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Silencing of *Bcl-XL* Expression in Human MGC-803 Gastric Cancer Cells by siRNA

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Abstract To investigate the inhibitory effect of the Bcl-XL small interfering RNA (siRNA) on *Bcl-XL* gene expression in the human gastric cancer cell line MGC-803, green fluorescent protein (GFP) siRNA was constructed and transfected into MGC-803 cells, together with GFP expression vector pTrace SV40. *GFP* expression levels were observed using fluorescence microscopy. Bcl-XL siRNA and negative siRNA were then constructed and stably transfected into MGC-803 cells. RT-PCR and immunofluorescence were used to detect the expression of Bcl-XL. Spontaneous apoptosis was detected by acridine orange (AO) and flow cytometry. Results were as follows: (1) 48 h after GFP expression vector and GFP siRNA co-transfection, the expression level of GFP in the GFP siRNA group was much lower than the negative siRNA group, according to fluorescence microscopy results. The mRNA and protein levels of *Bcl-XL* in Bcl-XL siRNA stable transfectants were reduced to almost background level compared with negative siRNA transfectants or untreated cells. (2) Changes in nucleus morphology was observed by AO staining nucleic and flow cytometry analysis, which showed that stable Bcl-XL siRNA transfectants have an increased spontaneous apoptosis (21.17%±1.26% vs. 1.19%±0.18% and 1.56%±0.15% respectively, $P<0.05$ vs. negative siRNA or untreated control). siRNA targeting *GFP* or *Bcl-XL* genes can specifically suppress *GFP* or *Bcl-XL* expression in MGC-803 cells, and Bcl-XL siRNA can increase spontaneous apoptosis. Bcl-XL siRNA may be a beneficial agent against human gastric adenocarcinoma.

Key words *Bcl-XL*; siRNA; MGC-803 cells

Gastric adenocarcinoma is the second leading cause of cancer mortality in the world and the leading cause of cancer mortality in China. There is still no effective treatment for patients with advanced gastric adenocarcinoma [1]. Chemotherapy has generally shown some clinical effect, but resistance to chemotherapeutical drugs is a big problem. Most of these drugs act primarily by inducing apoptosis. The development of resistance of cancer cells to cytotoxic drugs may be a result of resistance to apoptosis. Apoptosis is regulated in part by the Bcl-2 family including pro-apoptotic Bax and Bak and anti-apoptotic Bcl-2, Bcl-XL, and Mcl-1. The relative ratio of these proteins determines the sensitivity or resistance of cells to various apoptotic stimuli [2,3]. It was reported that most cancers show over-expression of anti-apoptotic proteins. It may be a good therapeutic method to down-regulate over-expressed anti-apoptotic genes, such as *Bcl-2* and *Bcl-XL*, or *IAP* and *Mcl-1* genes. In fact, down-regulation of *Bcl-2* expression by antisense oligonucleotides is currently at the final stage of clinical trial. But antisense oligonucleotide technology also faces many problems, including low absorption rates, non-specific inhibition effects, large effective dosage and toxicity [4, 5]. Recently, the successful use of small interfering RNAs (siRNAs) showed a promising therapeutic method. RNA interference (RNAi) is a cellular pathway of homologous

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gene silencing in a sequence-specific manner at the mRNA level. The basic mechanism of RNAi is that a double-stranded RNA (dsRNA) is broken into short pieces called short interfering RNA (siRNA), which trigger the activation of an RNA-cutting enzyme (ribonuclease) directed specifically to degrade just the messenger RNA related to the trigger by an identical sequence, whereas other genes remain unaffected [6–8]. RNAi may provide a new therapeutic technique for tumors such as leukemia, melanoma, breast, colon and cervical cancer [9–12]. Several studies have documented that successful down-regulation of *BCR-ABL*, *bcl-2*, *c-raf* and *xIAP* expression in human myeloid leukemia cells results in inducing apoptosis [13–15], and down-regulation of *MDR-1* results in up-regulating chemosensitization of human pancreatic and gastric cell lines [16].

