

# Ik6 expression provides a new strategy for the therapy of acute lymphoblastic leukemia

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**Abstract.** Our previous study demonstrated that the dominant-negative Ikaros isoform 6 (Ik6) is overexpressed in Chinese children with newly diagnosed B-acute lymphoblastic leukemia (B-ALL) and is strongly associated with a poor outcome. The purpose of the present study was to further explore the function of Ik6 in B-ALL. The association between Ik6 expression as detected by real-time PCR and efficacy of chemotherapy was evaluated. The effect of the alteration in Ik6 on leukemic cell lines was assessed by *in vitro* gain-of-function and loss-of-function techniques. PCR analysis showed that Ik6 expression was decreased when patients completed induction chemotherapy and reached complete remission. Ik6 expression was significantly increased when patients suffered relapse. Stable transfection of Ik6 into the Nalm-6 cell line revealed that Ik6 enhanced proliferation of Nalm-6 cells through the promotion of G0/G1-to-S-phase transition and enhanced chemoresistance to chemotherapeutics through anti-apoptotic effects. However, Ik6 expression did not affect the invasion of Nalm-6 cells. In contrast, silencing of Ik6 in Sup-B15 cells significantly inhibited proliferation and increased chemosensitivity. The present study suggests that Ik6 may be a biological marker of chemosensitivity and relapse and Ik6 may provide a potential therapeutic strategy for ALL.

## Introduction

In spite of continuous progress in the therapy of acute lymphoblastic leukemia (ALL), relapses still occur in up to 20% of children and most adults with ALL (1-4). Moreover, the outcome of relapsed ALL patients is extremely poor (3,5,6). To improve the survival of ALL patients, it is critical to identify new molecular biomarkers such as BCR/ABL which are

involved in the regulation of the malignant biological behavior of leukemia cells and are valuable in the prognosis and therapy of leukemia patients.

Ikaros is a lymphoid transcription factor that was identified as a hematological tumor suppressor (7,8). Only Ikaros isoforms that contain at least three DNA-binding zinc fingers possess functional activity, such as Ik1, Ik2 and Ik3. Isoforms lacking two or more zinc-finger domains cannot bind DNA and impair the function of Ikaros proteins in a dominant-negative manner (9). Dominant-negative Ikaros isoform 6, (Ik6), with a deletion of coding exons 3 through 6, is the most common and strongest transcriptional repressor in the Ikaros family (10-12). Ample evidence indicates that overexpression of Ik6 is associated with a poor prognosis of ALL patients (13-15). A recent study showed that the prognosis of Ph-negative patients with Ik6 was close to that of Ph-positive patients (16).

The clinical data available to date suggest that Ik6 should be evaluated as a prognostic marker for newly diagnosed ALL patients and may be involved in leukemogenesis. However, there are few studies concerning the role of Ik6 in the therapy of ALL. In the present study, in order to ascertain whether Ik6 is a marker of chemotherapeutic efficacy and is a potential therapeutic target, we investigated changes in Ik6 expression during treatment of ALL patients and assessed the effects *in vitro* of Ik6 expression on cell proliferation, cell cycle, chemosensitivity to vincristine (VCR), daunorubicin (DNR) and L-asparaginase (L-Asp) and invasion in ALL cell lines.

## Materials and methods

**Patients and samples.** The 25 patients included in the present study were diagnosed with Ik6-positive B lineage ALL between January 2009 and January 2012 and were treated at Wuhan Union Hospital, in accordance with the CCLG-ALL-2008 Protocol (Children's Cancer and Leukemia Group). All samples were bone marrow aspirates and were obtained following informed consent in strict accordance with the Declaration of Helsinki. The endpoint of the present study was January 2013, and the expression of Ik6 was measured at the point of initial diagnosis, end of induction therapy, at complete remission and at relapse.

