
14-3-3zeta-siRNA group	71.34 ± 3.75*,#	4.12 ± 0.34*,#	24.55 ± 2.07*,#

*P<0.05 *vs*. Blank control group, #P<0.05 *vs*. Control-siRNA group.

stern blotting were analyzed by *t*-tests, using GraphPad Prism 6.0 software. Significance was defined as P<0.05.

Results

14-3-3zeta siRNA inhibits proliferation of human gastric cancer cells

Following the successful transfection of human gastric cancer cells (**Figure 1**), the proliferation of the transferred cells was examined. Using CCK8, the continuous determination of the cell proliferation ability was evaluated, and the results indicated that the proliferation of *14-3-3zeta* siRNA cells in the experimental group was significantly lower than the blank control group and control-siRNA group (**Figure 2**). However, there was no difference between the blank control group and control-siRNA group (**Figure 2**).

14-3-3zeta siRNA blocks the cell cycle in G0/G1 phase

To investigate the effect of *14-3-3zeta* on the cell cycle of human gastric cells, the cell cycles in each group were observed via flow cytometry. Compared with the control group, the cell proportion at the G0/G1 phase in the *14-3-3zeta* siRNA group was significantly increased (P<0.05); however, there was no difference between the blank control group and control-siRNA group (P > 0.05) (**Figure 3** and **Table 2**). The cell proportions at the G2/M and S phase, which is the cell division phase, were significantly lower than those in the blank control group and control-siRNA group (P<0.05) (**Figure 3** and **Table 2**). These results indicated that after inference of *14-3-3zeta* expression, the cell cycle was mainly blocked at the G0/G1 phase.

14-3-3zeta siRNA induces apoptosis of human gastric cancer cells

After transfection, the apoptosis of human gastric cancer cells was investigated via the Annexin V-FICT/PI flow cytometry double staining method. As indicated in **Figure 4** and **Table 3**, the rate of apoptosis of SGC-7901 in the *14-3-3zeta* siRNA group was significantly higher than that of the blank control group and control-siRNA group (P<0.05), however, there was no difference between the blank control group and control-siRNA group (P > 0.05).

14-3-3zeta regulates proliferation and apoptosis of human gastric cancer cells potentially through the CyclinD1 and Bax/Bcl2 signaling pathways

To investigate the mechanism of cell proliferation and apoptosis after the interference of *14-3-3zeta*, the CyclinD1 and Bax/Bcl2 signaling pathways were assessed via Western Blotting. As indicated in **Figure 5**, expression of the cycle related protein CylinD1 in the *14-3-3zeta* siRNA group was significantly lower than

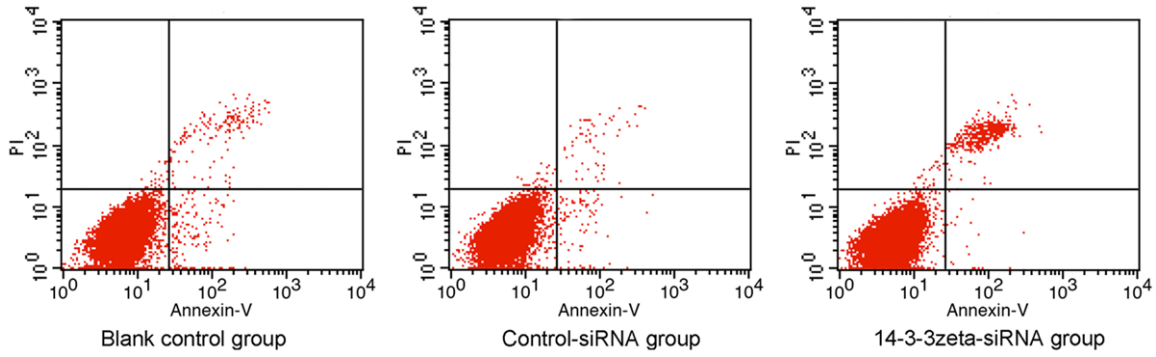

Figure 4. Representative flow cytometry results of apoptosis of transfected SGC-7901 cells and blank control cells.

