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Effect of silencing Bcl-2 expression by small interfering RNA on radiosensitivity of gastric cancer BGC823 cells

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ABSTRACT

Objective: To explore the influence of silencing Bcl-2 expression by small interfering RNA (siRNA) on Bcl-2 protein expression, cell apoptosis rate and radiosensitivity of gastric cancer BGC823 cells. **Methods:** siRNA segment for Bcl-2 gene was designed and synthesized, then was induced into gastric cancer BGC 823 cells by liposome transfection. Bcl-2 protein expression was detected by Western Blotting. After X radiation, flow cytometry and clone forming assay were used to determine the effects of RNA interference on BGC823 cell apoptosis rate and radiosensitivity. **Result:** After the transfection of Bcl-2 siRNA, the positive expression rate of Bcl-2 protein in BGC823 cells was (35.45±2.35)%. Compared with the control group and negative siRNA transfection group, the rate was significantly decreased ($P<0.01$). The apoptosis rate of BGC823-RNAi cell was (10.81±0.91)%, which was significantly higher than the control group and negative siRNA transfection group ($P<0.01$). After 48h X radiation, the apoptosis rate of BGC823-RNAi was (28.91±1.40)%, which was significantly higher than the control group and the group without radiation ($P<0.01$). During clone forming assay D_0 , D_q and SF_2 values in Bcl-2 siRNA1 transfection group were all lower than those in the control group. The radiosensitivity ratio was 1.28 (the ratio of D_0) and 1.60 (the ratio of D_q). **Conclusions:** Specific siRNA of Bcl-2 gene can effectively inhibit the expression of Bcl-2 gene, enhance the radiosensitivity and apoptosis of gastric cancer BGC823 cells, having good clinical application perspective.

1. Introduction

Radiotherapy is one of the main methods for treating gastric cancer, but the radiosensitivity varies in person. Inducing tumor cell apoptosis is one of the main mechanisms of radiotherapy, however, cell apoptosis was regulated by its related genes. Therefore, regarding apoptosis associated genes as the marker of radiosensitization has become the hot research spot[1]. Bcl-2, an important gene inhibiting apoptosis, plays an important role in maintaining cell proliferation, development and dynamic balance. The high expression of Bcl-2 gene can influence the tumor development in a certain degree and resist radiotherapy. Some researches[2–4] showed that the overexpression of Bcl-2 gene can inhibit tumor cell apoptosis induced by radiotherapy, and the inhibition of Bcl-2 gene expression may increase the radiosensitization to radiotherapy.

RNA interference (RNAi) is the new discovered and developed gene block technique, inhibiting the expression of the target gene through using double strand RNA to induce the degradation of host homologous mRNA[5,6]. This research used synthesized siRNA to transfect gastric cancer BGC823 cells and observed the influence of inhibiting Bcl-2 gene expression on the apoptosis and radiosensitivity, which provided theoretical basis for exploring the gene therapy for gastric cancer.

2. Materials and methods

2.1. Cell line and main reagents

The human gastric cancer BGC823 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Academia Sinica. RPMI-1640 culture fluid and fetal bovine serum were from America Gibco Company; siRNA order, siRNA transfection reagent and siRNA culture fluid from America Qiagen Company. Lipo-fectamine™ 2000 liposome was the product of Santa Cruz Company. Mouse anti-human monoclonal antibody and HRP marked goat anti-rat second

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antibody were purchased from Santa Cruz Company. All the primers were designed by the America Applied Biosystem Company and purchased from the Shanghai Boya Biology Co., Ltd. Annexin V-FITC apoptosis kits were from Beijing Baosai Biotech Co., Ltd.

2.2. Design and synthesis of siRNA

Positive-sense strand of Bcl-2 siRNA: 5'-CGGCCUCUGUUUGAUUUCU-3'; antisense strand: 5'-AGAAAUCAACAGAGGCCGTT-3'. The negative control was siRNA aimed at no target gene, the positive-sense strand of which: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense strand: 5'-ACGUGACACGUUCGGAGAATT-3'.

