Potential efficacy and prognosis of silencing the CRLF2-mediated AKT/mTOR pathway in pediatric acute B-cell lymphoblastic leukemia

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Abstract. Acute B-cell lymphoblastic leukemia (B-ALL) is a common type of blood cancer, which is associated with aberrant gene expression. Cytokine receptor-like factor 2 (CRLF2) serves a crucial role in the growth and allergic and inflammatory responses of dendritic cells and T cells. The purpose of the present study was to investigate the potential therapeutic and prognostic effect of silencing the CRLF2-mediated RAC-α serine/threonine-protein kinase (AKT)/serine/threonine-protein kinase mTOR (mTOR) pathway in B-ALL. In our study, bone marrow specimens were collected from 128 children with B-ALL and 26 healthy children. The expression of CRLF2 in bone marrow tissue was detected using immunohistochemistry. The survival rates were compared among the children with high and low CRLF2 expression levels. BaF3 leukemia cells were treated with CRLF2 short hairpin RNA knockdown and/or the AKT/mTOR pathway specific inhibitor LY294002. mRNA and protein expression associated with CRLF2 and the AKT/mTOR pathway in each group was detected by reverse transcription-quantitative polymerase chain reaction analysis and western blotting. The viability of BaF3 cells in all the groups was assessed by Cell Counting Kit-8 assay; the migration and invasion of BaF3 cells were determined by wound healing and Transwell invasion assays; and the sensitivity of BaF3 cells to the chemotherapeutic drug imatinib was detected using flow cytometry. The results demonstrated that CRLF2 overexpression is associated with a poor prognosis in B-ALL, and the CRLF2/AKT/mTOR pathway is involved in the migration, invasion and chemotherapeutic agent-induced apoptosis of BaF3 cells.

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Abbreviations: CRLF2, cytokine receptor-like factor 2; B-ALL, B-cell acute lymphoblastic leukemia; OS, overall survival; EFS, event-free survival; WBC, white blood cell count; HGB, hemoglobin; PLT, platelet count; LDH, lactate dehydrogenase

Key words: CRLF2, B-ALL, OS, EFS, WBC, HGB, PLT, LDH

Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is a common type of blood cancer (1). With advances in medical technologies, there has been a great improvement in the survival rate of children with B-ALL. However, certain children experience recurrence and a poor prognosis following treatment (2,3). Previous studies have confirmed that the onset of B-ALL is associated with aberrant gene expression (4,5). Genes that have been identified to be involved in the pathogenesis of ALL include ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1), ABL2, protein tyrosine kinase 2β, Janus kinase (JAK)2, cytokine receptor-like factor 2 (CRLF2) and colony stimulating factor 1 receptor (6). CRLF2 is located in the Xp22.3/Yp11.3 autosomal regions and is able to interact with interleukin-7 receptor-α. This protein not only serves an important role in the growth and allergic and inflammatory responses of dendritic cells and T cells, but is also able to regulate the proliferation and apoptosis of B cells, which affects the progression of B-ALL (7-9). Certain studies have reported that high CRLF2 expression is associated with recurrence and poor prognosis in children with B-ALL (10). It has been documented that the activation of the RAC-α serine/threonine-protein kinase (AKT)/serine/threonine-protein kinase mTOR (mTOR) signaling pathway is closely associated with the incidence of B-ALL, and targeted inhibition of AKT/mTOR is of great significance to the treatment of this disease (11). Certain researchers have reported that the CRLF2 gene is able to induce the incidence of B-ALL by affecting the proliferation and differentiation of lymphocytes, via activation

of the JAK signaling pathway (12). However, the association between CRLF2 and the AKT/mTOR signaling pathway has not been thoroughly examined. Therefore, the present study aimed to investigate the effects of CRLF2 on the invasion, migration and apoptosis of the BaF3 leukemia cell line via the AKT/mTOR pathway.

Materials and methods

Study participants. The present study was approved by the Ethics Committee of the Yichang Central People's Hospital (Yichang, China). A total of 128 children (72 males and 56 females) who were newly diagnosed with B-ALL and treated in Yichang Central People's Hospital between June 2005 and January 2012 were enrolled in the study. A further 26 healthy children, who were matched for sex and age, were included as controls. Children with positive BCR-ABL1 or Down's syndrome were excluded from the study. With the consent of the patients and their families, bone marrow specimens and peripheral blood were collected prior to the initial treatment. Subsequently, the children with B-ALL were graded and treated according to the Associazone Italiana Ematologia Oncologia Pediatrica-Berlin-Frankfurt-Münster ALL-2000 protocol (13). Follow-ups were conducted in the form of inpatient and outpatient visits and telephone calls. The 4-year overall survival (OS) and the 3-year event-free survival (EFS) rates of the children were documented during the follow-ups.