In this work, we observed the inhibitory effect of siRNA targeting *Bcl-XL* on the human gastric cancer cell line MGC-803 and the increased spontaneous apoptosis in cells transfected with Bcl-XL siRNA.

Materials and Methods

siRNA vector construction

pSilencer 3.1-H1 vector was purchased from Ambion (Austin, USA). The Bcl-XL siRNA inserting sequence had sense and antisense sequences as follows: *Bcl-XL* sense sequence 5′-CAGGGACAGCATATCAGAG-3′, antisense sequence 5′-CTCTGATATGCTGTCCCTG-3′. The Ambion web-based target sequence converter was used to convert siRNA target sites into double-stranded DNA fragments with *Bam*HI and *Hin*dIII sticky ends. The fragments were synthesized by Shanghai Sangon (Shanghai, China), annealed, and ligated into the linearized pSilencer vector. Negative control vector that expresses a hairpin siRNA with limited homology to any known sequences in the human genome and green fluorescent protein gene (*GFP*) control insert template were provided with the vector kit.

Cell lines and transfection

Human gastric adenocarcinoma cell line MGC-803, obtained from Sun Yat-Sen University (Guangzhou, China), was routinely maintained in phenol red-free Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, USA) containing 100 μl/ml fetal bovine serum (Hyclone, Logan, USA), 37 °C in a humidified atmosphere with 5% CO_2 in air. Cells grown in 6-well plates were transfected with Lipofectamine 2000 and harvested 2 d after the transfection, then cells were split at a ratio of 1:12 in 24 wells. After 24 h, geneticin (G418; Amresco, Solon, USA) at the final concentration 400 μg/ml was added to select transfected Bcl-XL siRNA and negative siRNA cells. The cultures were refreshed using G418-containing medium every 4 d. After 8 d, colonies were observed and picked to expand the culture. After 14 d, cells were harvested and examined. Plasmids encoding *GFP* and siRNAs were generally used at a ratio of 1:1. GFP siRNA and negative siRNA cells were directly detected under fluorescence microscope 48 h after GFP expression vector and GFPsiRNA or negative siRNA co-transfection.

RT-PCR

Bcl-XL siRNA or negative siRNA stably transfected cells and untreated control cells were harvested and washed with phosphate buffer saline (PBS), and total RNA was extracted from the cells using Trizol reagent (Gibco BRL) according to the manufacturer's protocol. 3 μg of total RNA was used for reverse transcription in a total volume of 20 μl with the Superscript preamplification system (Promega, Madison, USA). Aliquots of 1.5 μl cDNA were subsequently amplified in a total volume of 20 μl using the Gene amp PCR kit following conditions recommended by the manufacturer. The sense and antisense primers for *Bcl-XL* were 5′-TTGGACAATGGACTGGTTGA-3′ and 5′-GTAGAGTGGATGGTCAGTG-3′ (780 bp) respectively. The sense and antisense primers for the β-actin gene used as an internal control were 5′-GGTGGCACCTGTGGT-CCACCT-3′ and 5′-CTTCACTTGTGGCCCAGATAG-3′ (420 bp), respectively. The cycling conditions were 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were separated on the 1.5% agarose gel stained with ethidium bromide and viewed under ultraviolet light.

Immunofluorescence microscopy

Transfected and untreated cells were seeded and grown on cover slips in 6-well plates. After 24 h they were washed twice with PBS and fixed with methanol acetic acid (3:1) for 15 min at room temperature. The cells were permeabilized with PBS containing 0.25% Triton X-100 (Amresco) and 5% dimethyl sulfoxide (DMSO) (Sangon, Shanghai, China) for 30 min at 37 °C and washed twice with PBS containing 0.25% Triton X-100. Cells were then incubated with the Bcl-XL primary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) for 60 min at 37 °C.

The anti-Bcl-XL was used at the dilution of 1:100 in PBS. After washing for three times, the cells were incubated with the rabbit anti-mouse RPE-conjugated secondary antibodies (BD PharMingen, San Diego, USA) for 60 min at 37 °C and washed three times with PBS. The cover slips were directly observed under fluorescence microscope (Olympus Company, Ishikawa-cho, Hachioji-shi, Tokyo, Japan), and the data were acquired with Pixera Camera (Pixera Corporation, Los Gatos, USA).

