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BCL6 promotes the methotrexate-resistance by upregulating ZEB1 expression in children with acute B lymphocytic leukemia

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Abstract. – OBJECTIVE: To investigate the effect of BCL6 on methotrexate-resistant children with acute B lymphoblastic leukemia (B-ALL) and its underlying mechanism.

PATIENTS AND METHODS: Bone marrow samples of B-ALL children diagnosed at The Children & Women's Healthcare of Laiwu City from June 2014 to February 2017 were collected. Subjects were assigned into methotrexate-resistant group (n=8) and non-resistant group (n=32) according to the follow-up outcome. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed to detect expressions of BCL6 and E-cadherin in bone marrow tissues. Cell cycle and apoptosis of methotrexate-resistant B-ALL cells (BALL-1/MTXR cells) were detected after overexpression or inhibition of BCL6, respectively. Western blot was performed to determine the protein levels of E-cadherin and BCL6. The direct binding of BCL6 in the ZEB1 promoter region was verified by the ChIP (chromatin immunoprecipitation) assay.

RESULTS: QRT-PCR showed a higher BCL6 expression in bone marrow samples of methotrexate-resistant group than that of the non-resistant group. Moreover, BCL6 was upregulated in BALL-1/MTXR cells than that of untreated B-ALL cells. After knockdown of BCL6 expression, we observed a decreased IC50, increased apoptosis, and arrested the cell cycle in BALL-1/MTXR cells. In addition, increased expression of E-cadherin was found in BALL-1/MTXR cells, which could be reversed by ZEB1 overexpression. ChIP assay suggested that BCL6 bound to the promoter region of ZEB1, so as to promote ZEB1 expression.

CONCLUSIONS: BCL6 is overexpressed in the bone marrow of methotrexate-resistant children with B-ALL, which is capable of attenuating the sensitivity of B-ALL to methotrexate via promoting ZEB1 expression.

Key Words:
Acute B lymphoblastic leukemia, BCL6, Methotrexate resistance, ZEB1.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children of our country. ALL is a malignant proliferative disease that originates from B-lineage or T-lineage lymphoid progenitor cells. It is mainly composed of primitive and immature lymphocyte clones with a high specificity[1]. Proliferated lymphoid progenitor cells invade into various organs and inhibit normal hematopoiesis, thereafter resulting in a series of clinical manifestations, such as hepatosplenomegaly, fever, anemia, infection, bone pain, etc.[2]. Acute B lymphoblastic leukemia (B-ALL) accounts for about 80% to 90% of ALL in children[3].

Treatment strategies for leukemia include systemic chemotherapy, local radiation therapy and surgical treatment, of which chemotherapy is currently the most important clinical method. In recent years, the single-agent and low-dose chemotherapy have improved to multi-drug and high-dose therapy, which has greatly elevated the treatment efficacy in ALL children[4]. Current chemotherapy drugs for B-ALL include methotrexate and etoposide. Although the disease condition of most B-ALL children can be alleviated after treatment, about 20% of the children still cannot be relieved due to the presence of resistant leukemia cells, which brings great pain to children and their families[5]. Therefore, in-depth researches to explore the mechanism of B-ALL drug resistance have important social and medical values.

Despite the diversity of signaling molecules involved in tumor progression, studies on drug resistance in acute B-ALL have focused on BCL6[6]. BCL6 is involved in germinal center B cells and progression of diffuse large B cell lymphoma as a proto-oncogene[7]. It is reported

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that BCL6 is capable of regulating proliferation, maturation, and resistance to DNA damage in B cells[8,9]. Recent studies have shown that overexpressed BCL6 in chronic myeloid leukemia (CML) and ALL protects leukemia cells from chemotherapy-induced DNA damage by inhibiting p53-induced apoptosis[10]. This evidence has pointed out that BCL6 could affect the phenotype of leukemic cells *via* regulating differentiation and cell cycle.