**Cell culture.** Sup-B15 and Nalm-6, human B-cell precursor leukemia cell lines, were used for the study. Both cell lines

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were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Gibco-BRL, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Overexpression or silencing of Ik6 in acute lymphoblastic leukemia cell lines.** Nalm-6 cells stably overexpressing Ik6 were obtained as follows. The complete Ik6 coding sequence was amplified by PCR from Sup-B15 cells and cloned into the lentiviral expression vector pHR-SIN-CSIGW. The vector pHR-CSIGW-Ik6 and the viral packaging system (containing an optimized mixture of two packaging plasmids, pMD2.G and psPAX2) were co-transfected into 293T cells to produce competent lentivirus. The viral supernatant was harvested at 48 h post-transfection and was used to infect Nalm-6 cells. The pHR-CSIGW-mock vector was also packaged and used as a negative control. For transfection, 1x10<sup>6</sup> Nalm-6 cells were collected on day 2 and resuspended in 1 ml complete medium (RPMI-1640 medium supplemented with 10% FBS). Cells were transfected with pHR-CSIGW-Ik6 or pHR-CSIGW-mock at a multiplicity of infection (MOI) of 50 for 8 h. Then half of the above medium was replaced with 1 ml fresh medium. Thereafter, cells were cultured for another 64 h and analyzed for expression of GFP by flow cytometry. The expression of Ik6 protein was further confirmed by western blotting.

Sup-B15 cells with stably silenced Ik6 were obtained through a similar procedure. Firstly, we designed several small interfering RNAs (siRNAs) and screened the most effective one. The target sequence for Ik6 was 5'-GCTACGAGAAGGAGAACA-3' and the negative control sequence was 5'-TTC TCCGAAGTGTACAGT-3'. Then, the small hairpin RNA (shRNA) was cloned into the self-inactivating lentiviral vector (GeneChem, Shanghai, China) containing a CMV-driven GFP reporter and a U6 promoter upstream of the cloning sites (*Age*I and *Eco*RI).

**Real-time RT-PCR assay.** Total cellular RNA was extracted from cells using TRIzol reagent and converted to single-stranded cDNA using the Toyobo kit. Real-time PCR amplification was performed using the SYBR-Green Master Mix (Toyobo, Japan) and the StepOnePlus™ Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The primers for Ik6, vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (Flt-1), placenta growth factor fragment (PlGF), angiogenin-1 (Ang-1), angiogenin-2 (Ang-2), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are shown in Table I. Cycling conditions were 95°C for 30 sec followed by 40 cycles of 95°C for 10 sec and 60°C for 35 sec. The relative levels of mRNA expression were quantified by comparison with the internal control (GAPDH). All the samples were performed in triplicate, and the results were analyzed using the 2<sup>-ΔΔC<sub>t</sub></sup> method.

**Western blotting.** Cells were lysed using a nuclear and cytoplasmic protein extraction kit (Beyotime, China) according to the manufacturer's instructions. The extracts were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was collected. A BCA protein assay kit (Pierce, Rockford, IL, USA) was used to determine the protein concentrations. Samples

Table I. Sequences of primers used in real-time PCR.

Gene		Primer sequences
Ik6	F	ccctgaagcgatactccag
	R	ttgtcccccacgact
VEGF	F	atcttcaagccatctgtgtgc
	R	gtcaccgcctcggtgtgt
Flt-1	F	atcattccgaagcaaggtgtg
	R	aaaccatttgacacatctgt
PlGF	F	cacttcccctgttcttctgaa
	R	caagcaaatggcaagtgtga
Ang-1	F	ttcctttcctttgtcttcctc
	R	ctgcagagcgtttgtgtgt
Ang-2	F	aacatcccagtcacacgtag
	R	ggcttctgttggctccgta
MMP-2	F	ggaagcatcaatcgactg
	R	ggcgaggagaaagtagca
MMP-9	F	cccacttacttggaaacg
	R	gaagatgaatggaatacgc
GAPDH	F	cctccaaggagtaagacccc
	R	agggtgtctacatggcaactg

F, forward; R, reverse; Ik6, Ikaros isoform 6; VEGF, vascular endothelial growth factor; Flt-1, vascular endothelial growth factor receptor; PlGF, placenta growth factor fragment; Ang-1, angiogenin-1; Ang-2, angiogenin-2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9.

were resolved in 10% SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad). After being blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20, proteins were detected by respective antibodies using an ECL kit (Pierce) and exposed to X-ray film. Blots were visualized with a western blotting detection system (Bio-Rad).