Apoptosis analysis

Cell apoptosis was identified by fluorescence staining with acridine orange (AO; Sigma, St. Louis, USA). Cells were collected from the above group and washed once, resuspended in PBS, then 25 μl of the cell suspension was mixed with 1 μl of a dye mixture containing AO (100 μg/ml) in PBS. One drop of the stained cell suspension was placed on a microscope slide and observed under fluorescence microscope.

Apoptosis was also determined by flow cytometry analysis. Cells were washed twice with 0.01 M PBS and fixed with 70% ethanol. The cells were then washed once with PBS, digested by 200 μl RNase (1 mg/ml) at 37 °C for 30 min, and stained with 800 μl propidium iodide (50 μg/ml) at room temperature for 30 min. Cells were subject to flow cytometry analysis (EPICS-XL, Beckman Coulter, Fullerton, USA) and data were analyzed with Multipcycle software.

Statistical analysis

Statistical analysis was performed using SPSS software (Release 11.0, SPSS Inc., Chicago, USA). Data were expressed as mean±SD and analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) test; and $P<0.05$ was considered significant.

Results

siRNA synthesized from DNA templates efficiently inhibited the transfected *GFP* gene and endogenous *Bcl-XL* gene in mammalian cells

Extracted plasmids were primarily confirmed by agarose gel electrophoresis [**Fig. 1(A)**]. We then used sequencing to verify the GFP or Bcl-XL siRNA inserted templates [**Fig. 1(B)**].

First, the GFP siRNA or negative siRNA together with GFP expression vector were transfected into MGC-803 cells. After 48 h, cells were collected and washed twice with PBS, and directly observed under a fluorescence microscope. No significant changes of *GFP* expression were found in cells transfected with negative siRNA vector [**Fig. 2(A)**, 1]. In contrast, the GFP siRNA vector greatly diminished its expression [**Fig. 2(A)**, 2]. The lack of complete inhibition may be due, in part, to the high level of *GFP* expression that was directed by a strong CMV promoter.

Next, we used vector-expressing siRNA to repress the endogenous *Bcl-XL* gene. MGC-803 cells were transfected with either the negative vector or Bcl-XL siRNA vector which directs synthesis of Bcl-XL siRNA. After 48 h,