Immunohistochemistry. Frozen bone marrow specimens extracted from children with B-ALL and healthy normal children were embedded in paraffin at 12-26˚C for 10 h, and cut into sections at 5 μm thickness. The paraffin sections were dried in an oven at 60˚C for 30 min and underwent routine dewaxing and hydration (treated with toluene I, xylene II, 100 alcohol, 95 alcohol, 85 alcohol and 80% alcohol for 5 min, respectively) followed by rinsing with water for 2 min. Subsequently, antigen retrieval was performed in 1 mM Tris-EDTA (pH 8.0) with microwave irradiation at 100˚C. The sections were cooled to room temperature and rinsed with PBS three times (5 min/wash). Following this, 3% H_2O_2-methanol was added to blocking endogenous peroxidase activity at room temperature for 10 min, followed by washing twice with PBS (5 min/wash). The primary antibody against CRLF2 (rabbit anti-human; 5 μg/ml; cat. no. ab109626; Abcam, Cambridge, UK) was added, while IgG was used as a negative control. Samples were placed in a refrigerator at 4˚C overnight. On the following day, samples were washed in 0.1% PBS with Tween-20 (PBST) three times (5 min/wash) and were incubated at room temperature for 20 min following the addition of streptavidin biotin-peroxidase complex reagent (SABC). The sections were washed in 0.1% PBST three times (5 min/wash) and horseradish peroxidase (HRP)-conjugated secondary IgG antibodies (goat anti-rabbit; cat. no. ab205718; Abcam) were added for a 30-min incubation at room temperature, prior to washing again in PBS three times. Subsequently, the samples were incubated with SABC for 20 min and were rinsed with 0.1% PBST three times (5 min/wash). 3,3'-Diaminobenzidine staining was conducted for 5 min at room temperature, followed by a rinse with distilled water to stop the reaction. The samples were counterstained with hematoxylin at room temperature for 3 min, rinsed, differentiated in 1% hydrochloric acid alcohol for 20 sec, and rinsed again until the samples turned blue. Dehydration was conducted and neutral balsam was used for mounting. A total of 500 cells were counted under the light microscope (Olympus CX41; Olympus Corporation, Tokyo, Japan); brown cells were identified as positive, and samples were graded according to the percentage of positive cells in all tumor cells. The criteria for immunohistochemical scoring were as follows: Negative expression, with a positive cell percentage of <10% (0 point); a positive cell percentage of 10-25% (1 point); a positive cell percentage of 25-50% (2 points); and a positive cell percentage of >50% (3 points). A total score of 0-1 indicated negative expression, 2-4 indicated low expression, 5-8 indicated moderate expression and 9-12 indicated high expression. Children with B-ALL were subdivided into a high expression group and a low expression group (including children with negative, low and moderate CRRF2 expression), and the CRLF2 protein expression was compared among B-ALL patients with high and low CRRF2 expression and healthy normal children.

Clinical data. The white blood cell count (WBC), hemoglobin (HGB) and platelet count (PLT) in the peripheral blood of patients (prior to the initial treatment) were measured using an automatic blood analyzer (cat. no. SK9000; Shenzhen Sinothinker Technology, Co., Ltd., Shenzhen, China). The concentration of lactate dehydrogenase (LDH) was determined using an LDH kit (cat. no. MAK066-1KT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). MRI was utilized to examine the degree of tumor infiltration in the liver and spleen. All these indices were analyzed and compared between the children with high CRLF2 expression and children with low CRLF2 expression.

Cell culture. BaF3 leukemia cell lines (cat. no. YB-ATCC-2781) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The BaF3 cells were first thawed and resuscitated, and subsequently placed into a centrifuge tube containing RPMI-1640 medium without fetal bovine serum (FBS) (cat. no. PM150110; Procell Life Science and Technology Co., Ltd., Wuhan, China) for resuspension and centrifugation for 5 min (377 x g; 4˚C). The supernatant was removed, and the cells were rinsed once or twice and seeded in RPMI-1640 cell culture medium containing 15% FBS (Sigma-Aldrich; Merck KGaA). The cells were incubated at 37˚C in an atmosphere of 5% CO_2. The cells were routinely sub-cultured. Only cells in the exponential phase were selected for the experiments.

Cell treatment and grouping. BaF3 cells were subdivided into the following five groups: The Blank group (untreated), the NC group (transfected with a negative control sequence), the short hairpin (sh)CRLF2 group (transfected with a CRLF2 shRNA plasmid), the LY294002 group (treated with an AKT/mTOR pathway-specific inhibitor) and the siCRLF2 + LY294002 group. The CRLF2 shRNA sequence (5'-CACCGGAGTTCC GTTATGGTACTGGCGAACCAGTACCATAACGGAACT CC-3') was designed online with the BLOCK-iT™ RNAi Designer (https://rnaidesigner.thermofisher.com/rnaiexpress/design.do). BaF3 cells were seeded in 6-well plates at a

density of $3x10^5$/ml. Once the cells reached 90% confluence, they were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). During this process, 4 μg target plasmid (negative control plasmid and CRLF2 shRNA plasmid; Shanghai GenePharma Co., Ltd., Shanghai, China) and 10 μl Lipofectamine 2000 were separately diluted in 250 μl serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) and mixed gently. After being placed at room temperature for 5 min, the above two solutions were mixed. The mixture was subsequently added to the culture wells after 20 min and incubated at 37˚C in an atmosphere of 5% CO_2. A total of 6 h subsequently, the medium was replaced, and the mixture was cultured for a further 48 h prior to collecting the cells. CRLF2 shRNA and negative control double-stranded RNA were synthesized by Shanghai GenePharma Co., Ltd. In addition, the cells were cultured in RPMI-1640 cell culture medium containing LY294002 (50 μmol/l) when they reached 80% confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from BaF3 cells in each group using TRIzol® reagent (cat. no. 15596026; Thermo Fisher Scientific, Inc.). Ultraviolet spectrophotometry and agarose gel electrophoresis were performed to detect the RNA concentration, purity and integrity. One-step RT-qPCR was performed using a SuperScript® III Platinum® One-Step RT-qPCR kit (cat. no. 11732088; Thermo Fisher Scientific, Inc.). The reaction system included SuperScript™ III RT/Platinum™ Taq Mix (1 μl), 2X Reaction Mix (25 μl), forward primer (10 μM), reverse primer (10 μM), fluorogenic probe (10 μM), template (1 μl) and diethyl pyrocarbonate-treated water (50 μl). Running parameters were set as follows: One cycle at 50˚C for 15 min; one cycle at 95˚C for 2 min; and 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. GAPDH was used as an internal control and the mRNA expression was quantified using the $2^{-\Delta\Delta Cq}$ method (14). Primer sequences are listed in Table I.