**Proliferation assay.** To investigate the effects of the alteration in expression Ik6 on leukemia cells, exponentially growing cells (2x10<sup>4</sup>/well) were seeded in quintuplicate in 96-well plates. After seeding for 1, 2, 3 and 4 days, the quantity of viable cells was determined. Ten microliters of CCK-8 Cell Counting Reagent (Dojindo Molecular Technologies, Inc., Japan) was added directly to each well. The plates were sequentially incubated for 4 h at 37°C, and the WST-8 formazan product was measured at 490 nm using a microplate reader (Tecan Sunrise, Switzerland). To investigate the effects of the alteration in Ik6 expression combined with chemotherapeutics on leukemia cells, cells were incubated with culture medium containing various concentrations of the chemotherapeutics [2.5 to 50 ng/ml for vincristine (VCR); 2.5 to 50 ng/ml for daunorubicin (DNR) and 0.1 to 2.5 IU/ml for L-asparaginase (L-Asp)], respectively. After allowing cells to grow for 24 h at 37°C, the viable cell population in each well was reflected by the OD values. Then the fraction of surviving cells was calculated and the IC<sub>50</sub> was determined by nonlinear regression analysis using SPSS 11.5 software.

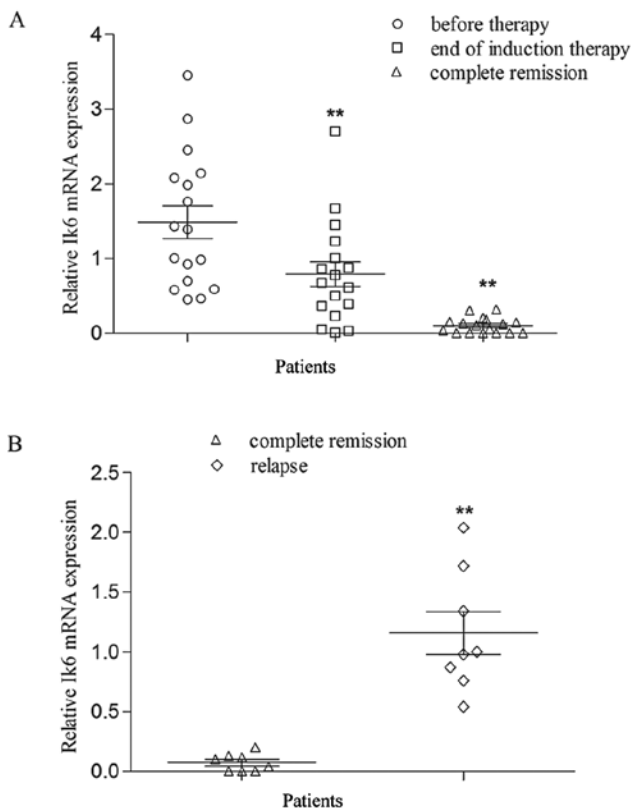


Figure 1. Ik6 expression of 25 B-ALL children during chemotherapy. (A) Ik6 expression was decreased when patients completed induction chemotherapy (\*\* $P<0.01$ ,  $n=17$ ), or reached complete remission (\*\* $P<0.01$ ,  $n=17$ ). (B) In contrast, Ik6 expression was increased when patients suffered relapse (\*\* $P<0.01$ ,  $n=8$ ). Ik6, Ikaros isoform 6; B-ALL, B-acute lymphoblastic leukemia.

**Cell cycle analysis.** The cells ( $10^6$  cells) were collected and fixed with ice-cold 70% ethanol overnight at 4°C. After washing with PBS and resuspension, fixed cells were treated with 50  $\mu\text{g/ml}$  RNase A (Amresco Inc., Solon, OH, USA) for 15 min at 37°C, and then incubated with 5  $\mu\text{g/ml}$  propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature in the dark. The cell cycle distribution was detected by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

**Analysis of apoptosis.** The apoptosis detection kit was from KeyGen Biotech (Nanjing, China). Cells were incubated with culture medium containing final concentrations of 5.0 ng/ml VCR, 0.5 ng/ml DNR and 0.1 IU/ml L-Asp, respectively, for 24 h. Treated cells were stained with propidium iodide and Annexin V-FITC for 15 min according to the manufacturer's instructions. The stained cells were subjected to flow cytometric analysis.