2.3. Cell culture and transfection

Gastric cancer BGC823 cells were cultured in the RPMI1640 culture fluid containing 10% fetal calf serum under the condition of 37 °C, 5% CO₂ and saturated humidity. One day before transfection, BGC823 cells in log phase were inoculated to 6-well culture plate at 5×10⁵ cells/well. 24 h later when the cell was proliferated to 50%–70%, transfection was conducted according to the Lipo-fectamine™ 2000 instruction. The transfection efficiency was detected by fluorescently-labeled siRNA. Three groups were control group: only liposome was added without any siRNA; negative control group: siRNA aimed at no target gene was transfected; Bcl-2 siRNA group: siRNA targeted at Bcl-2 gene was transfected.

2.4. Bcl-2 protein expression detected by Western blotting

The transfected cells were put in the cell lysis buffer. The protein concentration was detected by the Coomassie brilliant blue G-250 dying method. 10% SDS-PAGE electrophoresis was conducted. The products were electrotransferred to NC membrane. After washed by Tris buffer solution containing 2% BSA and sealed for 1 h, the first antibody-Bcl-2 mouse anti-human monoclonal antibody was added (The working fluid was diluted according to 1:10), then the second antibody- HRP marked goat anti-mouse IgG was added (The working fluid was diluted according to 1:10). Then incubation was conducted for 1h at 37°C. DAB was used for coloration.

2.5. Cell irradiation

The cells were irradiated by 6MV X-ray produced by linear accelerator (dose rate: 4 Gy/min). The cell side of the culture flask was put upward with 1 cm compensator when conducting irradiation, and the source-skin distance was 100 cm.

2.6. Apoptosis detection

After 48 h irradiation, the cells in 3 groups were washed twice by PBS and suspended in the 100 μL AnnexinV binding buffer. Then the cell concentration was rejusted to 1×10⁶/mL by PBS. 10 μL Annexin V-FITC and 5 μL PI were added and well mixed. 15 min incubation was conducted at room temperature away from light. After adding 300 μL combining buffer, the cell apoptosis rate was detected by flow cytometry immediately.

2.7. Clone forming assay

After digesting the transfected and untransfected cells in log phase into single cell suspension, the cells were inoculated to 35mm corning culture dish according to 150, 300, 600, 1 200, 1 500, 2 000 cells/dish. The cells were respectively given 0, 2, 4, 6, 8, 10 Gy X-ray irradiation after 24h culture. The cells were continuously given 10–14 d conventional culture after irradiation. After Giemsa's staining, the clone number of cells was counted by naked eye.

2.8. Statistical processing

SPSS15.0 software was used for statistical processing. The measurement data were expressed by mean±standard deviation ($\bar{x}\pm s$). *t* test or single factor analysis of variance were adopted. A *P*<0.05 was taken to indicate a difference of statistical significance.

3. Results

3.1. Cell transfection of siRNA

siRNA was successfully transferred into gastric cancer BGC823 cell. The transfected BGC823 cell can be seen in green fluorescence under the fluorescent microscope. Cell in the shape of polygon was successfully transfected (Figure 1). The transfection efficacy was highest (92%) when the final concentration of siRNA was 100 nM.

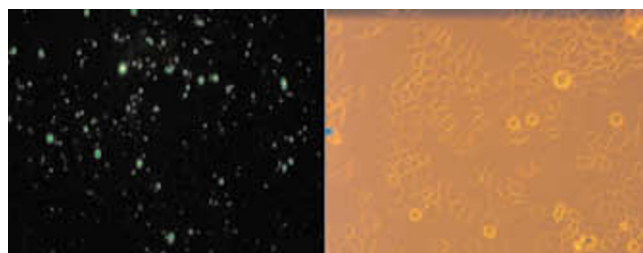


Figure 1. Fluorescent microscope image (×200) and inverted microscope image (×100) of siRNA transfected gastric cancer BGC823 cell.