Western blotting. BaF3 cells from each group were washed with PBS, resuspended and centrifuged. The supernatant was collected and radioimmunoprecipitation lysis buffer was added (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China) and 0.1 mM phenylmethylsulfonyl fluoride. Following gentle shaking and resuspension, the cells were incubated on ice for 30 min and centrifuged for 10 min (241 x g; 4˚C). The total cellular protein, which remained in the supernatant, was subsequently collected. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) and the protein concentration was adjusted to 4 μg/μl. Total cellular protein (30 μg) was extracted for 10% SDS-PAGE, followed by transferring to the nitrocellulose (NC) membranes by 50 V wet electrotransfer and blocked in 5% skim milk [TBS with Tween-20 (TBST) formulation] for 1.5 h. at room temperature. Antibodies were diluted in primary antibody diluents, according to the manufacturer's protocol. Primary antibodies included CRLF2 (rabbit anti-human; 1 μg/ml; cat. no. ab109626; Abcam, Cambridge, UK), phosphorylated (p)-AKT (rabbit anti-human; 1:1,000; cat. no. ab52192; Abcam), p-mTOR (rabbit anti-human; 1:1,000; cat. no. ab109268; Abcam), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1; rabbit anti-human; 1:5,000; cat. no. ab32024; Abcam), ribosomal protein S6 kinase β-1 (S6K1; rabbit anti-human; 1:5,000; cat. no. ab32529; Abcam), and GAPDH (rabbit anti-human; 1:10,000; cat. no. ab181602; Abcam). The blocked NC membranes were placed in a plastic container into which the above antibodies were added, followed by agitation and storage at 4˚C for 24 h. The membranes were washed in TBST three times (15 min/wash) and were subsequently incubated at room temperature for 2 h following the addition of diluted HRP-labeled secondary antibodies IgG (goat anti-rabbit; 1:5,000; cat. no. ab205718; Abcam). The membranes were washed in TBST three times (15 min/wash) and treated with enhanced chemiluminescence (ECL western blotting substrate kit; Thermo Fisher Scientific, Inc.). Samples were photographed using SmartView Pro 2000 (UVCI-2100; Major Science, Saratoga, CA, USA). Protein banding gray-scale analysis was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Gene		Primer sequence (5'→3')
CRLF2	Forward	GTTCAGTAGCGGAGCCCCTTC
	Reverse	GTTTGGGAGGCGTTGGTGTC
AKT	Forward	TTTATTGGCTACAAGGAACG
	Reverse	AGTCTGAATGGCGGTGGT
mTOR	Forward	ATGACCAGACCCAGGCTAAG
	Reverse	GCCAGTCCTCTACAATACGC
4EBP1	Forward	GACCTGCCAACCATTCCAG
	Reverse	CACCTGCCCGCTTATCTTC
S6K1	Forward	CGTGGAGTCTGCGGCGGGTC
	Reverse	CGCTCTGCTTTCGTGTGGGC
GAPDH	Forward	GACATCAAGAAGGTGGTGAAGC
	Reverse	TGTCATTGAGAGCAATGCCAGC

CRLF2, cytokine receptor-like factor 2; AKT, RAC-α serine/threonine-protein kinase; mTOR, serine/threonine-protein kinase mTOR; S6K1, ribosomal protein S6 kinase β-1; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

Cell Counting Kit-8 (CCK-8) assay. BaF3 cells from each group were washed with PBS twice, digested with 0.25% trypsin and dissociated into single-cell suspensions. Following cell counting, the cells were seeded in triplicate in 96-well plates at a density of $1x10^4$ cells/well. At 24, 48 and 72 h of incubation, respectively, 10 μl CCK-8 reagent (cat. no. 40203ES60; Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) was added to each well for a 4-h incubation. The optical density (OD) of each well was measured at 450 nm with an automated quantitative microplate reader (MK3; Thermo Fisher Scientific, Inc.). The cell viability curves were plotted with time on the x-axis and the OD value on the y-axis. Each test was repeated three times.

Wound healing assay. A total of 48 h after transfection, culture was resumed with BaF3 cells extracted from each group. When the cells reached 80-90% confluence, scratches were made with a 200 μl pipette tip using a ruler. The cells were rinsed with PBS three times to remove detached cells, and RPMI-1640 medium containing 10% FBS was added. Samples were subsequently incubated at 37˚C in an atmosphere of 5% CO_2. The cells were observed and photographed at 0 and 48 h under a phase contrast microscope (Olympus Corporation) at x100 magnification. The wound healing was observed dynamically and the scratch width of the cells in each group was measured. The migration of cells was calculated as follows: Migration rate = (scratch width at 0 h - scratch width at 48 h)/scratch width at 0 h.

Transwell invasion assay. The Transwell invasion assay was performed according to the instruction of the Costar 24-Well Transwell™ (EMD Millipore, Billerica, MA, USA). Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) which was previously stored at -20˚C was defrosted at 4˚C. The Matrigel was placed on ice and diluted 1:1 using serum-free medium. Following mixing, the mixture was added into the Transwell chamber (Costar; EMD Millipore.) with 15 μl per well and coated at 37˚C for 1 h, followed by three washes with serum-free medium. Following complete digestion, BaF3 cells were washed twice in serum-free medium and counted. The cell suspensions (containing $1x10^5$ cells), along with serum-free DMEM were added into the upper compartment and diluted to 400 μl, and triplicate wells were set up for each group. Complete DMEM (600 μl) containing 15% FBS was added into the lower compartment. Samples were incubated at 37˚C in an atmosphere of 5% CO_2 for 24 h. Subsequently, the liquid in the compartment was removed and the cells in the compartment were wiped away using cotton swabs. The compartment was immersed in fixation solution (50% methanol) at 4˚C for 15 min and washed with PBS three times. Following staining with crystal violet for 30 min at room temperature and air-drying, six fields were randomly selected in each well for photographing and cell counting under an inverted phase contrast microscope (Olympus Corporation) at x200 magnification. The mean cell count in each field was calculated.

Cell sensitivity to chemotherapeutic agents. The concentration of BaF3 cells in each group was adjusted to $0.5x10^{10}$/l by adding RPMI-1640 medium. Cells were cultured *in vitro* for 24 h following the addition of 25 μmol/l imatinib in each group. Subsequently, the cells were harvested and the apoptosis of BaF3 cells in each group was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining kit (cat. no. 556547; Shanghai FuShen Biotechnology Co., Ltd., Shanghai, China). The procedure for the test was as follows: 10X binding buffer was diluted 10 times using deionized water and the cells from each group were centrifuged for 5 min (670 x g; 4˚C); subsequently, the cells were harvested and resuspended with pre-cooled 1X PBS, followed by centrifugation at for 5-10 min (670 x g; 4˚C); next, the cells were washed and suspended with 1X binding buffer (300 μl); 5 μl Annexin V-FITC was added and mixed, and the mixture was incubated away from light at room temperature for 15 min; 5 min prior to the cell analysis by flow cytometry (Cube 6; Sysmex Partec GmbH, Görlitz, Germany), 5 μl PI was added and the cells were placed in an ice bath away from light for 5 min. The excitation wavelength was 480 nm; FITC was detected at 530 nm and PI was detected at a wavelength >575 nm.

