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ORIGINAL ARTICLE

Effect of Bcl-xL gene expression silenced by RNA interference on invasion of human colorectal cancer cells

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Summary

Purpose: *To investigate the effect and possible mechanisms of Bcl-xL gene on the invasive capacity of human colon cancer cells.*

Methods: *HT29 human colorectal carcinoma cell line was transfected by small interfering RNA (siRNA) of Bcl-xL gene. Quantitative real-time (RT)-PCR and Western blot were used to detect the transfection, and soft agar colony culture experiments and Boyden chamber model test were used for cancer cell proliferation and invasion, respectively. Western blot was used to detect the protein changes of urokinase-type plasminogen activator (uPA) in cancer cells.*

Results: *Compared with the control group, the number of soft agar colonies and the number of penetrating membrane cells significantly reduced in the siRNA transfection group, and had dose-dependent characteristics; the uPA protein decreased significantly in the siRNA transfected cells.*

Conclusion: *Bcl-xL gene may play an important role in the invasion of colon cancer cells, and the mechanism may be related to regulation of uPA expression.*

Key words: *Bcl-xL, colorectal cancer, invasion, urokinase plasminogen activator*

Introduction

Although the clinical efficacy of most mAbs is Colorectal cancer is the third most common malignant neoplasm worldwide [1] and the second leading cause of cancer-related deaths [2]. Despite recent advances in diagnosis and treatment, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Enhanced understanding of the signaling mechanisms that regulate metastasis of colon cancer may provide important insights for more effective therapeutic strategies.

Bcl-xL gene is an important inhibitor of apoptosis of the Bcl-2 family. Research shows that the high expression of Bcl-xL gene in a variety of malignant tumors is closely related to the occurrence, development and prognosis of tumors [1-3]. Many studies have found that high expression of Bcl-xL gene has important relationship with cell invasion and metastasis of some malignant tumors such as squamous cell carcinoma, liver cancer, colorectal cancer and breast cancer [2-6]. *In vitro* tests have shown that cancer cell invasion might also increase or decrease in parallel with the increase and decrease of the expression level of Bcl-xL gene [7-10]. Hence, Bcl-xL gene is closely related to the invasion of some cancer cells.

Previous studies on the subject found that the Bcl-xL gene was overexpressed in colorectal carcinoma, and its high expression was closely related to the pathological grade, lymph node metastasis and Dukes stage [6]. It is suggested that Bcl-xL gene plays an important role in the invasive ca-

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pacity of colorectal cancer cells, but the mechanism is not clear.

Based on a previous work [11], this study adopted the RNA interfering (RNAi) technology, using Bcl-xL siRNA chemical synthesis transfecting colorectal cancer HT29 cell line, to observe its effect on the invasion and to explore the possible mechanism from the aspect of uPA. In the present study, we investigated the possible correlation of Bcl-xL and the invasion capacity of the HT29 human colon cancer cell line *in vivo* and *in vitro*.

Methods

Bcl-xL siRNAs sequence

The siRNA (5'-CTCTGATATGCTGTCCCTG-3', antisense sequence) corresponding to Bcl-xL mRNA with dTdT on 3'-overhangs was designed and chemically synthesized according to the recommendation of the manufacturer (Dharmacon Research, Luisiana, USA), and its sequences were 5'-UUCUCCGAACGUGUCACGUTdTd-3' and 5'-ACGUGACACGUUCGGAGAATdTdT-3'.

Cell culture and transfection

Colorectal cancer HT29 cells (purchased from Biochemistry and Cell Biology Institute of Shanghai Life Science Academy) were cultured in RBMI-1640 medium, containing 10% fetal bovine serum, at 37°C, 5% CO_2, under saturated humidity environment. One day before transfection, cells of 1×10^5/mL concentration were seeded in 24- or 6-well culture plates, cultured overnight, and transfected the next day. Cells were divided into three groups: 1) the blank control group (Con-A): colorectal cancer cells without any treatment; 2) the empty vector control group (Con-B): group treated only with liposome treatment; 3) siRNA groups of different concentrations (3.12, 6.25, 12.5 and 25 nmol/L) of siRNA, entrapped in liposomes were maintained in RBMI-1640 with 10% fetal bovine serum. After transfection, cells were digested by trypsin and collected at different time intervals to undergo the following test.