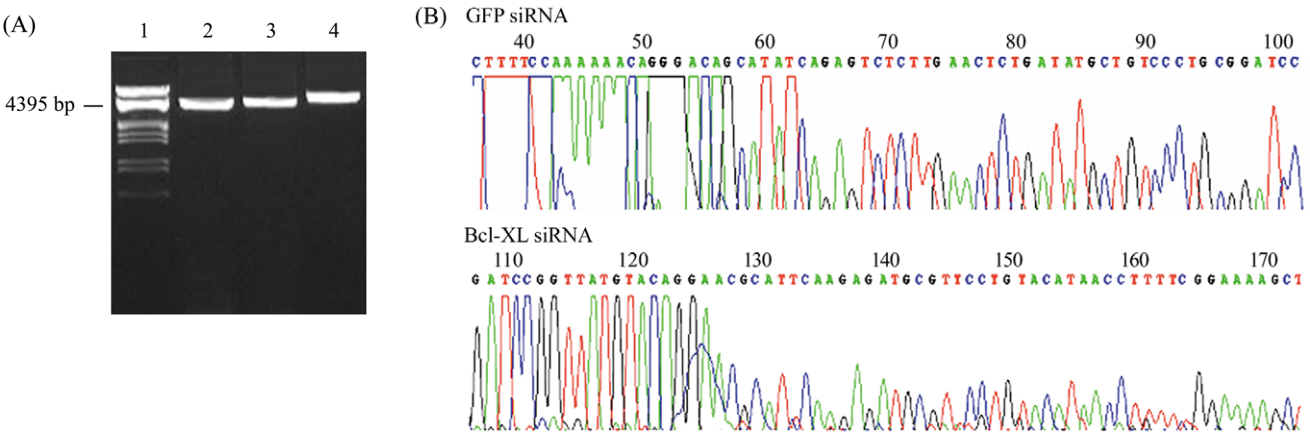

Fig. 1 Identifying GFP siRNA and Bcl-XL siRNA expression vectors

(A) Agarose gel electrophoresis confirmed the extracted plasmids. 1, marker; 2, GFP siRNA vector; 3, Bcl-XL siRNA vector; 4, pTrace SV40 GFP expressing vector. (B) Sequencing confirmed the insert sequence.

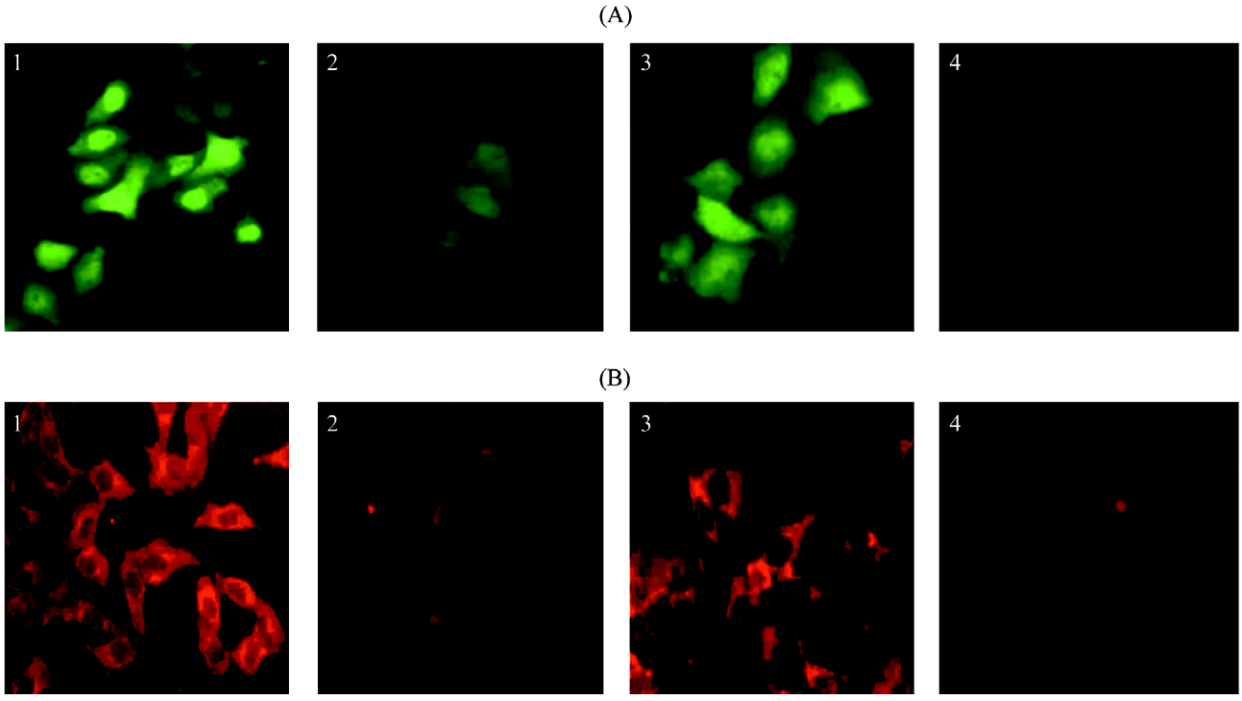

Fig. 2 Fluorescence analysis of the effect of siRNA on *GFP* and *Bcl-XL* gene expression

(A) GFP siRNA reduced *GFP* expression. 1, *GFP* expression cells; 2, *GFP* expression together with GFP siRNA cells; 3, *GFP* expression together with negative siRNA cells; 4, cells only added lipofectamine. Magnification, 40×. (B) The Bcl-XL protein level was observed by fluorescence microscopy. 1, control cells of Bcl-XL protein; 2, level of Bcl-XL protein in cells transfected with Bcl-XL siRNA; 3, level of Bcl-XL protein in cells transfected with negative siRNA; 4, level of fluorescent background of cells only stained with RPE-conjugated rabbit anti-mouse secondary antibody (representative of three independent experiments). Magnification, 40×.