Statistical analysis. SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Each experiment was run in triplicate. Measurement data are expressed as mean ± standard deviation. Comparisons between the two groups were conducted by t-test and comparisons across multiple groups were conducted by one-way analysis of variance with the Bonferroni post hoc test. The OS and EFS in children with high CRLF2 expression and children with low CRLF2 expression were compared using a Kaplan-Meier survival curve and the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CRLF2 expression levels in bone marrow. The immunohistochemical results in bone marrow tissues from healthy normal children and children with B-ALL are presented in Fig. 1A. The CRLF2 expression was hardly visible in the normal bone marrow tissues. By contrast, the CRLF2 expression was increased in the bone marrow tissues from children with B-ALL. The histogram of positive cell rates of CRLF2 in each group is displayed in Fig. 1B. The CRLF2 expression in the bone marrow of children with B-ALL was significantly upregulated compared with that in healthy normal children ($P<0.05$). Likewise, among all the children with B-ALL, the upregulation of CRLF2 expression in the high expression group (n=95) was greater compared with that in the low expression group (n=33) ($P<0.05$).

Factors associated with CRLF2 high expression. It was identified that the age, sex, PLT count, HGB concentration, liver and lymph node infiltration indexes, as well as risk stage were similar between children with low and high CRLF2 expression (all $P>0.05$), whereas WBC count, LDH concentration and spleen infiltration indexes were different between these two groups (all $P<0.05$; Table II).

Association between CRLF2 expression and patient prognosis. The comparison of OS and EFS in patients with high CRLF2 expression and low CRLF2 expression is displayed in Fig. 2A and B, respectively. The results of the analysis indicated that B-ALL patients with high CRLF2 expression had lower OS and EFS rates compared with those with low expression (45.45 vs. 72.63%, $P<0.001$; 48.48 vs. 78.95%, $P<0.001$, respectively), suggesting that CRLF2 overexpression was associated with poor prognosis ($P<0.001$).

Detection of mRNA and protein expression in BaF3 cell by RT-qPCR and western blotting. The results of the relative mRNA expression in each group as assessed by RT-qPCR are presented in Fig. 3A; results of the relative protein expression in each group, assessed by RT-qPCR, are presented in Fig. 3B; results of the protein bands in each group, assessed

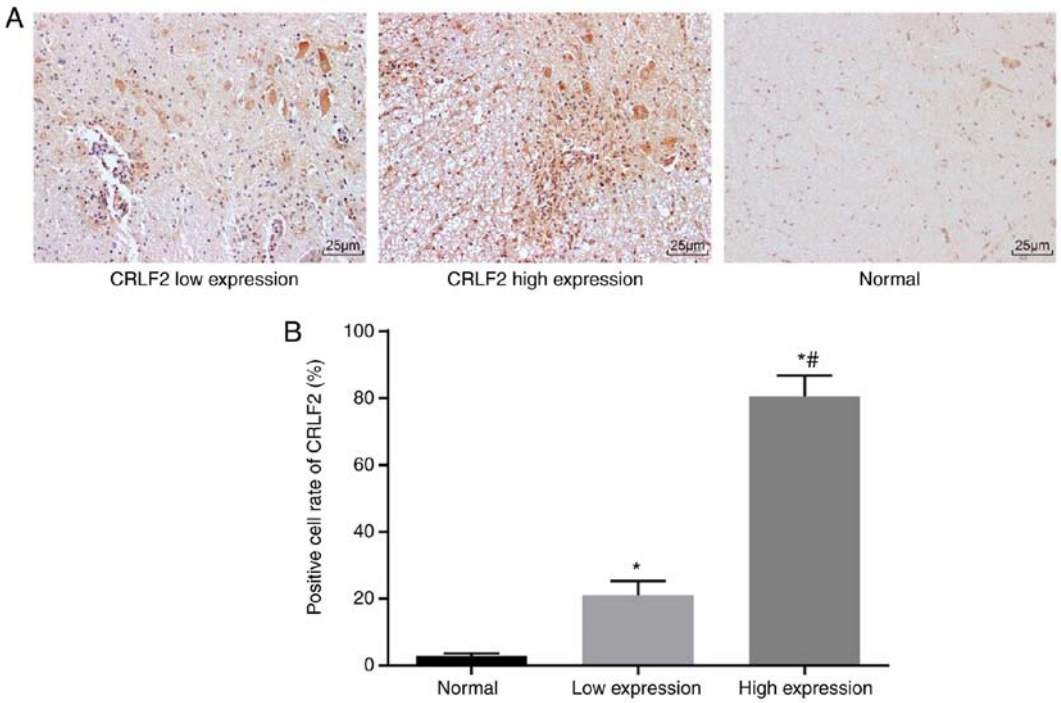

Figure 1. Analysis of CRLF2 expression in bone marrow through immunohistochemistry. (A) Immunohistochemical staining of bone marrow tissues from normal healthy children and children with B-cell acute lymphoblastic leukemia. (B) Histogram of the positive cell rate of CRLF2 in each group. *P<0.05 vs. the normal group; #P<0.05 vs. the low expression group. CRLF2, cytokine receptor-like factor 2.