Patients and Methods

Bone Marrow Tissue Samples

Bone marrow specimens of B-ALL patients treated in the Hematology Department of the Children & Women's Healthcare of Laiwu City from June 2014 to February 2017 were collected. This study was approved by The Children & Women's Healthcare of Laiwu City Medical Ethics Committee. The informed consent was provided for all patients and their families. A total of 40 children who were diagnosed as B-ALL according to the classification criteria of French-America-British (FAB) and World Health Organization (WHO) were included. All patients were initially treated without medical history of other malignant tumors. There were 29 males and 11 females in this study, with a median age of 8.1 years (4.3 to 17.2 years).

Cell Culture

Acute B-lymphocytic leukemia cell line BALL-1 was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator at 5% CO_2. The cell line was provided by the American Type Culture Collection Center (ATCC, Manassas, VA, USA). Cells were passaged when the cell confluence was up to 80%.

Transfection of siRNA and pcDNA

Cells were seeded in 6-well plates, and siRNA or pcDNA was transfected respectively when cell confluence was up to 60%. A total of 10 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or 10 μL of 20 nM siRNA-BCL6/siRNA-ZEB1 (or 2.68 μg of pcDNA-BCL6) was mixed in 500 μL of serum-free suspension and incubated for 15 min, respectively. The mixture was added to a Petri dish with 1.5 ml of 1640 medium (Gibco, Grand Island, NY, USA) and cultured for 6 h. Isodose Lipofectamine 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and siRNA-control (or pcDNA-control) were added to the control group.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted according to the instructions of TRIzol (Invitrogen, Carlsbad, CA, USA), and 50 μL of the reaction system was prepared according to qRT-PCR specification. The reverse transcription reaction was performed using the following conditions: 50°C for 30 min, and 92°C for 3 min. The resulting complementary Deoxyribose Nucleic Acid (cDNA) was subjected to a PCR amplification reaction under the following conditions: 92°C for 10 s, 55°C for 20 s, and 68°C for 20 s, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression. The primers used were as follows: GAPDH-F: GACCTGCCGTCTAGAAAAACCTGC, GAPDH-R: TCGCTGTTGAAGTCAGAGGAGACC; E-cadherin-F: TCAGCCAAGATCCTGAGCTCCCT, E-cadherin-R: AGGTCAGCAGCTTGAACCACCAG; BCL6-F: GTCCTGCAGCAGTAAGAATGCCTG, BCL6-R: GGCTGTTGAGCACGATGAACTTGT; ZEB1-F: CCAGACAGTGTTACCAGGGAGGAG, ZEB1-R: TGCCCTTCCTTTCCTGTGTCATCC.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 96-well plates with 6 replicates in each group at a density of 5×10^3/well. After 6 h, 20 μL of CCK-8 (Dojindo, Kumamoto, Japan) solution was added to each well and placed in a 37°C, 5% CO_2 incubator for 2-3 h. Thereafter, cell viability was measured at 24 h, 48 h, 72 h, and 96 h, respectively. Absorbance at the wavelength of 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer in the presence of a protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) to harvest total cellular protein. The protein concentration of each cell lysate was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein sample was loaded onto a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide

gel electrophoresis) gel and then transferred to a PVDF (polyvinylidene difluoride) membrane after being separated. After blocking with skim milk, membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then incubated with HRP (horseradish peroxidase) conjugated secondary antibody for 2-3 h at room temperature. Finally, an image of the protein band was captured by the Tanon detection system using enhanced chemiluminescence (ECL) reagent (Thermo, Waltham, MA, USA).

Flow Cytometry

After the cell supernatant was collected in labeled flow tubes, the cells were digested with trypsin without Ethylene Diamine Tetraacetic Acid (EDTA) and prepared as single cell suspensions. Cell suspensions were then washed with phosphate-buffered saline (PBS) and centrifuged twice for collecting the cell pellet. 200 μL of calcium-containing binding buffer and the target antibody were added, followed by detection in dark using flow cytometry (Partec AG, Arlesheim, Switzerland).

