Effect of Bcl-2-siRNA on proliferation and apoptosis of pediatric acute B lymphoblastic leukemia (A-BLL) cells

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ABSTRACT. This study analyzed the effect of small interfering RNA specific for the *Bcl-2* gene (*siRNA Bcl-2*) on the proliferation and chemotherapeutic sensitivity of pediatric A-BLL cells. Marrow samples were obtained from sixty newly-diagnosed A-BLL pediatric patients. The *Bcl-2* mRNA expression in these samples was quantified by real time polymerase chain reaction. The *Bcl-2* mRNA re-expression was analyzed by RNA interference using *Bcl-2-siRNA*. Cellular proliferation was detected using the MTT (Thiazolyl Blue Tetrazolium Bromide) assay. The cell apoptosis was quantified by flow cytometry. The *Bcl-2* mRNA expression was significantly higher in the drug-resistance group than in the chemotherapy sensitivity group prior to chemotherapy ($P < 0.05$). In addition, the *Bcl-2* mRNA expression in the chemotherapy sensitivity group was significantly higher before chemotherapy than that after chemotherapy ($P < 0.05$). The *Bcl-2* mRNA expression significantly decreased in the leukemic cells of the *Bcl-2-siRNA*

transfection group. We observed statistically significant differences in the relative mRNA expression levels among the Bcl-2-siRNA transfection, blank control, liposome empty transfection, and unrelated sequence oligonucleotide groups (P < 0.05). The rate of apoptosis in pediatric A-BLL leukemic cells was observed to increase significantly after transfection with *Bcl-2-siRNA* compared to the control, liposome empty transfection, and unrelated sequence oligonucleotide groups (P < 0.05). Therefore, we concluded that *Bcl-2-siRNA* can successfully inhibit the multiplicative capacity of A-BLL leukemic cells and promote apoptosis.

Key words: *Bcl-2-siRNA* gene; Cell proliferation; Cell apoptosis; Pediatric acute B lymphocytic leukemia

INTRODUCTION

The B-cell lymphoma/leukemia-2 (Bcl-2) gene is closely associated with apoptosis and tumor incidence and progression. It is a critical regulatory factor for apoptosis, and has been the subject of widespread attention. Its anti-apoptotic effect plays an important role in the incidence of apoptosis (Kaparou et al., 2013). It is also associated with the incidence of malignant tumor, and determines the applicability of the specific chemotherapeutic drugs based on tumor prognosis (Wang et al., 2014). Acute B lymphocytic leukemia (A-BLL) is the most common pediatric acute leukemia with a high rate of fatality; A-BLL is known to severely threaten the life of pediatric patients (Singh et al., 2011). So far, its specific pathogenesis remains unclear. A few recent reports have indicated a correlation between Bcl-2 expression and drug resistance of clinical chemotherapy for pediatric A-BLL.

The expression of the *Bcl-2* gene in leukemic tumor cells can be reduced and apoptosis of the leukemic cells can be promoted by interrupting the *Bcl-2* gene during transcription and translation (Chaber et al., 2013). Therefore, the mRNA of the *Bcl-2* gene can be targeted through the synthesized small interefering RNA (siRNA), thereby interrupting the translation and reducing the expression of the *Bcl-2* gene (Vazquez-Franco et al., 2012). *Bcl-2* plays an important role in the incidence, progression, and drug resistance of pediatric A-BLL (Amirghofran et al., 2011). Therefore, in this study, siRNA specific for *Bcl-2* mRNA was designed and synthesized, and the effect of *Bcl-2-siRNA* on the proliferation and apoptosis of pediatric A-BLL cells was determined via RNA interference.

MATERIAL AND METHODS

Subjects

Sixty A-BLL pediatric patients aged between 1 and 18 years (average 8.7 years; 26 male and 24 female patients) newly diagnosed at the Affiliated Hospital of Inner Mongolia Medical University between January 2013 and June 2014 were tested in this study. A definite diagnosis was made based on the results of cytomorphological, immunophenotype, and molecular biological diagnostic criteria. The A-BLL-positive patients tested positive for CD79a, CDl9, CD10, and HLA-DR and negative for SmIg and CyIg via flow cytometry. The pediatric patients were subjected

to the vincristine, daunorubicin, L-asparaginase, and dexamethasone (VDCD) chemotherapeutic regimen recommended by the Chinese Children's Leukemia Group. Marrow was collected from all patients before and after chemotherapy.