**Cell migration and invasion assays.** Cell migration was evaluated using an uncoated Transwell assay. Cells ( $2 \times 10^5$ ) were suspended in 200  $\mu\text{l}$  of serum-free RPMI-1640 medium and placed in the upper chambers of the Transwell plate (Corning, Cambridge, MA, USA). RPMI medium plus 10% FBS (250  $\mu\text{l}$ ) and NIH3T3-conditioned medium (250  $\mu\text{l}$ ) were added to the lower chambers. Plates were incubated at 37°C for 8 h. The cells of the lower compartments were counted, and the rate of

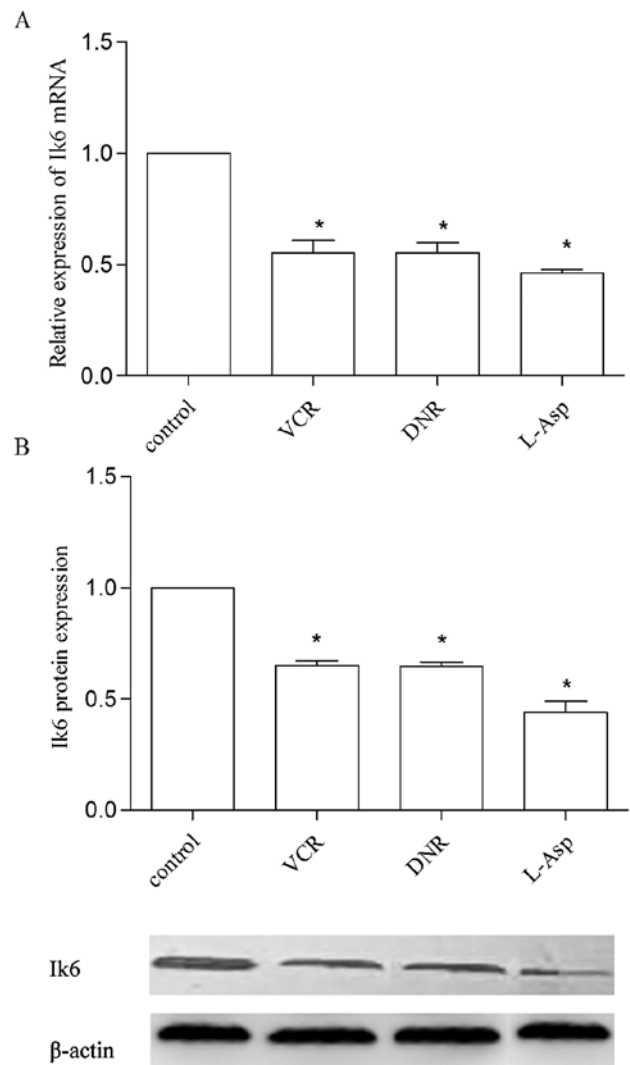


Figure 2. Effect of chemotherapeutics on the expression of Ik6 in Sup-15 cells. Treatment with VCR, DNR and L-Asp significantly decreased the (A) mRNA and (B) protein expression of Ik6 (\* $P<0.05$ ,  $n=3$ ) in Sup-15 cells. Ik6, Ikaros isoform 6; VCR, vincristine; DNR, daunorubicin; L-Asp, L-asparaginase.

migration was expressed as a percentage of the total number of cells added to each well. The cell invasion assay was similar to the migration assay but a Matrigel-coated Transwell was used.

**Statistical analysis.** Data are presented as means  $\pm$  SD. Comparisons between groups were carried out by the Student's t-test with software SPSS 11.5. Differences were considered to be statistical significant at  $P<0.05$ .

## Results

**Expression of Ik6 during chemotherapy.** In the 25 patients with Ik6-positive expression, 8 responded poorly to chemotherapy. Seventeen patients achieved complete remission but 8 patients of these 17 patients later suffered from relapse. After induction chemotherapy, the Ik6 expression was significantly downregulated compared with that before treatment ( $P<0.01$ ). However, in the children with relapse, Ik6 expression was again increased ( $P<0.01$ ) (Fig. 1). We also determined the alteration