protein expression was observed under a fluorescence microscope. Both the untreated control group and the negative siRNA group showed similar fluorescence expression [**Fig. 2(B)**, 1 and 3]. But *Bcl-XL* expression in cells transfected with Bcl-XL siRNA vector was reduced to the control level with the secondary antibody alone [**Fig. 2(B)**, 2 and 4]. Consistent with this, the RT-PCR results showed that siRNA greatly reduced the mRNA of *Bcl-XL* (**Fig. 3**).

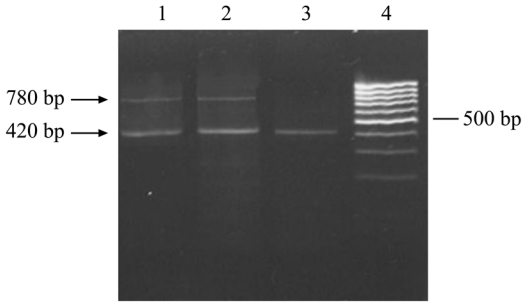

Fig. 3 RT-PCR analysis of the effect of siRNA on *GFP* and *Bcl-XL* gene expression

The *Bcl-XL* mRNA level was assessed by RT-PCR. 1, level of *Bcl-XL* mRNA in the cells transfected with negative siRNA; 2, control level of *Bcl-XL* mRNA; 3, level of *Bcl-XL* in cells transfected with Bcl-XL siRNA; 4, marker. The PCR products were indicated in arrows: the 420 bp band is the β-actin fragment; the 780 bp band is *Bcl-XL*.

Down-regulation of Bcl-XL increases the spontaneous apoptosis of cells

Having demonstrated that Bcl-XL siRNA can down-regulate the expression of Bcl-XL, we asked whether there is higher spontaneous apoptosis in cells transfected with Bcl-XL siRNA. First, we examined the morphological changes in the cells. The nuclei of Bcl-XL siRNA transfectants exhibited bright condensed chromatin or were fragmented, and some cells were blebbed. In contrast, the untreated cells and negative siRNA transfectants did not show these apoptotic features [**Fig. 4(A)**]. We then analyzed the Bcl-XL siRNA cell cycle by flow cytometry. The Bcl-XL siRNA group had a higher proportion of cells