ChIP Assay

The cells were fixed in 1% formaldehyde for 10 min. Anti-RNA polymerase, normal mouse IgG and BCL6 antibodies were then immunoprecipitated overnight at 4°C. Protein G agarose (Cell Signaling Technology, Danvers, MA, USA) was added to collect immune complexes. The beads were resuspended in elution buffer and incubated overnight at 65°C before DNA extraction. The DNA was purified using a spin column and quantified using qRT-PCR. Primers used to amplify specific regions of the indicated genes were as follows: ZEB1-F: TATTCGAAGGAGGTGGGAAGCAGG; ZEB1-R: CGTGCAGGACCTTAAGGCAAGAAG.

Statistical Analysis

Statistical product and service solutions (SPSS22.0, IBM, Armonk, NY, USA) statistical software was used for data analysis and GraphPad Prism 6.0 (La Jolla, CA, USA) was used for image editing. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared using the *t*-test. Chi-square test was performed to test the classification data. $p < 0.05$ considered the difference was statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

BCL6 Was Upregulated in Bone Marrow and BALL-1/MTXR Cells

We collected bone marrow samples from children who were diagnosed as B-ALL in the Children & Women's Healthcare of Laiwu City from June 2014 to February 2017. According to the follow-up outcome after treatment for 6 months, B-ALL children were assigned into methotrexate-resistant group (n=8) and non-resistant group (n=32). PCR results showed a higher expression level of BCL6 in the methotrexate-resistant group than that of the non-resistant group (Figure 1A). The expression level of BCL6 was remarkably increased after treatment in the methotrexate-resistant group compared with that before treatment. However, no significant difference before and after treatment was observed in the non-resistant group (Figure 1B). Subsequently, we utilized methotrexate to screen BALL-1/MTXR cells. CCK-8 results showed that the selected BALL-1/MTXR cells were remarkably resistant to methotrexate than those of the parent cells treated with different concentrations of methotrexate (Figure 1C). Besides, higher protein level of BCL6 was observed in BALL-1/MTXR cells than that of the parental cells (Figure 1D). Overall, BCL6 was down-regulated in methotrexate-resistant tissues and BALL-1/MTXR cells.

Upregulation of BCL6 in BALL-1/MTXR Cells Reversed the Sensitivity to Methotrexate

To investigate the effect of BCL6 on the cell sensitivity to methotrexate, we transfected BCL6 overexpression plasmid into BALL-1/MTXR cells. Transfection efficiency was verified by qRT-PCR (Figure 2A). Next, we measured the IC_{50} of BALL-1/MTXR cells after overexpression of BCL6. The results showed that the IC_{50} in BALL-1/MTXR cells was remarkably lower than that of the control group after BCL6 was overexpressed (Figure 2B). The above data indicated that enhanced BCL6 expression in BALL-1/MTXR cells could increase the sensitivity of BALL-1 cells to methotrexate.

Cell Cycle of BALL-1/MTXR Cells Was Arrested After BCL6 Overexpression

To further explore the effect of BCL6 on BALL-1/MTXR cells, we performed CCK-8 assay and colony formation assay, respectively. Our results suggested that the proliferation ability in

