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

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Abstract: Background: Preliminary studies have indicated that there is abnormal expression of the 14-3-3-zeta gene in gastric cancer cells; however, the effect and mechanism of this abnormal expression are unknown. Purpose: To investigate the effect of the 14-3-3-zeta gene on proliferation and apoptosis in human gastric cancer cells. Methods: 14-3-3-zeta-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, CyclinD1, PUMA, Bax and Bcl2 were analyzed by Western blot. Results: Compared with the control groups, down-regulation of 14-3-3-zeta 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 14-3-3-zeta siRNA cells. Furthermore, down-regulation of 14-3-3-zeta increased expression of the p53, PUMA and Bax proteins, whereas it reduced expression of the CyclinD1 and Bcl2 proteins. Conclusions: Down-regulation of 14-3-3-zeta 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. 14-3-3-zeta has the potential to serve as an early marker of gastric cancer.

Keywords: 14-3-3-zeta, 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 14-3-3-zeta 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 14-3-3-zeta 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 14-3-3-zeta gene of the human gastric cancer cell line SGC-7901. The proliferation and apoptosis of gastric cancer cells were sub-
p53 is the target of 14-3-3zeta

Table 1. Details of primary antibodies used in this study

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

Subsequently 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% CO₂ 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 (SYBR II 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^ΔΔCt 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 FLUOstar 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 resuspend 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.
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 14-3-3-zeta 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 14-3-3-zeta 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 1. After transfection, the 14-3-3-zeta gene expression was tested by RT-qPCR and Western Blot. A. Relative folds of 14-3-3-zeta 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 14-3-3-zeta protein extracts from transfected SGC-7901 cells. β-actin was used as a loading control. C. Protein signals of 14-3-3-zeta 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).
p53 is the target of 14-3-3zeta

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, mean ± s)

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

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

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 (P > 0.05) (Figure 3 and Table 2). These results indicated that after interference 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-FI/C/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 CyclinD1 in the 14-3-3zeta siRNA group was significantly lower than...
p53 is the target of 14-3-3zeta


...that in the blank control group and control-siRNA group (P<0.05). However, expression of the apoptosis related pathway proteins p53, PUMA, Bax and Bcl2 was significantly increased (P<0.05).

...that in the blank control group and control-siRNA group (P<0.05). However, expression of the apoptosis related pathway proteins p53, PUMA, Bax and Bcl2 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**

14-3-3 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 detected 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 cell 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 cell 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.
p53 is the target of 14-3-3zeta

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