Grouping criterion

The remission criterion was in accordance with the diagnosis and treatment criterion presented by the Hematology Group of the Pediatrics Branch of the Chinese Medical Association. The patients in complete remission were sensitive to chemotherapy and the patients not meeting the complete remission criterion were deemed to be chemotherapy-resistant (Amirghofran et al., 2009).

Materials and reagents

The INTERFERin kits were purchased from Ruixianghe Biotechnology (Beijing, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining apoptosis detection kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from ComWin Biotech Co., Ltd. (Beijing, China). Real time PCR Master Mix was purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) and MTT were purchased from Boyan Biotechnology Co., Ltd (Shanghai, China). Reverse transcription reagents and the dNTP mix were purchased from Bioleaf Biotech Co., Ltd. (Shanghai, China). Taq DNA polymerase was purchased from Fermentas (MBI Fermentas, MA, USA). The DNA marker was purchased from the Amresco (Solon, OH, USA). All primers were purchased from Weijing Biotech Co., Ltd. (Shanghai, China).

The flow cytometer was purchased from Labnet International Inc. (Edison, NJ, USA), while the UV-2000 ultraviolet analyzer was purchased from Heraeus (Hanau, Germany). The constant-current and constant-voltage electrophoresis apparatus was purchased from Thermo-Scientific Fisher (Waltham, MA, USA). The tabletop high-speed centrifuge was purchased from Jingli Centrifuge Co., Ltd. (Beijing, China). The -70°C ultra low temperature refrigerator was purchased from Medica Corporation (Bedford, MA, USA). The PCR amplifier was purchased from Hach (Loveland, CO, USA).

Cell culture

The leukemic cells were collected and centrifuged at 157 rcf (g) for 5 min. This was pipetted well to obtain a cell suspension after the addition of 1X phosphate buffered saline (PBS). One hundred microliter of the cell suspension and 100 μL Evans blue were added to the centrifuge tube and mixed well. The solution was allowed to sit overnight. Several droplets of the staining solution were pipetted and added to the counting chamber along the edge of the cover slip, in order to fill the gap between the counting plate and the cover slip. The cell body was observed under the microscope to be complete, transparent, and unstained. The cell suspension was diluted to the desired concentration (determined based on the experiment objective) after determining the cell concentration by counting.

Culture of leukemic cells and transfection of synthesized siRNA

RPMI-1640 medium (KEYGEN, Nanjing, China) containing 10% fetal bovine serum, penicillin (100U/ml), and streptomycin (100U/ml) was added to a 24-well microplate filled with

leukemic cells. The cells were cultured in an incubator containing 5% CO_2 at 37°C, until a cell coverage rate of 50% was achieved. The culture solution was pipetted. The cells were then washed once with the RPMI-1640 culture medium without penicillin, streptomycin, or fetal bovine serum. The cells were divided into the blank control, liposome empty transfection, unrelated sequence oligonucleotide, and *Bcl-2-siRNA* groups. The cells were then prepared using the INTERFERin kit, as per the manufacturer protocols.

Detection of *Bcl-2* mRNA

Total DNA was extracted using TRIzol reagent, in accordance with the manufacturer instructions. The polymerase chain reaction (PCR) solution contained the template DNA (4 µL), 10X PCR reaction buffer (5 µL), 50 mM $MgCl_2$ (1.5 µL), 10 mM dNTP mixture (l µL), 10 pM upstream primer (l µL), 10 pM downstream primer (1 µL), Taq enzyme (0.3 µL), and water, at a final volume of 50 µL. The reaction conditions were set as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, 30 cycles of annealing at 60°C for 30 s, and another round of annealing at 72°C for 30 s, and a final extension at 72°C for 10 min. The amplification product (10 µL) was electrophoresed on a 1.5% agarose gel; the results were observed, scanned, and analyzed using an ultraviolet analyzer.