Table 3. Proportion of cell apoptosis by FCM (n=5, $\bar{x} \pm s$)

Group name	Early apoptosis rate (%)	Late apoptosis rate (%)	Total apoptosis rate (%)
Blank control group	3.42 ± 0.37	4.18 ± 0.40	7.72 ± 0.73
Control-siRNA group	3.66 ± 0.41	4.48 ± 0.51	8.15 ± 0.80
14-3-3zeta-siRNA group	6.71 ± 0.73*,#	7.56 ± 0.92*,#	14.28 ± 1.33*,#

*P<0.05 *vs*. Blank control group, #P<0.05 *vs*. Control-siRNA group.

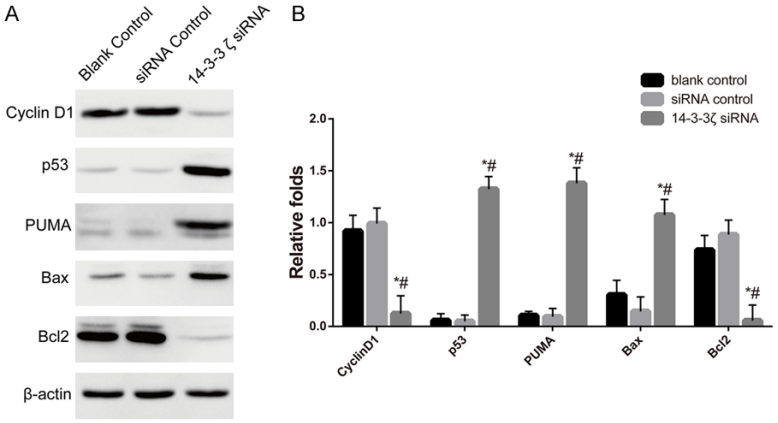

Figure 5. Potential mechanism of cell proliferation and apoptosis after *14-3-3zeta* siRNA. A. Representative Western blot results of CyclinD1, p53, PUMA, Bax and Bcl2 protein extracts from transfected SGC-7901 cells and blank control group. β-actin was used as a loading control. B. Quantification of Western Blots. Data are reported as the mean ± standard deviation (*P<0.01 *vs*. blank control group, #P<0.001 *vs*. siRNA control group).

that in the blank control group and control-siRNA group (P<0.05). However, expression of the apoptosis related pathway proteins p53, PUMA and Bax was significantly increased (P<0.05). Furthermore, expression of the Bcl2 protein, which is an inhibitor of apoptosis, was significantly reduced (P<0.05). There was no difference in the protein expression between the blank control group and control-siRNA group.

Discussion

14-4-3 protein is a highly conserved protein family in eukaryotic cells, which has been shown to exist in 7 subtypes, including β, γ, ε, zeta, η, σ and τ [10]. Many studies have indicated that 14-3-3 protein regulates the interaction between other proteins, which is also closely related to the occurrence and development of tumors [18]. A preliminary study indicated that the *14-3-3zeta* gene plays a significant role in promoting the proliferation of human gastric adenocarcinoma cells; however, its specific mechanism has not previously been clarified. In this study, the interference technique of siRNA was used to decrease expression of the *14-3-3zeta* gene in the human gastric cancer cell line SGC-7901; the cell proliferation, cell cycle and cell apoptosis were subsequently examined and analyzed. The potential molecular mechanism is discussed.