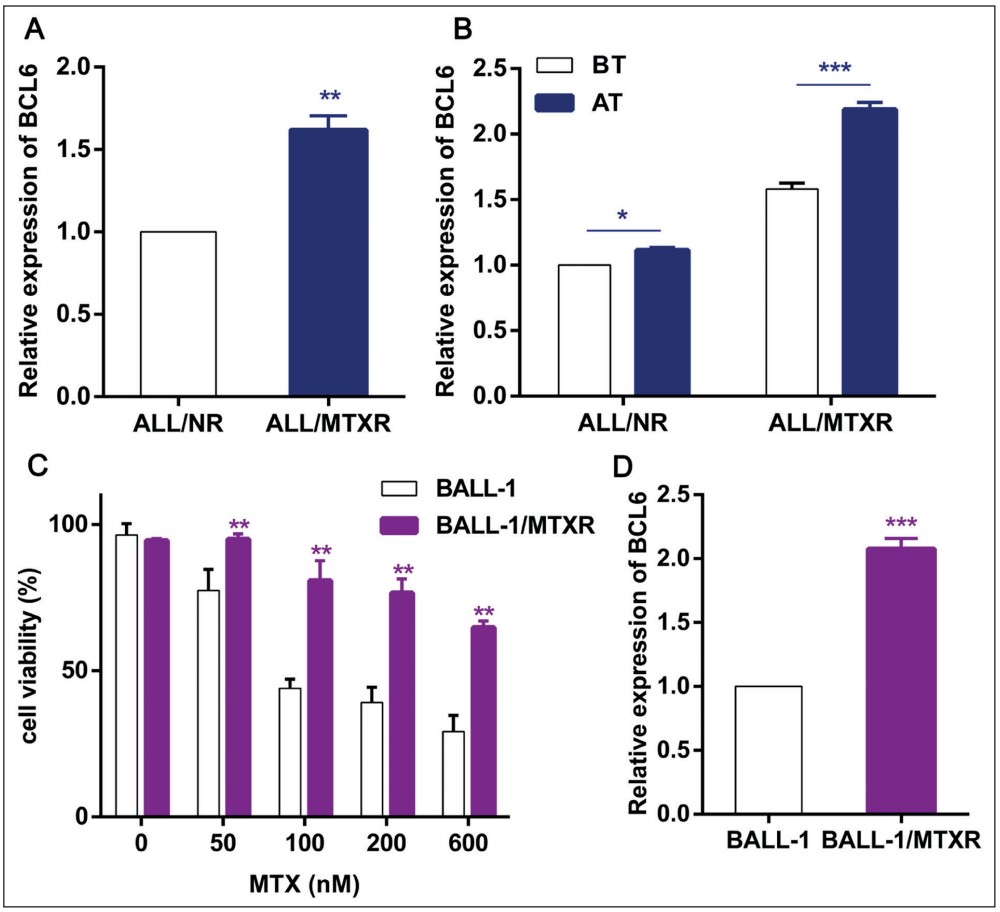

Figure 1. The expression of BCL6 was increased in bone marrow in B-ALL children and BALL-1/MTXR cells. ***A,*** The expression of BCL6 in bone marrow in the methotrexate-resistant group was significantly higher than in the non-resistant group. ***B,*** The expression of BCL6 in the methotrexate-resistant group after treatment was significantly higher than before treatment. ***C,*** Tolerance to methotrexate was significantly higher in resistant cells than that in parental cells treated with different concentrations of methotrexate. ***D,*** BCL6 expression of BALL-1/MTXR cells was significantly higher than of the parental cell BALL-1/NR.