Detection of Bcl-xL mRNA level

According to the Bcl-xL, siRNA antisense 5'-CTCTGATATGCTGTCCCTG-3' was synthesized by the Dharmacon Company. Primer sequences were: the upstream 5'-TCCTTGTCTACGCTTTCCACG-3' and downstream 5'-GGTCGCATTGTGGCCTTT-3'. FAM probe was 5'-CC AGGAGACCATCCGCGTCACC-3'-TAMRA. RNA was extracted by TRIzol, and underwent cDNA synthesis according to Kunze et al. method [10]. Bcl-xL gene mRNA levels were detected by quantitative RT-PCR fluorescence detection (TRIzol, RNase inhibitor, reverse transcriptase SSRT II, Taq enzyme and Oligofectamine transfection reagent were purchased from American Invitrogen Co (California, USA). Quantitative PCR reaction instrument 7700 Sequence Detector was purchased from the ABI PRISMTM (PE Applied Bioscience, Foster City, CA).

Bcl-xL protein expression

Routine Western blot was used to detect Bcl-xL protein expression with β-actin as internal control. Briefly, this method is as follows: collecting cells from cell culture plate, washing them with precooled phosphate buffer saline twice, adding in cell lysate, putting it static on ice for 10-20 min, after stirring with a cell scraper, centrifuging the homogenate at 12000 g at 4°C for 10 min, and obtaining the supernatant, joining in the 1/3 volume of 4 × the above sample buffer, and boiling for 10 min to achieve protein denaturation. Sample protein was separated by gel electrophoresis and electrotransfered to nitrocellulose membrane; after staying at room temperature for 1 h and adding in Bcl-xL antibody (purchased from Santa Cruz, USA), it was incubated at 4°C overnight. After3 washes with TBS-T solution for 10 min, the membranes underwent hybridization with a goat anti-rabbit IgG secondary antibody (1:1000) at 37°C for 1 h. After further washing, Bcl-xL level was visualized using an ECL chemiluminescence kit.

Soft agar colony formation

According to Fun et al. [8] of soft agar colony culture test, the effect of Bcl-xL siRNA on anchorage independent proliferation of colorectal cancer cell was observed.

Invasion assay

According to a previous method [11], the experiment was performed in the modified Boyden chamber. The number of cells passing through the polycarbonate membrane was counted under light microscope (x400), and the number of invasive cells represented the tumor cell invasion ability. The number of cells was randomly counted under 10 fields, the mean value was statistically processed, and 3 samples of each group were counted for a total of 3 times.

Detection of uPA protein level

Bcl-xL gene transfected cancer cells in different times and different concentrations were collected, cell protein was extracted with the β-actin as internal control, and routine Western blot (uPA monoclonal antibody, Santa Cruz Co, USA) was carried out to detect the changes of uPA protein levels. Identical to the above method, using the Kodak Digital Science 1D Image Analysis Software (Eastman Kodak Co, USA), Western blot strip net gray value was determined, which was compared with the results of the internal control β-actin, and the ratio was calculated. uPA protein level detection and strip net gray value calculation were repeated for 3 times.

Statistics

SPSS 16 statistical software package was used. To achieve statistical significance, experiments were performed in triplicate (each individual experiment was performed at least 3 times). Statistical analyses used Student's t-test to compare differences between two groups and one-way ANOVA to compare differences between multiple groups. Results are given as mean±SD. Statistical significance was set at $p<0.05$.

Results

Effect of siRNA on Bcl-xL gene expression

In the blank control group and empty vector group, the mRNA and protein levels did not change, while in the siRNA group the mRNA and protein levels were significantly lower (p=0.0001, p=0.0002; Figure 1 and 2), which indicated siRNA could effectively inhibit Bcl-xL gene.