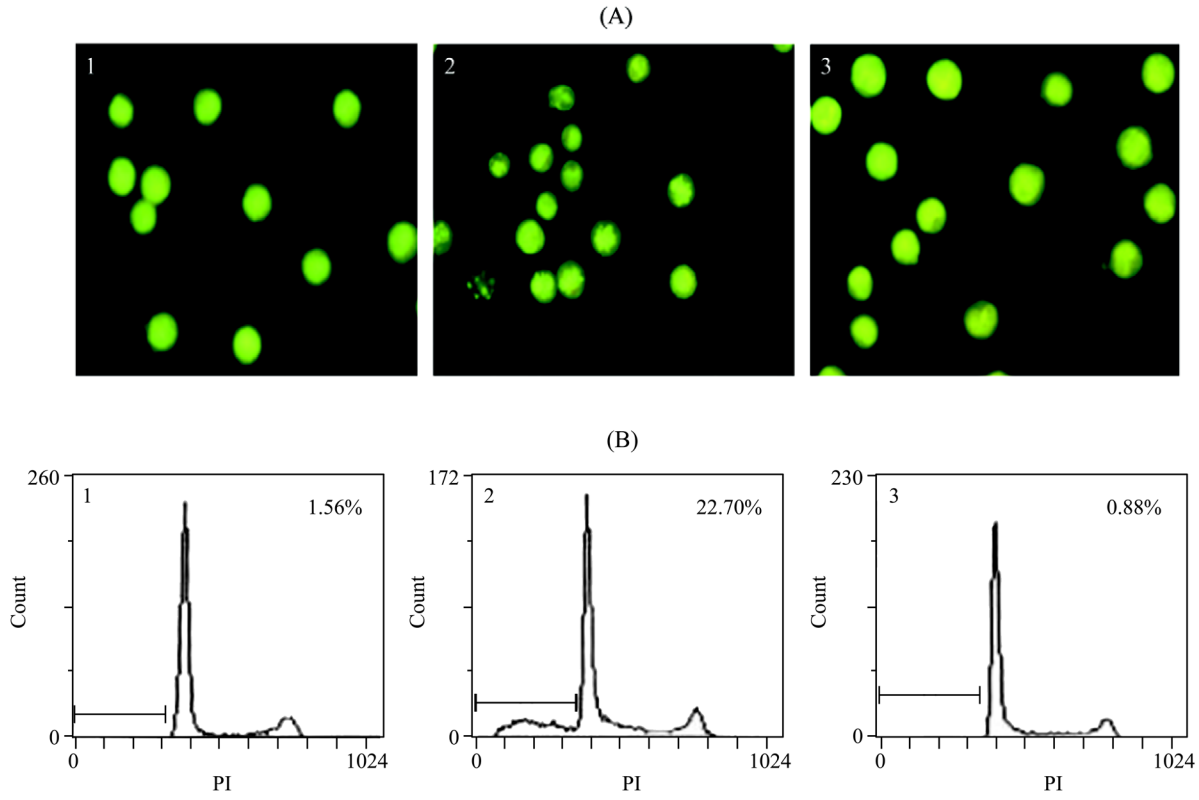

Fig. 4 Bcl-XL siRNA induced apoptosis

(A) Morphological study with fluorescence microscopy. 1, control cells; 2, Bcl-XL siRNA cells; 3, negative siRNA cells. Magnification, 40×. (B) Flow cytometry analysis of propidium iodide (PI) -stained cells. The percentage of hypodiploid cells is depicted in each histogram (representative of three independent experiments). 1, control cells; 2, Bcl-XL siRNA cells; 3, negative siRNA cells.

in the sub-G_1 population than the negative siRNA group or the untreated group, 21.17%±1.26% vs. 1.19%±0.18% and 1.56%±0.15% respectively, $P<0.05$ vs. negative siRNA or untreated control [**Fig. 4(B)**].

Discussion

The results presented in this study show that RNAi is effective in treating the human gastric cancer cell line MGC-803 and that this phenomenon has potential applicability as a therapeutic approach to gastric cancer treatment. So far, there are several methods to produce siRNA, including chemical synthesis, *in vitro* transcriptional synthesis, and vector-expressing siRNA [17–19]. Here, we used the vector-expressing hairpin siRNA method. First, we transiently co-transfected GFP siRNA vector and GFP vector, observed the down-regulation of the exogenous *GFP* gene expression, and demonstrated that it is feasible to use the vector-expressing siRNA method in MGC-803 cells. Then we stably transfected the Bcl-XL siRNA vector to MGC-803 cells.

Cancer chemotherapy mitigate the adverse side effects of drugs by molecular targeting [20]. It has been reported that Bcl-XL is an anti-apoptotic factor, in fact, cells over-expressing Bcl-XL showed resistance against a variety of cellular stress [21–23], so we chose *Bcl-XL* as the target gene to observe whether Bcl-XL siRNA could increase spontaneous apoptosis. From morphological study to cell cycle analysis, our results demonstrated that Bcl-XL siRNA indeed increased spontaneous cellular apoptosis.

In conclusion, the study attempted to explore the functionality of the RNAi pathway in gastric cancer cell lines MGC-803 and to evaluate the biological impact of the phenomenon. Further research is necessary to improve the efficacy of the vector expressing siRNA delivery and action, to investigate the drug sensitivity of cells by Bcl-XL siRNA combined with chemotherapeutic drugs, and to determine the optimal therapeutic action by combined RNAi for several different anti-apoptotic genes.

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