The results of this study indicate that in the cells after transfection, expression of *14-3-3zeta* mRNA and protein significantly decreased, which indicated that the transfection was

successful. To investigate its effects and mechanism, the cell cycle and apoptosis were detect via flow cytometry. After the *14-3-3zeta* genes down-regulated, the G0/G1 phase of human gastric cancer cells was increased, whereas the G2/M and S phase cells were decreased, which indicated that the normal cell division was inhibited. Furthermore, after expression of the *14-3-3zeta* gene was inhibited, apoptosis of human gastric cancer cells was significantly increased, which also indicates the key role of the *14-3-3zeta* gene in the promotion of cell proliferation and inhibition of apoptosis [19, 20]. To further investigate its mechanism, Western Blotting was used to detect expression of p53, cell cyclin protein CyclinD1, apoptosis promoting protein PUMA and Bax as well as inhibition of the apoptosis protein Bcl2. The results showed that in the *14-3-3zeta* siRNA group, the p53 protein expression significantly increased, whereas the p53 expression was significantly lower in the blank control group and control-siRNA group. Further investigation of the p53 protein downstream of the cycle protein CyclinD1 indicated that it was significantly lower in the *14-3-3zeta* siRNA interference group. CyclinD1 is one of the main proteins that regulate the cell division cycle G1, which promotes the cell cycle from G1 to S phase [19, 21, 22]. In this study, the protein expression of CyclinD1 also indicated that the human gastric cancer cells from the G1/G2 phase to the S phase transformation, that is, from the stationary phase to the division phase of the transformation, were inhibited. These results were consistent with the flow cytometry results. They also indicated that 14-3-3zeta can regulate the cell cycle via CyclinD1, which may also be one of the mechanisms to promote the occurrence of tumors. Moreover, the investigation of the apoptosis pathway related proteins indicated that the PUMA protein and Bax protein, which promote apoptosis, were significantly up-regulated, and the protein Bcl2, which inhibits apoptosis, was significantly down-regulated. These findings indicate that down-regulation of 14-3-3zeta can increase expression of p53 and subsequently activate the downstream Bax/Bcl2 apoptosis pathway as well as up-regulate expression of the PUMA protein, which promotes cell apoptosis.

As an important tumor suppressor gene, p53 can inhibit the growth of tumor cells by inhibiting the cell cycle progression and activation of cell apoptosis [23, 24]; moreover, its role occurs mainly through the transcriptional activation of its downstream target genes [13, 15]. Abnormal p53 expression may lead to the occurrence of tumors. In this study, we determined that the *14-3-3zeta* gene can interfere with the normal expression of p53, which is consistent with the previous point of view. PUMA, that is, p53 up-regulated modulator of apoptosis factors, as well as Bax and Bcl2 belong to the Bcl2 family members and regulate cell apoptosis at the level of the mitochondria. Because PUMA has only one BH3 domain, it must be combined with other proteins of the Bcl2 family to exercise its biological function [25]. *PUMA* is a downstream target gene of *p53*; moreover, PUMA is a key protein in the p53 induced apoptosis pathway, which strictly regulates expression of p53. As previously stated, PUMA induced apoptosis also requires direct or indirect effects of Bax and/or Bcl2, increased expression of Bax and down-regulation of Bcl2 expression to achieve the function of promoting the apoptosis of gastric cancer cells. Thus, the results of this study are consistent with the previously described reports [26, 27].

In conclusion, the experiment using the siRNA interference technique successfully interfered with expression of the *14-3-3zeta* gene in human gastric cancer cells, and the cell proliferation was inhibited. Low expression of the *14-3-3zeta* gene regulation of the p53 signaling pathway further down-regulated the cycle related protein CyclinD1, so that the cell division was blocked in the G0/G1 phase to prevent human gastric cancer cell division. Moreover, it promoted apoptosis through up-regulation of its downstream proteins PUMA and Bax and down-regulation of the inhibitor of the apoptosis protein Bcl2. This study investigated the regulation of the *14-3-3zeta* gene on human gastric cancer cell proliferation and apoptosis. The results indicated that the *14-3-3zeta* gene may represent a potential target for the diagnosis and treatment of gastric cancer. It is necessary to further investigate the role of the *14-3-3zeta* gene *in vivo* and the effect of other pathways, as well as the effects on other tumor biological characteristics, such as invasion and other effects.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhonghua Wang, Department of Cardiothoracic Surgery, General Hospital of Xinjiang Military Region, Urumchi 830000, Xinjiang Uygur Autonomous Region, China. Tel: +86 15299050273; E-mail: wangzh_cn11@163.com

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