Soft agar colony formation

Soft agar colony formation test results showed that colorectal cancer HT29 cell could spontaneously form colonies in *in vitro* semisolid culture system, while the number of siRNA transfected cell colony growth significantly reduced. When the concentration of siRNA was 3.125, 6.25, 12.5 nM, the colony numbers were 18.9±1.8, 12.5±1.6, 7.8±1.5, compared with 25.2±1.9 of Con-A group. Soft agar colony formations of the siRNA group showed dose-dependent decrease ($p<0.05$).

Effect of Bcl-xL siRNA on invasion of cells

When the concentration of siRNA was 3.125, 6.25, 12.5 nM, the number of cells passing through the membrane was 36.8±1.8, 23.8±1.5, 12.5±1.6, compared with 47.8±1.9 of Con-A group. The number of cells of the siRNA group passing through the membrane decreased significantly in a dose-dependent manner ($p<0.01$).

Effect of Bcl-xL siRNA transfection on uPA protein level

The uPA protein levels of Bcl-xL transfection group were significantly reduced in a time- and concentration-dependent manner ($p<0.05$) (Figure 3).

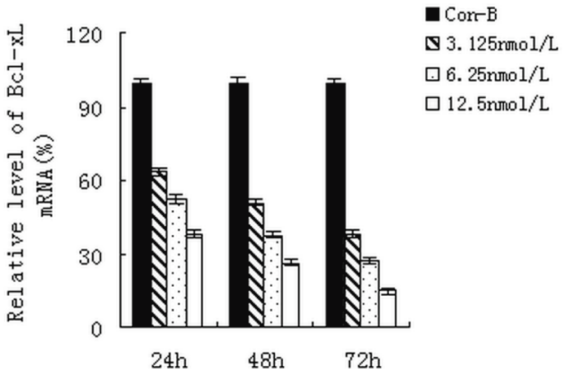

Figure 1. Effect of siRNA on Bcl-xL mRNA level of colon cancer HT29 cells (N = 3).

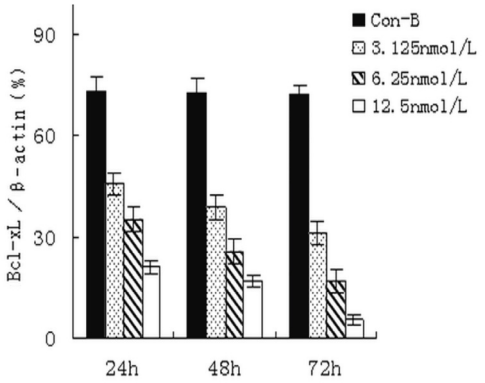

Figure 2. Effect of siRNA on Bcl-xL protein level of colon cancer HT29 cells (N = 3).

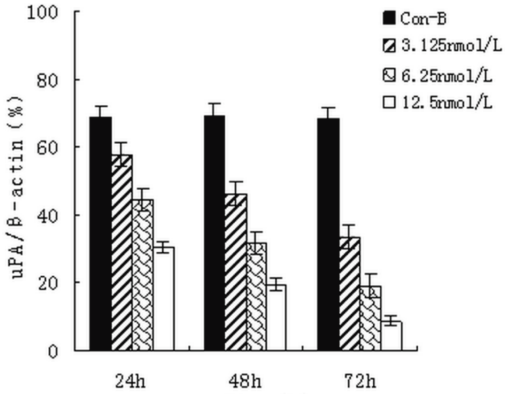

Figure 3. Effect of Bcl-xL siRNA transfection on uPA protein level of HT29 colon cancer cells.

Discussion

In recent years, the incidence of colorectal cancer is on the increase. Metastasis is one of the important problems in the treatment of colorectal cancer. A previous study found that the expression of Bcl-xL gene is high in colorectal cancer cells, and its high expression is closely related to invasion of cancer cells in lymph nodes [6]. Adenovirus-mediated siRNA targeting Bcl-xL could significantly inhibit the proliferation and colony formation of CRC cells, and Ad/shBcl-xL could significantly suppress the migration and invasion of CRC cells [12], but the mechanism is not clear.