Detection of cell proliferation by MTT assay

The cells were divided into control (blank), liposome empty transfection, unrelated sequence oligonucleotide, and *Bcl-2-siRNA* transfection groups. The leukemic cells were inoculated to a 96-well microplate at a concentration of 2.0 x 10^8/L (at 100 µL/well) and cultured for 48 h. The cells were cultured for 4 h at 37°C after the removal of the culture plate; twenty microliter of the MTT solution (5.0 g/L) was added to each well. The supernatant was pipetted out and 150 µL DMSO was added to the wells. The plate was shaken well using a plate shaking table, and the absorbance (A) of each well was determined at a wavelength of 570 nm. The result was expressed as the inhibition ratio (Gaizo Moore et al., 2008)..Computational formula for inhibition ratio:

$$\text{inhibition ratio (\%)} = \frac{(\text{average A value of the control group} - \text{average A value of the experimental group})}{\text{average A value of the control group}} \times 100\%$$

Determination of apoptosis characteristics using a flow cytometer

The Annexin V-FITC/PI kit was used to analyze apoptosis in the transfected leukemic cells. The cells were centrifuged at 2000 rpm for 5 min. The suspended cells were collected and washed twice with PBS. The cells were suspended with 400 µL 1X binding buffer. The concentration was set to approximately 1 x 10^6 cells/mL. The cell suspension was shaken gently after addition of 5 µL Annexin V-FITC, and incubated for 15 min at 2°-8°C. Ten microliter propidium iodide was added to the cells; the cells were incubated again for 5 min at 2°-8°C. The solution was detected using a flow cytometer within 1 h. Annexin V-FITC+/PI- signifies the presence of apoptotic cells at an early stage. Formula for the apoptosis rate = number of (FITC + PI -) cells/total number of cells (High et al., 2010).

Statistical analysis

All experimental data was statistically analyzed using the SPSS v.15.0 software platform (IBM, Armonk, NY, USA). The measurement data was expressed as mean ± standard deviation (mean ± SD). The data was subjected to one-way analysis of variance (ANOVA) in order to compare among multiple groups. P value < 0.05 indicated statistically significant differences.

RESULTS

Expression of *Bcl-2* mRNA in the different groups of A-BLL pediatric patients

The level of expression of *Bcl-2* mRNA in the drug-resistance group was (statistically) significantly higher than that in the concurrent chemotherapy sensitivity group (P < 0.05); the level of expression of *Bcl-2* mRNA in the chemotherapy sensitivity group was significantly higher before chemotherapy than that after chemotherapy; this difference was also statistically significant (P < 0.05). On the other hand, the level of expression of *Bcl-2* mRNA in the drug-resistance group did not alter significantly before and after chemotherapy (Figures 1 and 2).
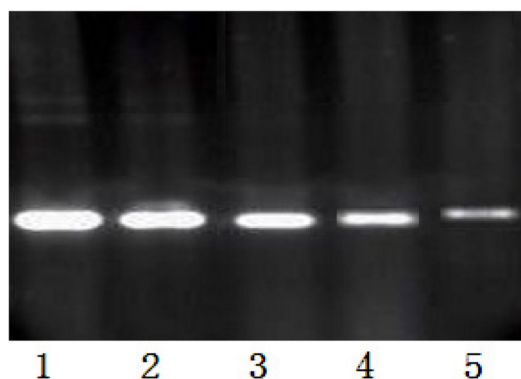

Figure 1. Expression levels of *Bcl-2* mRNA in various groups. *Lane 1* = drug-resistance group before chemotherapy; *lane 2* = drug-resistance group after chemotherapy; *lane 3* = sensitivity group before chemotherapy; *lane 4* = sensitivity group after chemotherapy; *lane 5* = control group.
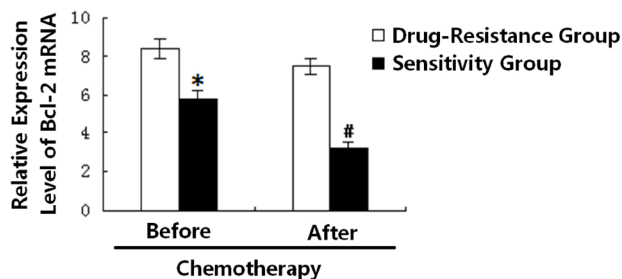

Figure 2. Comparison of relative expression levels of *Bcl-2* mRNA among various groups. The *Bcl-2* mRNA expression levels in the drug-resistance group before chemotherapy and in the concurrent chemotherapy sensitivity group were significantly different; *P < 0.05. The mRNA expression levels in the chemotherapy sensitivity group before and after chemotherapy were also significantly different; #P < 0.05.