3.2. Expression change of Bcl-2 protein after siRNA transfection

The Bcl-2 protein expression in BGC823 cell detected by Western-Blotting indicated that the Bcl-2/β-action expression level of the control group, negative control group and Bcl-2 siRNA group was 85.41±1.63, 82.68±3.74 and 35.45±2.35. There was no statistical significance between the former two groups (*t*=1.03, *P*>0.05). There was statistical significance when comparing the Bcl-2 siRNA group with the other two groups (*t*=24.53, *t*=18.52, *P*<0.01). This indicated that siRNA targeted interference can significantly inhibit the expression level of the Bcl-2 protein.

3.3. Change of cell apoptosis rate before and after the X-ray irradiation

Before X-ray irradiation, the apoptosis rate of the BGC823-RNAi cell group was $(10.81 \pm 0.91)\%$ which was significantly increased comparing with the BGC823 cell group and the BGC823-Neg cell group ($t=8.09$, $t=7.21$, $P < 0.01$). After 48 h X-ray irradiation, the apoptosis rate of the BGC823-RNAi cell group reached $(28.91 \pm 1.40)\%$, and there was statistical significance comparing with the other two groups after irradiation ($t=9.76$, $t=8.64$, $P < 0.01$). The apoptosis rate of the BGC823-RNAi cell group after irradiation was obviously increased comparing with that before irradiation ($t=18.78$, $P < 0.01$). All these indicated that Bcl-2 siRNA can promote BGC823 cell apoptosis and improve the cell's radiosensitivity (Table 1, Figure 2).

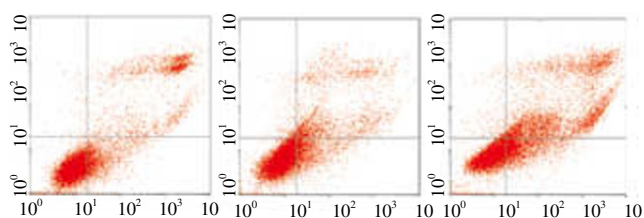


Figure 2. BGC823 cell apoptosis of Bcl-2 siRNA transfection combined with X-ray irradiation.

A: Simple X-ray group.

B: Negative siRNA transfection +X-ray group.

C: Bcl-2 siRNA1 transfection +X-ray group.

3.4. Analysis of the cell clone formation

The cell survival curve after Bcl-2 siRNA1 combined with X-ray irradiation at different doses showed that the cell survival curve obviously decreased in Bcl-2 siRNA1 group, while the cell survival curve in negative siRNA transfection group decreased unobviously (Figure 3). Radiobiological parameters were in Table 2 and SER was 1.28 (ratio of D_0), 1.60 (ratio of D_q), which indicated that there was certain radiosensitivity effect after the BGC823 cells were transfected by Bcl-2 siRNA.

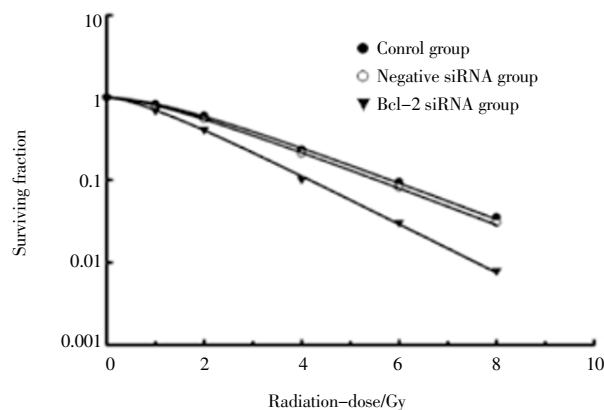


Figure 3. Influence of Bcl-2 siRNA transfection combined with X-ray irradiation on the cell survival curve.

4. Discussion

RNAi, a new technique developed in recent years having the characteristics of high degree of specificity and efficiency, is a silencing course after the transcription of specific homologous target gene induced by double-strand RNA. In the cells, siRNA distinguishes and combines the corresponding target mRNA strand through base complementarity, leading nuclease to cutting and digesting this mRNA strand. Finally, it leads to the silencing of target gene after transcription[7,8]. The recent studies showed that RNAi was widely used in experimental study as a regulation or close gene expression tool[9]. The researchers have successfully decreased the expression of many kinds of molecules in tumor cells by using RNAi technique. This research successfully decreased Bcl-2 protein expression in gastric cancer BGC823 cells, which verified the efficacy of RNA interference again.