In this study, with the help of RNAi technology, we discuss the role of Bcl-xL in human colorectal cancer cells and the possible mechanism.

Although there is lot of evidence about apoptosis and Bcl-xL gene, the relationship between Bcl-xL and invasion in malignant tumors remains rare. Our previous study showed that STAT3 siRNA transfection can result in inhibition of the invasive ability in human colon cancer cell line HT29 [8]. Interestingly, there was a significant inhibition of Bcl-xL protein in transfected cancer cells and we presumed that Bcl-xL contributed to the invasion of human colon cancer cell. In order to verify this hypothesis, the relationship of between Bcl-xL and invasion in human colon cancer was studied *in vitro*.

RNAi is an innovative technique that can significantly regulate the expression of oncogenes involved in cancer , thus constituting a promising epigenetic approach to cancer therapy [13]. While siRNA is about 21-25 nucleotides of small double-stranded RNA, which is generated in the RNAi process and is an important intermediate-effect molecular mechanism of RNAi function, there are synthetic siRNAs, which have obvious effect in removing corresponding genes mRNAs [10,13]. Based on the characteristics of Bcl-xL gene, we designed and synthesized siRNA to transfect colorectal cancer HT29 cells, and using fluorescence quantitative RT-PCR in colon cancer cells we detected mRNA and protein levels. In the transfected group, the mRNA gene and protein levels were significantly inhibited in a time- and concentration-dependent manner, which in turn indicated that Bcl-xL siRNA had been successfully transfected into colon cancer cells.

Anchorage dependence of growth blocks cell proliferation in inappropriate environments, thereby inhibiting cancer cell invasion and metastasis. Inhibition of growth regulatory pathways, including Rac, Erk and PtdIns 3-kinase in non-adherent cells mediates this effect [14]. Cell proliferation and invasion were evaluated using soft agar colony formation assays, and the more the colony numbers, the stronger the invasion capacity [15]. This study found that, after being transfected with different concentrations of Bcl-xL siRNA, the number of tumor cells that formed colony was significantly reduced in a dose-dependent manner. Boyden chamber model detection test showed that, after being transfected by siRNA, the number of colorectal cancer cells passing through the membrane was significantly reduced in a dose-dependent manner, which indicated that downregulation of Bcl-xL gene expression could inhibit the anchorage independence and invasion ability of colorectal cancer cells to a certain extent. The results of this study are similar with that of Weiler et al. [9], that is, after downregulation of Bcl-xL, the invasive ability of cancer cells may reduce.

Invasion and metastasis are the basic characteristics of malignant cells that involve very complicated mechanisms. During invasion and metastasis, the tumor cells must firstly and multiple times pass through the basement membrane and the extracellular matrix, an activity that may be related to the tumor cells producing and inducing a series of proteolytic enzymes to degrade the extracellular matrix and basement membrane, among which, metalloproteinases and serine proteases. uPA is an important member of the serine protease family. uPA, produced by tumor cells or epithelial cells, is secreted as inactive zymogen form, after being combined with the cell surface receptor uPAR, which is activated to uPA by cathepsin B, L serum enzymes or tumor cells, so as to achieve the plasminogen conversion to plasmin, which can degrade the extracellular matrix and basement membrane and promote invasion and metastasis of tumor cells [16,17]. Several authors have reported that the increase of uPA activity in colorectal cancer is closely related to invasion and metastasis [18-22]. This study found that uPA protein levels of the Bcl-xL siRNA transfected group were obviously downregulated, indicating that the Bcl-xL siRNA transfection functions to downregulate the uPA gene expression in colon cancer cells. At present, how the Bcl-xL regulates the uPA expression in cancer cells is not clear. Some authors speculated that Bcl-xL gene might play its role through some kind of internal mechanism to intervene the uPA receptor (uPAR) [23].

In conclusion, this study suggests that RNAi silencing of Bcl-xL gene inhibits the invasion of colorectal cancer cells by a mechanism which might be related to downregulation of uPA gene expression.

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