Effect of *Bcl-2-siRNA* on the expression of *Bcl-2* mRNA in pediatric A-BLL leukemic cells

The leukemic cells of A-BLL pediatric patients were transfected with *Bcl-2-siRNA*. The results indicated that the *Bcl-2* mRNA expression in the leukemic cells in the *Bcl-2-siRNA* transfection group decreased significantly. The differences among the relative expression levels of *Bcl-2 mRNA* in the *Bcl-2-siRNA* transfection, blank control, liposome empty transfection, and unrelated sequence oligonucleotide groups were not statistically significant ($P > 0.05$) (Figures 3 and 4).
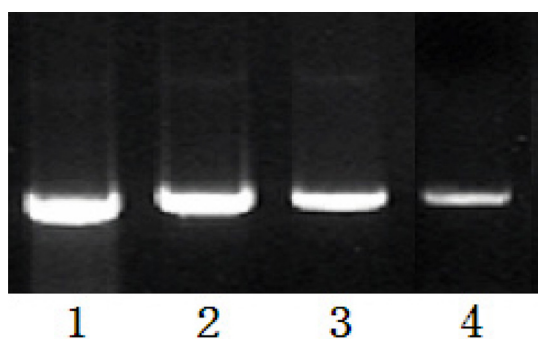

Figure 3. Effect of *Bcl-2-siRNA* on the level of expression of *Bcl-2* mRNA. Lane 1 = control group; *lane 2* = liposome empty transfection group; *lane 3* = unrelated sequence oligonucleotide group; *lane 4* = *Bcl-2-siRNA* transfection group.
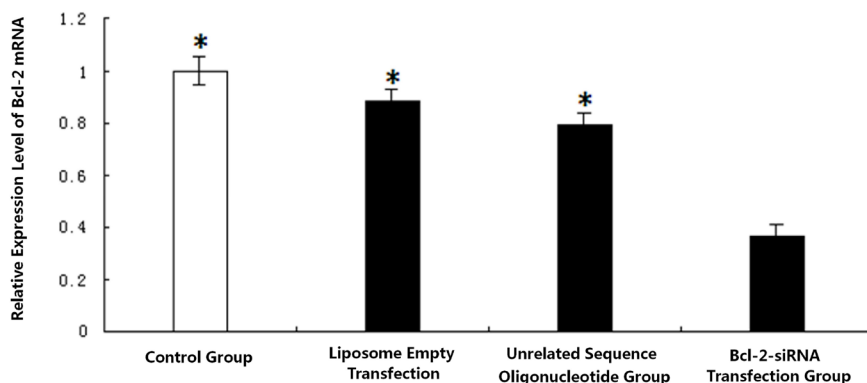

Figure 4. Differences in the levels of *Bcl-2* mRNA expression in the different transfection groups. *$P < 0.05$ compared to the *Bcl-2-siRNA* transfection group.

Effect of *Bcl-2-siRNA* on the proliferative capacity of pediatric A-BLL leukemic cells