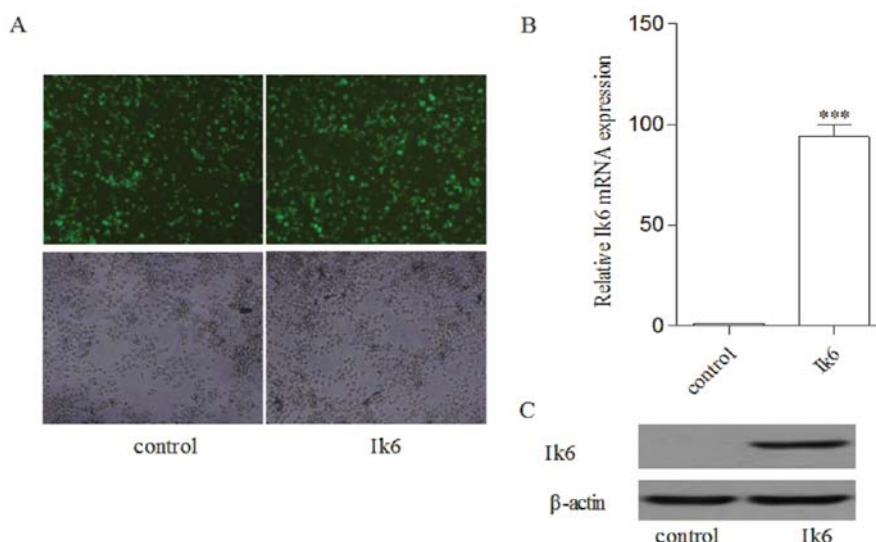


Figure 3. Lentiviral-mediated expression of Ik6 in Nalm-6 cells. (A) Expression of GFP in Nalm-6 cells transfected with Ik6 or control for 72 h. (B and C) The expression levels of mRNA and protein of Ik6 were confirmed by real-time RT-PCR and western blotting (\*\*\*P<0.001, n=3). Ik6, Ikaros isoform 6.

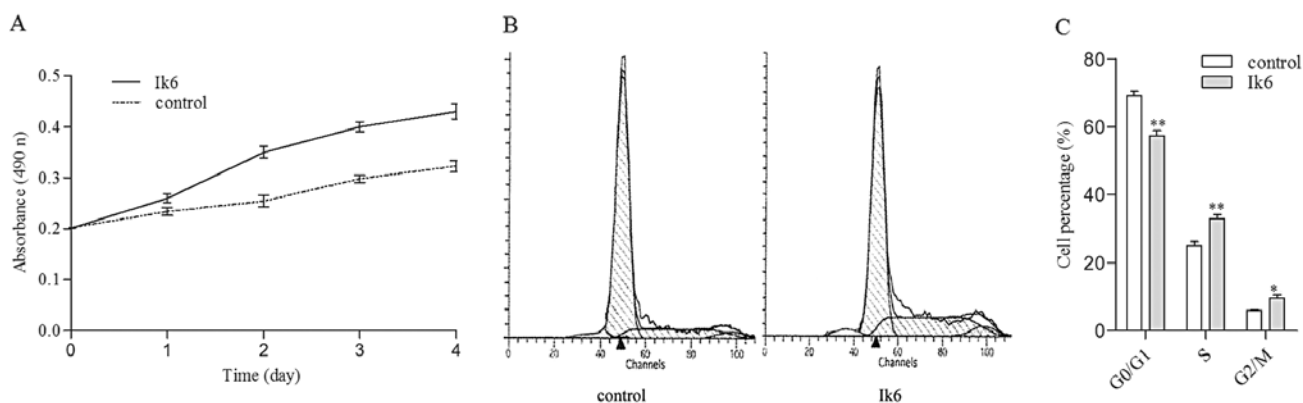


Figure 4. Ik6 overexpression promotes the proliferation of Nalm-6 cells. (A) The CCK-8 assay indicated that the percentage of viable cells in the Nalm-6/Ik6 group was significantly higher than those in the control group during the culture period (P<0.05, n=5). (B and C) The result of the cell cycle analysis showed that Nalm-6/Ik6 cells exhibited a decreased proportion of cells in the static phase (G0/G1) and an increased proportion in the synthesis (S) and mitotic phase (G2/M) of the cell cycle. (\*P<0.05, \*\*P<0.01, n=3). Ik6, Ikaros isoform 6.

in Ik6 expression in the Sup-B15 cell line after co-culture with different chemotherapeutics. A similar result was found in that VCR, DNR and L-Asp treatment markedly decreased the Ik6 mRNA and protein expression (P<0.05) (Fig. 2).