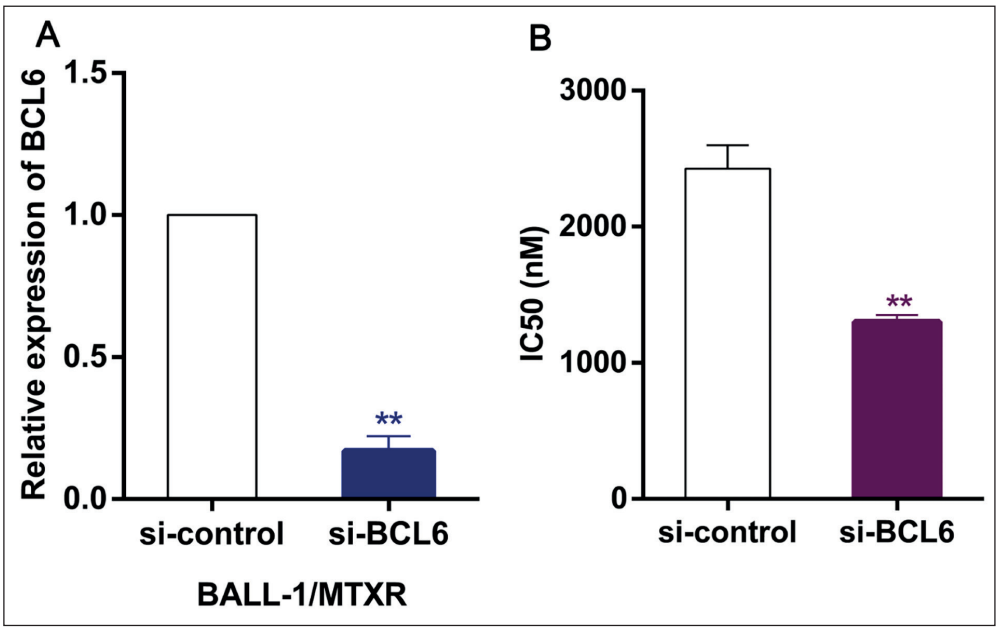

Figure 2. Role of BCL6 in the sensitivity of BALL-1 to methotrexate. ***A,*** After transfection of pc-BCL6, the expression of BCL6 in BALL cell lines was significantly increased. ***B,*** After overexpression of BCL6, the IC_{50} of BALL-1/MTXR cells was lower than that of the control group.

BALL-1/MTXR cells after BCL6 knockdown was remarkably weaker than that of the control group (Figure 3A, 3B). Flow cytometry results showed that the cell cycle of BALL-1/MTXR cells was arrested in the G1 phase after BCL6 knockdown (Figure 3C), illustrating the role of BCL6 in the proliferation of BALL-1/MTXR cells.

BCL6 Inhibited Proliferation of BALL-1/MTXR Cells by Downregulating ZEB1

The process of epithelial to mesenchymal transition (EMT) exists in a variety of malignant tumors. Recently, EMT has been reported to be closely related to tumor recurrence, metastasis, and drug resistance[11,12]. Therefore, we detected E-cadherin expression in BALL-1/MTXR cells. Western blot results showed that E-cadherin was downregulated in BALL-1/MTXR cells when BCL6 was overexpressed (Figure 4A). ZEB1 exerts an essential role in the resistance of tumor cells[13]. In our study, ZEB1 expression was remarkably increased in BALL-1/MTXR cells after BCL6 overexpression (Figure 4A). Knockdown of ZEB1 in BALL-1/MTXR cells resulted in inhibited proliferative ability (Figure 4B). Our ChIP study further validated that BCL6 could bind to promoter region of ZEB1 (Figure 4C-D).

Discussion

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, which accounts for about 30% of pediatric cancers[14]. About 80-85% of ALL in children are derived from B cells malignancy, that is, B-ALL. Although the treatment has been greatly progressed in recent years, ALL is still one of the leading causes of death in children with tumors[15]. ALL is still the fifth common pediatric malignancy, which severely endangers children's health[16]. Therefore, it is especially important to determine the key factors affecting the development and drug resistance of B-ALL.