The results of this experiment indicated that the inhibition ratios of the blank control group, liposome empty transfection group, unrelated sequence oligonucleotide group, and the *Bcl-2-siRNA* group were 0, 8.31%, 13.57%, and 58.42%, respectively. The inhibition ratio of the *Bcl-2-siRNA* transfection group was significantly higher than those of the control, liposome empty transfection, and unrelated sequence oligonucleotide groups. This difference was statistically significant ($P < 0.05$) (Figure 5).
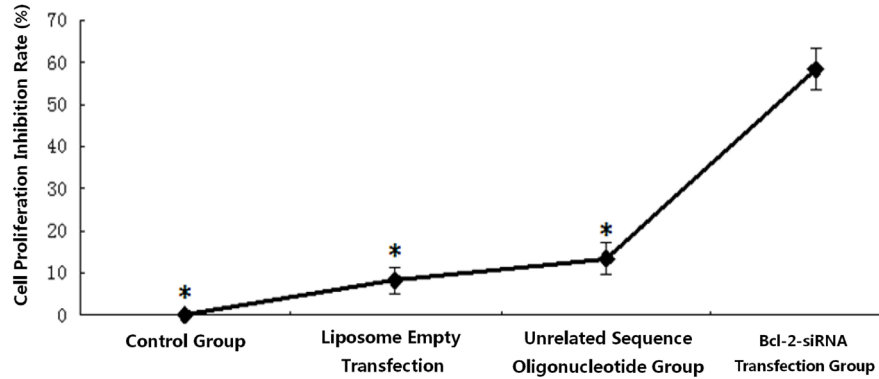
Figure 5. Effect of *Bcl-2-siRNA* on the proliferative capacity of acute B lymphocytic leukemia (leukemic) cells. *P < 0.05, compared to the *Bcl-2-siRNA* transfection group.

Effect of *Bcl-2-siRNA* on pediatric A-BLL leukemic cell apoptosis

The results of this study indicated that the apoptosis rates of the blank control, liposome empty transfection, unrelated sequence oligonucleotide, and *Bcl-2-siRNA* groups were 2.37 ± 0.82, 3.86 ± 1.52, 6.94 ± 1.83, and 37.91 ± 5.22, respectively. The apoptosis rate of the pediatric A-BLL leukemic cells was significantly higher than those of the control, liposome empty transfection, and unrelated sequence oligonucleotide groups ($P < 0.05$) (Figure 6).
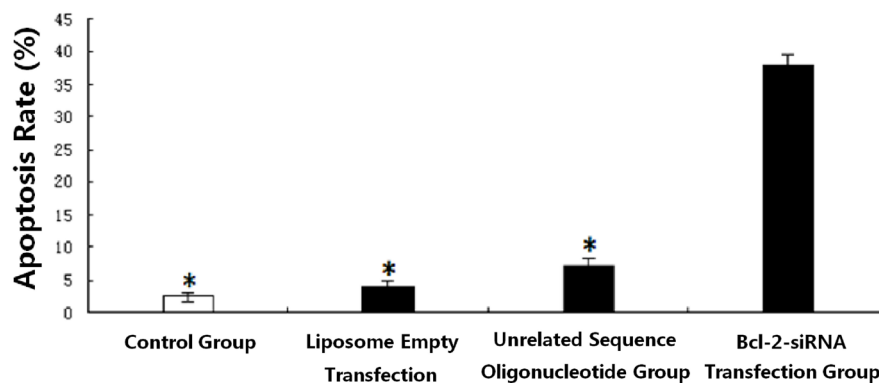

Figure 6. Effect of *Bcl-2-siRNA* on the apoptosis capacity of A-BLL leukemic cells. *P < 0.05, compared to the *Bcl-2-siRNA* transfection group.

DISCUSSION

Acute B lymphocytic leukemia (A-BLL) is the most common pediatric neoplastic disease, wherein the B lymphocytic cell clones proliferate abnormally, the normal hematopoietic components in the marrow are replaced by leukemic cells, and the leukemic cells spread through blood and affect the tissues and organs outside of the marrow (such as liver, spleen, and lymph node), thereby causing the corresponding clinical manifestations and severely threatening the life of pediatric

patients (high fatality rate) (Gupta et al., 2013; Avila et al., 2014; Morais et al., 2014). Radioactive rays, and chemicals, viruses, and genetic factors are known to trigger leukemia. In addition, adhesives, coating, and floor covering, which are used in decoration, release toxic substances, such as formaldehyde, lead, and benzene, which severely damage the hemopoietic system of the human body. This is an important cause leading to a high incidence of childhood leukemia over the past few years (Liu et al., 2014; Pana et al., 2014; Petersen et al., 2014). The VDLD therapeutic regime that has been used clinically is the first-line approach for treatment of A-BLL. However, recent research has indicated an increase in the rate of resistance to chemotherapeutic drugs; in addition, these drugs impart severe toxic and side effects, thereby restricting the clinical use of this therapeutic regime (Brackett et al., 2014; Melachuri et al., 2014).