**Overexpression of Ik6 in Nalm-6 cells enhances cell proliferation and alters cell cycle distribution.** Nalm-6 cells expressed GFP after being transfected with Ik6 or with the control, and GFP fluorescence was observed in almost 95% of the cells (Fig. 3A). Real-time PCR and immunoblotting showed a generally higher level of Ik6 mRNA and protein expression in Nalm-6 cells following Ik6 overexpression (Fig. 3B and C).

After transfection with Ik6, Nalm-6/Ik6 cells exhibited increased cell proliferation compared with the Nalm-6 cells lacking the protein (Fig. 4A, P<0.05, n=5). Cell cycle results showed that Ik6-expressing Nalm-6 cells exhibited a decreased proportion of cells in the static phase (G0/G1) and an increased proportion in the synthetic (S) and mitotic phases (G2/M) of the cell cycle (Fig. 4B and C). Therefore, expression

of Ik6 in Nalm-6 cells promoted cell cycle progression from the G0/G1 phase to the S and G2/M phase.

**Overexpression of Ik6 in Nalm-6 cells decreases sensitivity to chemotherapeutics through an anti-apoptotic effect.** The effects of VCR, DNR and L-Asp on the growth of leukemia cells were evaluated, and the results indicated that the resistance to VCR, DNR and L-Asp was increased in the Ik6 transfectants. The IC<sub>50</sub> values of VCR (34.94 vs. 20.51 ng/ml), DNR (12.25 vs. 1.89 ng/ml) and L-Asp (2.37 vs. 0.361 U/ml) were higher than that of the control (P<0.05) (Fig. 5). The results from flow cytometry and western blotting revealed that Ik6 decreased the drug-induced apoptosis together with the upregulation of the bcl-xl protein (Fig. 6A and B).

**Ik6 does not affect the invasiveness of Nalm-6 cells.** Real-time PCR was performed to measure the expression of genes regulating invasion, and the results revealed no significant differences in the expression levels for the mRNA coding of

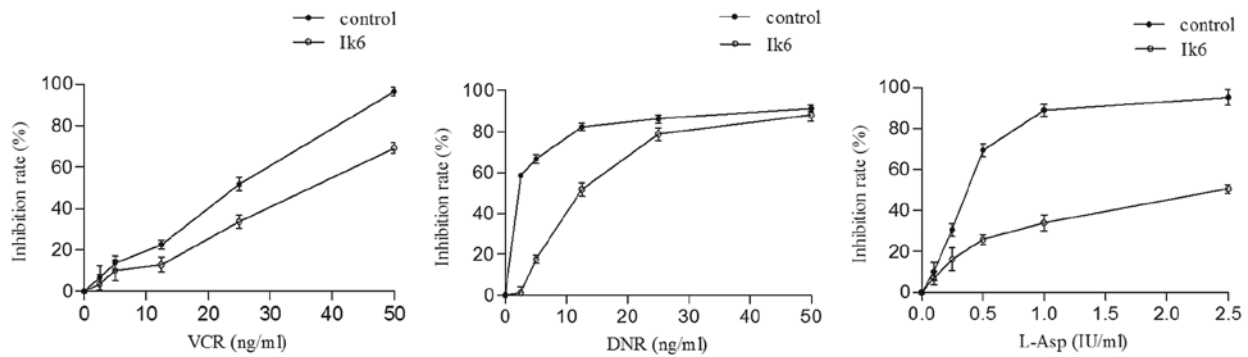


Figure 5. Ik6 overexpression enhances the chemoresistance of Nalm-6 cells. The CCK-8 assay revealed a dose-dependent growth inhibition, and the growth inhibition rate of Nalm-6/Ik6 cells was lower than that of the control cells following treatment with the same concentrations of VCR, DNR or L-Asp ( $P < 0.05$ ,  $n = 5$ ). Ik6, Ikaros isoform 6; VCR, vincristine; DNR, daunorubicin; L-Asp, L-asparaginase.