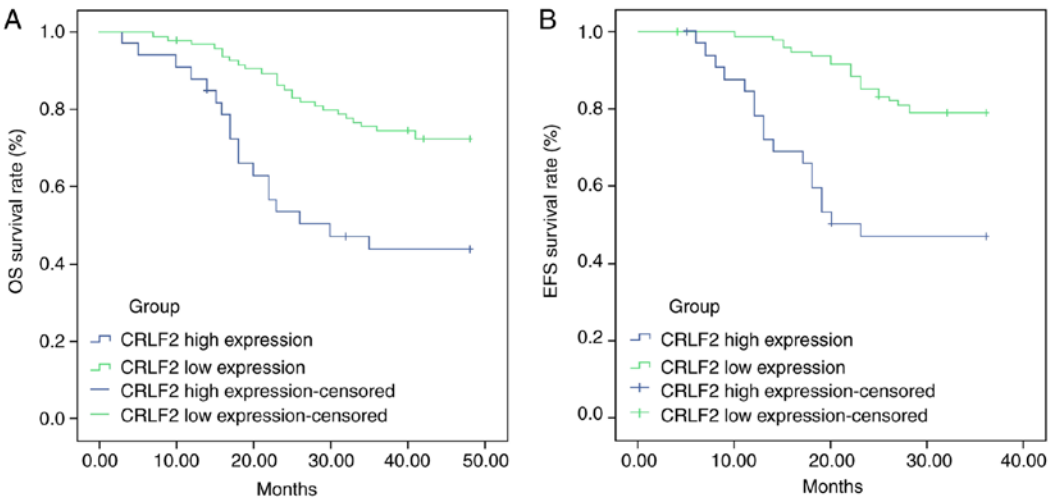

Figure 2. CRLF2 expression and the prognosis of the patients. (A) Comparison of OS in patients with high CRLF2 expression and low CRLF2 expression. (B) Comparison of EFS in patients with high CRLF2 expression and low CRLF2 expression. OS, overall survival; EFS, event-free survival; CRLF2, cytokine receptor-like factor 2.

by western blotting, are displayed in Fig. 3C. The findings revealed that compared with the blank group, the expression of CRLF2 protein and mRNA was significantly downregulated in the shCRLF2 and the shCRLF2+LY294002 groups (both P<0.05), whereas the expression levels in the LY94002 group and the NC group were similar to those in the blank group (both P>0.05).

A downregulation of the expression levels of AKT, mTOR and S6K1 mRNAs, and phosphorylated AKT and mTOR proteins, in addition to an evident upregulation of the expression levels of 4EBP1 mRNA and protein, were observed in the shCRLF2, LY294002 and shCRLF2+LY294002 groups compared with the blank group (all P<0.05). The same patterns were observed in both the shCRLF2 and the LY294002 group compared with the shCRLF2+LY294002 group (all P<0.05). No intergroup differences were observed between the blank group and the NC group for these indices (all P>0.05; Fig. 3).

BaF3 cell viability in each group measured by CCK-8 assay. The viability of BaF3 cells at different time points was measured by CCK-8 assay. The results indicated that the cell viability in each group increased with the prolongation of culture time. The cell viability in the shCRLF2, LY294002 and shCRLF2+LY294002 groups was inhibited at 24, 48 and 72 h compared with that in the

Table II. Correlation between CRLF2 expression and patients' characteristics.

Clinical characteristic	High expression of CRLF2, n=33 (mean ± standard deviation)	Low expression of CRLF2, n=95 (mean ± standard deviation)	P-value
Age, years	8±2	9±4	0.1715
Sex			
Male	21	53	0.4320
Female	12	42	
WBC, 10^9/l	47.4±6.3	31.2±2.1	<0.0001
LDH, μg/l	565±87	845±59	<0.0001
PLT, 10^9/l	38.4±9.2	36.1±8.1	0.1771
HGB, g/dl	11.2±3.2	10.9±1.6	0.4858
Extramedullary infiltration, %			
Liver	15.2±4.7	14.8±5.4	0.7058
Spleen	66.4±5.2	30.5±4.3	<0.0001
Lymph node	48.2±6.1	45.7±7.5	0.3055
Risk stage			
Standard risk	19	63	0.3670
High-risk	14	32	

CRLF2, cytokine receptor-like factor 2; B-ALL, acute B-cell lymphoblastic leukemia; WBC, white blood cell count; HGB, hemoglobin; PLT, platelet count; LDH, lactate dehydrogenase.

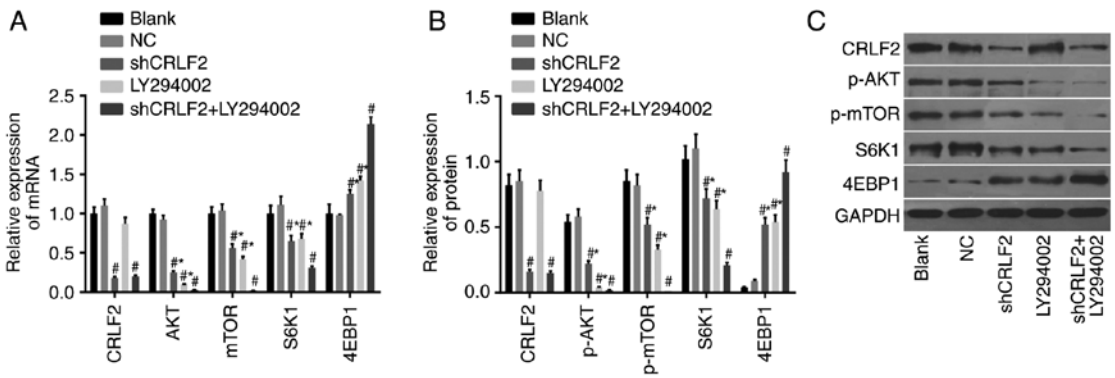

Figure 3. Reverse transcription-quantitative polymerase chain reaction and western blotting results in each group. (A) Relative mRNA expression in each group. (B) Relative protein expression in each group. (C) Electrophoresis bands. #P<0.05 vs. respective blank group; *P<0.05 vs. respective shCRLF2+LY294002 group. NC, negative control; sh, short hairpin; CRLF2, cytokine receptor-like factor 2; p, phosphorylated; AKT, RAC-α serine/threonine-protein kinase; mTOR, serine/threonine-protein kinase mTOR; S6K1, ribosomal protein S6 kinase β-1; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

blank group (all P<0.05). The cell viability was greatly increased at all time points in the shCRLF2 group and the LY294002 group compared with that in the shCRLF2+LY294002 group (all P<0.05). No intergroup difference was noted between the blank group and the NC group (P>0.05; Fig. 4).