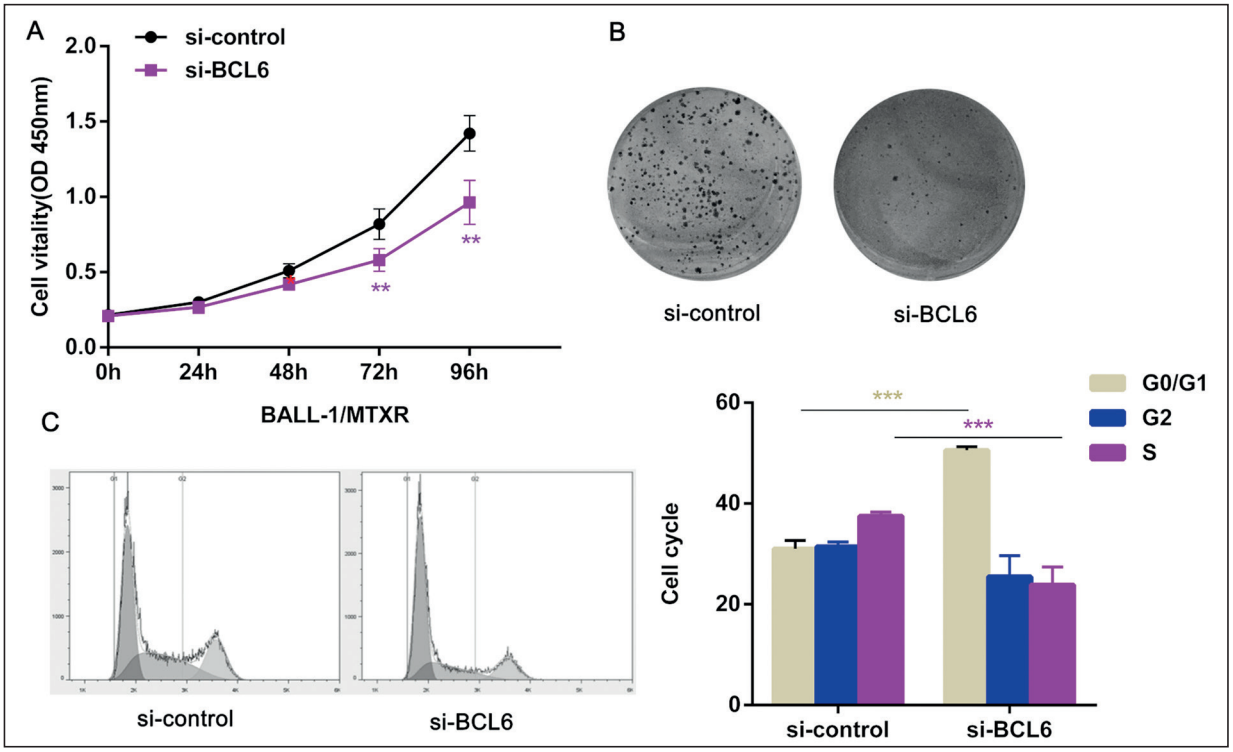

Figure 3. Effect of BCL6 on cell cycle and apoptosis of BALL-1/MTXR cells. ***A,*** CCK-8 results showed that BCL-1/MTXR cell proliferation in resistant cells was significantly weaker than that of the control group after BCL6 knockdown. ***B,*** The results of clone formation showed that the ability of BALL-1/MTXR cells to form clones in resistant cells was significantly weaker than that of the control group after BCL6 knockdown. ***C,*** Flow cytometry results showed that BCL-1/MTXR cells were arrested in the G1 phase after BCL6 expression was reduced.