In this study, we tested the changes in the expression of the *Bcl-2* gene before and after chemotherapy, divided the clinical A-BLL pediatric patients into the sensitivity and drug-resistant groups for analysis, and compared the differences in expression in different groups of pediatric patients suffering from A-BLL between January 2013 and June 2014. The results of this study have indicated that the A-BLL pediatric patients in the drug-resistance group show significantly higher *Bcl-2* mRNA expression, compared to those in the chemotherapy sensitivity group. In addition, the *Bcl-2* mRNA expression in the A-BLL pediatric patients classified into the chemotherapy sensitivity group decreased significantly after chemotherapy, while the expression of *Bcl-2* mRNA in the A-BLL pediatric patients in the chemotherapy drug-resistance group did not alter significantly before and after chemotherapy. A literature review revealed that *Bcl-2* may be an important factor affecting the incidence, progression, and prognosis of pediatric A-BLL; in addition, high Bcl-2 expression may be a leading cause for chemotherapeutic drug resistance in leukemic cells, and may affect the therapeutic potential of the drug (Brumatti et al., 2013; Hitzler et al., 2014; Liu et al., 2014). However, further research must be conducted to demonstrate the involvement of *Bcl-2,* and specifically define the roles and mechanism of *Bcl-2* in incidence, progression, and drug-resistance of pediatric A-BLL.

The emergence of advanced techniques, such as gene cloning and sequencing, and the rapid development and maturity of the RNA interference technique, has led to the focus on siRNA (designed for a specific target gene) as the new breakthrough for the treatment of various diseases, including cancers, at the molecular level (Park et al., 2013; Vitagliano et al., 2013). Moreover, RNA interference, a gene therapy-based treatment method, has many advantages, including a high specificity, high biological activity, high efficiency, low toxicity, and high safety, compared to the traditional treatment methods. Therefore, it has been subjected to increasing attention by researchers throughout the world (Patel et al., 2014).

The *Bcl-2* gene, which is a potential inhibitor regulating apoptosis, can inhibit or block apoptosis resulting from several factors (Davids et al., 2013; Samuel et al., 2013). Inhibition of *Bcl-2* gene expression in malignant tumors could promote apoptosis in tumor cells. Therefore, a siRNA was designed to specifically inhibit the expression of the *Bcl-2* gene via RNA interference. This can be used to inhibit the growth of tumors expressing a high level of the *Bcl-2* gene (Kaparou et al., 2013). Current RNA interference research on the *Bcl-2* gene has successfully improved the sensitivity of many drug-resistant tumors to radiotherapy and chemotherapy, as well as the prognosis of tumor patients. The *Bcl-2* siRNA can inhibit tumor growth and reverse drug-resistance arising from high *Bcl-2* expression. Previous research has demonstrated that RNA interference has a good therapeutic effect (Lagadinou et al., 2013; Takahashi et al., 2013) on non-Hodgkin's lymphoma (among hematological tumors). In addition, it has been shown to exhibit a high level of safety and efficacy, without any toxic or side effects (Ishitsuka et al., 2012).

However, the expression of *Bcl-2* in pediatric A-BLL remains to be quantified; in addition, the effect of RNA interference on resistance to chemotherapeutic drugs against leukemia remains to be investigated. Based on the results of previous research, the authors utilized RNA interference to transfect *Bcl-2-siRNA* into the leukemic cells of pediatric patients with A-BLL in this study. In addition, an experimental model was successfully designed to further demonstrate that *Bcl-2-siRNA* significantly reduces the proliferative capacity of pediatric A-BLL leukemic cells and increases the apoptosis rate. Moreover, this study suggested that *Bcl-2-siRNA* can inhibit the proliferation of tumor cells, and induce apoptosis in tumor cells.

Conflicts of interest

The authors declare no conflict of interest.

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