BaF3 cell migration in each group measured by wound healing assay. The migration of BaF3 cells in each group was measured by wound healing assay. The results indicated that the migration of the cells was considerably inhibited in the shCRLF2 (44.20±3.41%), LY294002 (35.40±2.51%) and shCRLF2+LY294002 (14.60±1.52%) groups compared with the blank group (82.30±8.10%; all P<0.05). In addition, the migration of the cells was increased considerably in the shCRLF2 and LY294002 groups compared with the shCRLF2+LY294002

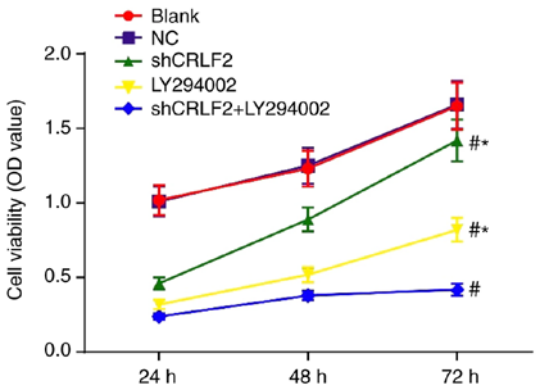

Figure 4. BaF3 cell viability in each group. #P<0.05 vs. the respective blank group; *P<0.05 vs. respective shCRLF2+LY294002 group. OD, optical density; NC, negative control; sh, short hairpin; CRLF2, cytokine receptor-like factor 2.

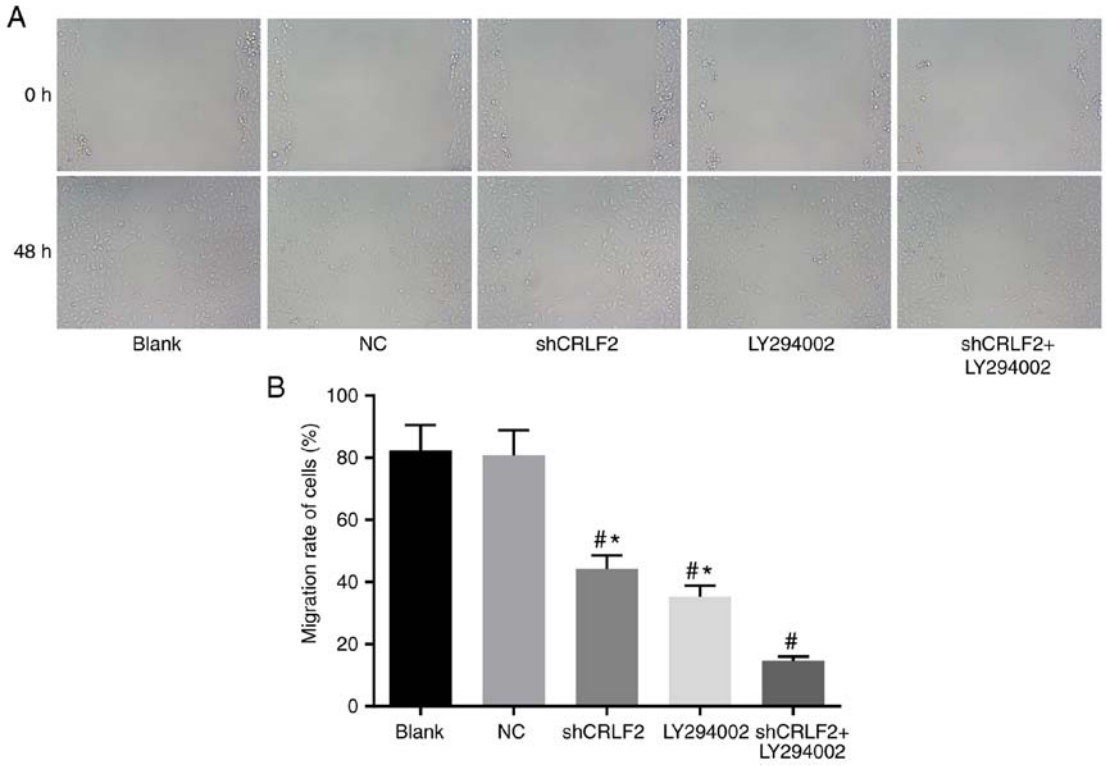

Figure 5. Results of the wound healing assay in each group. (A) Image of wound healing assay in BaF3 cells. magnification, x100. (B) BaF3 cell migration rate. #P<0.05 vs. the blank group; *P<0.05 vs. the shCRLF2+LY294002 group. NC, negative control; sh, short hairpin; CRLF2, cytokine receptor-like factor 2.