Bcl-2 gene, a kind of apoptosis inhibition gene which has been studied lucidly so far, plays a key role in inducing apoptosis[10]. Bcl-2 protein prevents the release of cytochrome C from chondriosome through regulating endoplasmic reticulum Ca^{2+} -involved intranuclear and extranuclear matter translocation and permeability transition (PT). Furthermore, Bcl-2 interacts with Apaf-1, procaspase-9. Finally, it inhibits the apoptosis cascade reaction process caused by Caspase-9 and Caspase-3.

Table 1

Change of cell apoptosis before and after X-ray irradiation ($\bar{x} \pm s$, $n=3$).

	BGC823 (%)	BGC823-Neg (%)	BGC823-RNAi (%)
Before radiation	5.30 ± 0.75	5.82 ± 0.78	10.81 ± 0.91
After radiation	18.95 ± 1.08	19.48 ± 1.27	28.91 ± 1.40

Note: Compared with before radiation, $P < 0.01$; Compared with BGC823, $\Delta P < 0.01$; Compared with BGC823-Neg, $\blacktriangle P < 0.01$

Table 2

Radiobiological parameters of BGC823 cell multitarget simple hit model after fitting.

Group	D_0	D_q	SF ₂
Control group	1.92	1.33	0.62
Negative siRNA transfection group	1.91	1.15	0.52
Bcl-2 siRNA transfection group	1.50	0.83	0.38

Besides, Bcl-2 can inhibit the activation of many caspase hydratase molecules, which finally prevents the occurrence of apoptosis^[11-13]. Studies indicated that many tumors high express Bcl-2 protein^[14-16], including gastric cancer. And Bcl-2 protein is related to the repellence of tumor cells to chemotherapy and radiotherapy^[17-19]. It is a good target of anti-tumor molecule. Currently, many experimental studies on Bcl-2 have been reported in domestic and abroad. These experimental studies have certain positive effect on increasing radiation sensitivity of tumor and improving apoptosis rate of tumor cells. Yang *et al*^[20] used adenovirus mediated siRNA to inhibit Bcl-2 protein expression in colon cancer cell, which significantly increases the radiation sensitivity of colon cancer cells. Kim *et al*^[21] reported that using RNA to interfere Bcl-2 gene expression can inhibit the proliferation of tumor carneus cell line. The studies of Hara *et al*^[22] verified that Bcl-2 antisense oligonucleotides can combine with the target DNA or mRNA in cells and prevent its transcription or translation process. As a result, it can inhibit Bcl-2 protein expression. In this study, the Bcl-2 specific siRNA was induced into BGC823 cells through liposome transfection technique, which successfully decreased Bcl-2 protein expression and induced the apoptosis of BGC823 cells.

This research showed that compared with the control group and the negative siRNA transfection group, after the transfection of Bcl-2 siRNA, the sensitivity of BGC823 cells to X-ray irradiation obviously increased ($P < 0.01$), apoptosis and death rate significantly increased, the clone forming rate significantly decreased and the cell survival curve obviously declined, the radiosensitization ratio was 1.28 (ratio of the D_0 value) and 1.60 (ratio of the D_q value). It is believed that after BGC823 cells are transfected by siRNA, Bcl-2 protein expression in cells decreases and the threshold value of cells to apoptosis signals significantly decreases. When the apoptosis signal is given to X-ray irradiation, the death rate of cells significantly increases. Because simple Bcl-2 siRNA interference has increased apoptosis, the death of tumor cells is increased to a greater degree when it combines X-ray irradiation. Therefore, the tumor inhibition rate is increased.

The results of this study showed that using RNAi technique can decrease Bcl-2 protein expression in gastric cancer cell- BGC823 and the sensitivity of gastric cancer cell to X-ray irradiation was significantly increased, which provided a certain theoretical basis for the application of Bcl-2 gene specific siRNA in clinical treatment of gastric cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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