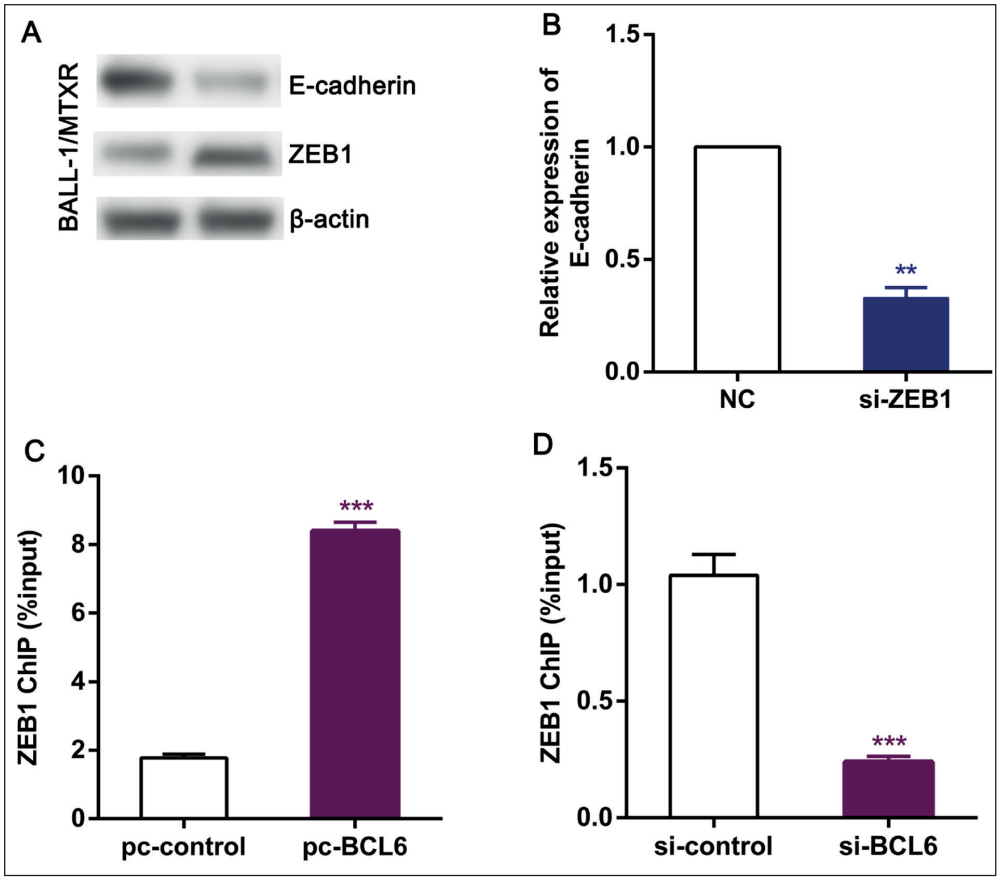

Figure 4. Relationship between BCL6 and ZEB1 in BALL-1/MTXR cells. ***A,*** When BCL6 was over-expressed, E-cadherin in BALL-1/MTXR cells was significantly reduced and ZEB1 expression was significantly increased. ***B,*** Knockdown of ZEB1 showed suppression of the proliferation of resistant strain BALL-1/MTXR cells synchronized with MTX. ***C,*** The binding rate of BCL6 to the ZEB1 promoter was increased when BCL6 was up-regulated. ***D,*** The binding rate of BCL6 to the ZEB1 promoter was decreased when BCL6 was down-regulated.

BCL6, located in the region of chromosome 3q27, contains 10 exons and 7 introns with a length of 26 kb. BCL6 participates in various biological processes, such as cell activation, differentiation, cell cycle regulation, and DNA damage repair. However, the carcinogenetic mechanism of BCL6 is still unclear. It is proposed that the consistent expression of BCL6 in the late stage of B cell differentiation interrupts terminal differentiation, which contributes to increased cell survival, and phenotype transformation[17]. BCL6 induces B cell differentiation into plasma cells by inhibiting Blimp1 and c-Myc expression, resulting in the arrest of B cell differentiation and normal cell cycle[18]. BCL6 also inhibits the expression of the tumor suppressor p53, thus preventing p53-mediated cell cycle arrest and apoptotic responses[19]. Notably, BCL6 directly inhibits p21 expression and cell cycle arrest in germinal center B cells through binding to Miz-1[20,21]. The inhibitory effect of BCL6 on cell cycle regulators may partly explain the pathological role of BCL6 in the development of B-ALL, while little research has been carried out on BCL6 in B-ALL drug resistance[22].

Our study showed that BCL6 expression in the methotrexate-resistant group was remarkably higher than that of the non-resistant group. Moreover, the relative expression of BCL6 in the drug-resistant group was increased after treatment. BCL6 expression in BALL-1/MTXR cells screened by methotrexate was also higher than that of the untreated B-ALL cells. Knockdown of BCL6 in BALL-1/MTXR cells resulted in the increased cell sensitivity to methotrexate, as well as rescued cell cycle arrest and apoptosis. Besides, we also found that BCL6 promoted EMT *via* increasing ZEB1 expression. ZEB1 is a zinc

finger protein transcription factor and is closely related to the occurrence and development of various tumors and drug resistance in previous studies[23]. Our data revealed that BCL6 promotes ZEB1 expression by binding to the promoter region of ZEB1.

Conclusions

We showed that BCL6 is overexpressed in the bone marrow of methotrexate-resistant children with B-ALL, which is capable of attenuating the sensitivity of B-ALL to methotrexate *via* promoting ZEB1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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