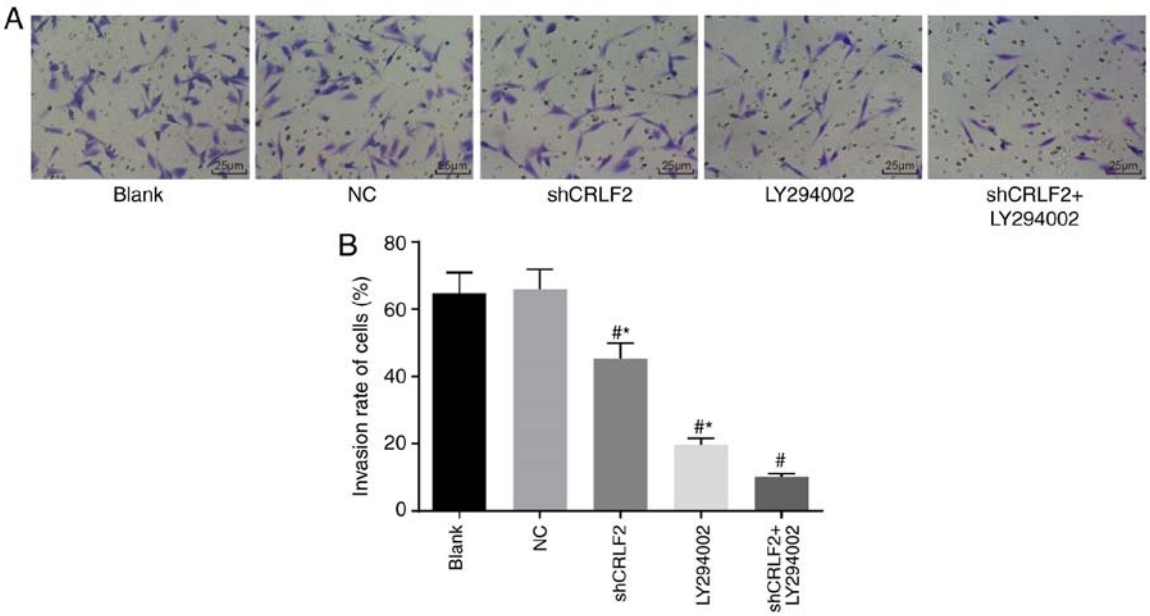

Figure 6. BaF3 cell invasion in each group. (A) BaF3 cell invasion in each group. (B) Histogram of BaF3 cell invasion rate in each group. #P<0.05 vs. the blank group; *P<0.05 vs. the shCRLF2+LY294002 group. NC, negative control; sh, short hairpin; CRLF2, cytokine receptor-like factor 2.

group (all P<0.05). No intergroup difference was observed between the blank group and the NC group (P>0.05; Fig. 5).

BaF3 cell invasion in each group detected by Transwell invasion assay. The invasion of BaF3 cells in each group may be observed in Fig. 6A and the histogram of the BaF3 cell invasion rate in each group is presented in Fig. 6B. The results documented that the invasion of BaF3 cells was markedly suppressed in the shCRLF2 (45.40±5.28%), LY294002 (19.70±2.41%) and shCRLF2 ± LY294002 (10.20±1.66%) groups compared with the blank group (64.70±5.25%; all P<0.05). The invasion of cells was significantly enhanced in the shCRLF2 and LY294002 groups compared with the shCRLF2+LY294002 group (all P<0.05; Fig. 6).

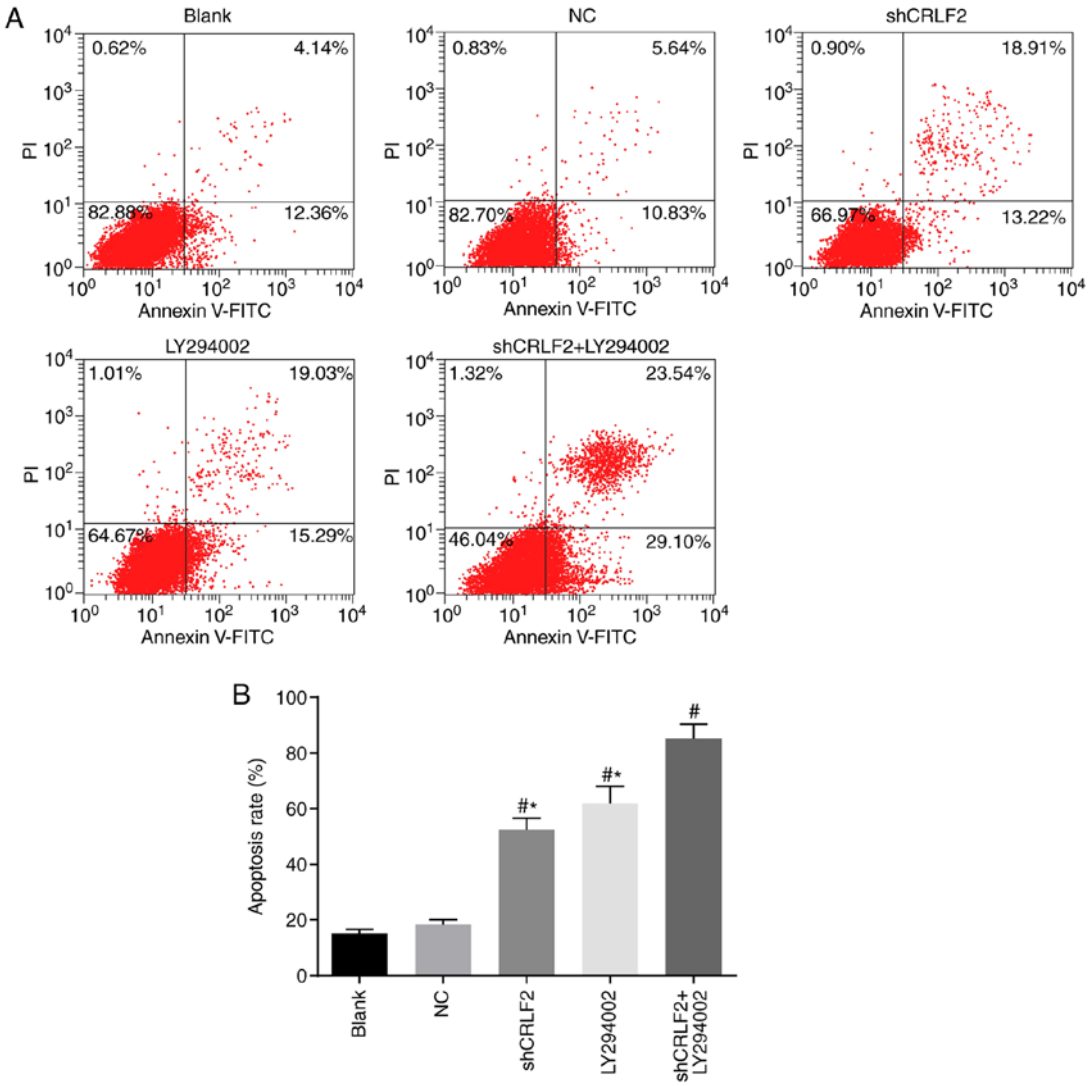

Figure 7. BaF3 cell sensitivity to chemotherapeutic agents. (A) BaF3 cell apoptosis in each group. (B) Histogram of the BaF3 cell apoptosis rate in each group. #P<0.05 vs. the blank group, *P<0.05 vs. the shCRLF2+LY294002 group. NC, negative control; sh, short hairpin; CRLF2, cytokine receptor-like factor 2; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Cell apoptosis in each group, assessed by Annexin V-FITC/PI double staining assay. The Annexin V-FITC/PI double staining assay was performed to detect the drug-induced apoptosis of imatinib-treated cells. The results of the BaF3 cell apoptosis and apoptosis rates in each group are presented in Fig. 7A and B, respectively. The results demonstrated in the shCRLF2 group, LY294002 group and shCRLF2+LY294002 group there was an increase in the sensitivity of BaF3 cells to chemotherapeutic agents compared with the blank group. Furthermore, the shCRLF2+LY294002 group achieved even better results compared with the shCRLF2 and LY294002 groups; the cell apoptosis rates in the shCRLF2 and LY294002 groups were decreased compared with the shCRLF2+LY294002 group (both P<0.05; Fig. 7).

Discussion

Certain studies have confirmed that CRLF2 serves a key role in the development of B lymphocytes, and mutations in the CRLF2 gene may further activate the CRLF2/thymic stromal lymphopoietin (TSLP) signaling pathway and induce B-ALL (15-17). Moreover, CRLF2 is a marker for Ph-like ALL. The abnormal expression of CRLF2 is closely associated with the occurrence, recurrence and poor prognosis of ALL (18). There was a study in which CRLF2 expression was observed in TSLP humanized transplantation mouse models following injection of human HS27 cells expressing the human TSLP gene, and the results demonstrated that CRLF2 is able to promote the differentiation and growth of B cells, in addition to the proliferation of lymphocytes at the early stage in TSLP mouse models (19). The AKT/mTOR signaling pathway has been proven to serve an essential role in cell proliferation and growth, and the abnormal activation of this signaling pathway has been observed in studies on T-cell ALL (20). Certain studies have compared the effects of different signaling pathway inhibitors on B-ALL cells and have reported that AKT/mTOR signaling pathway inhibitors can significantly inhibit the B-ALL cell cycle and reduce the cell survival rate (21). The present study performed a further investigation based on these findings and identified that

silencing the CRLF2 gene may suppress the activation of the AKT/mTOR signaling pathway, which may have protective effects on B-ALL.

In the present study, significant upregulation of CRLF2 expression in the bone marrow of B-ALL patients was observed, implying that CRLF2 may be implicated in the pathogenesis of B-ALL. In order to investigate the association between the CRLF2 gene and the pathogenesis of B-ALL, the present study divided BaF3 cell lines into different groups and examined the expression levels of mRNAs and proteins associated with the CRLF2 and AKT/mTOR pathways, the cell viability and cell sensitivity to chemotherapeutic agents. The results indicated that the mRNA and protein expression levels of CLRF2 were downregulated in the shCRLF2 and shCRLF2 + LY294002 groups as compared with the Blank and NC groups, however, no significant difference was noted in the LY294002 group. Moreover, the expression levels of AKT, mTOR and S6K1 mRNAs and associated phosphorylated proteins, cell viability, cell migration and cell invasion in the shCRLF2, LY294002, and shCRLF2 + LY294002 groups were significantly decreased compared with the Blank and NC groups, indicating that CLRF2 gene could affect the protein expression, cell migration and cell invasion in BaF3 cells via AKT, mTOR and other signaling pathways. The results revealed that silencing CRLF2 expression or inhibiting the AKT/mTOR signaling pathway could reduce the cell viability, migration and invasion in B-ALL and promote cell apoptosis. Other studies have stated that >80% of patients with ALL have abnormal activation of the AKT/mTOR pathway, which further confirms the findings in the present study (22,23).

Gene rearrangement refers to the movement of a gene from a site distal to the promoter to a site proximal to the promoter, thereby influencing gene transcription and expression. During the treatment of children with B-ALL, Raghunathan *et al* (24) identified that children with CRLF2 rearrangement had improved treatment responses and 4-year recurrence-free survival rates compared with those without CRLF2 rearrangement, suggesting that the abnormal expression of CRLF2 induced by gene rearrangement may be highly correlated with the prognosis of B-ALL. In the present study, the Annexin V-FITC/PI double staining assay was used, and it was identified that the silencing of the CRLF2 gene and the use of the AKT/mTOR signaling pathway inhibitor significantly increased the sensitivity of BaF3 cells to chemotherapeutic agents against B-ALL compared with the blank group and NC group; furthermore, the combined use of these two methods achieved an even a better result. Survival analysis indicated that among all the children with B-ALL, those with high CRLF2 expression had much lower OS and EFS levels than those with low CRLF2 expression, implying that low CRLF2 expression may have a positive effect on the prognosis of B-ALL. Useful findings have been obtained in the present study by investigating the association between silencing of the CRLF2-mediated AKT/mTOR pathway and the treatment efficacy and prognosis of pediatric B-ALL. However, due to regional and individual differences, further studies are required in the future for verification.

The present study demonstrated that silencing the CRLF2 gene may inhibit the activation of the AKT/mTOR pathway, which is closely associated with the treatment effect and prognosis in children with B-ALL. CRLF2 is a poor prognostic factor for B-ALL and may be used as a key target in the management of this disease. The suppression of CRLF2 expression or the use of an AKT/mTOR signaling pathway inhibitor may be helpful in the treatment of B-ALL, and the results of the present study may provide a basis for the future study and treatment of this disease.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

MJ was the guarantor of the integrity of the entire study. MJ and XZ contributed to the study concepts, the definition of intellectual content, literature research, clinical studies, experimental studies, manuscript preparation and manuscript editing. XZ contributed to data acquisition, data analysis and statistical analysis. LL contributed to the study design. MJ and LL contributed to manuscript review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Yichang Central People's Hospital (Yichang, China), and consent was obtained from the patients and their families.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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