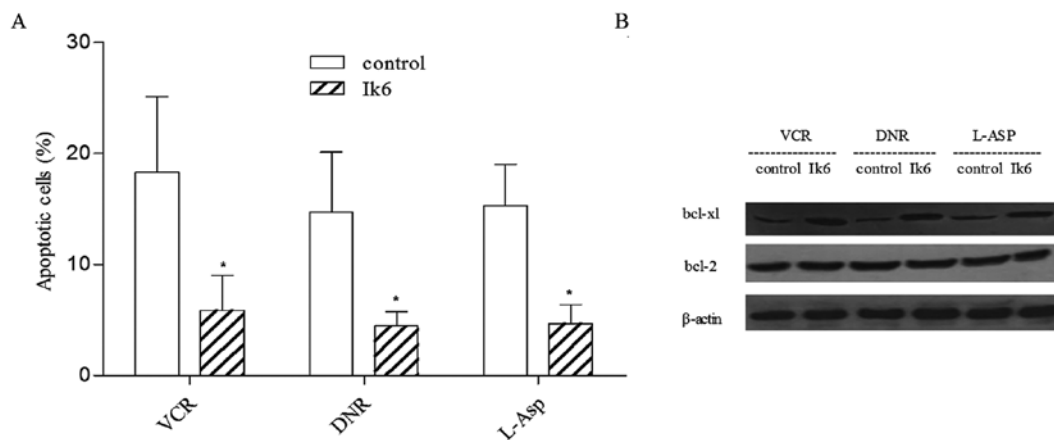


Figure 6. Ik6 overexpression decreases the drug-induced apoptosis of Nalm-6 cells. (A) The result of the flow cytometry revealed that, in contrast to the control cells, cell apoptosis induced by the drugs was markedly reduced in Nalm-6/Ik6 cells ( $P < 0.05$ ,  $n = 3$ ). (B) Western blot analysis of apoptosis-related proteins showed that the expression of bcl-xl in Nalm-6/Ik6 cells was upregulated. Ik6, Ikaros isoform 6.

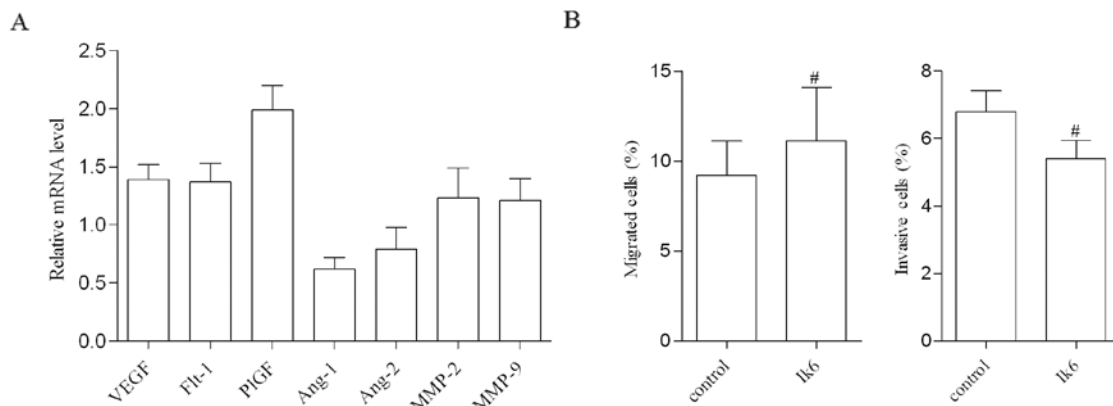


Figure 7. Ik6 overexpression has no effect on the invasive ability of leukemia cells. (A) The mRNA level of genes regulating invasion, VEGF, Flt-1, PlGF, Ang-1, Ang-2, MMP-2 and MMP-9, in Nalm-6/Ik6 cells was detected by real-time RT-PCR. Using Nalm-6/mock cells as the control, the result showed that there was no differences in the expression of these genes ( $n = 5$ ). (B) The migration and invasion assays showed that there was no difference in migratory and invasive abilities between the Nalm-6/Ik6 cells and control cells ( $P > 0.05$ ,  $n = 3$ ). Ik6, Ikaros isoform 6; VEGF, vascular endothelial growth factor; Flt-1, vascular endothelial growth factor receptor; PlGF, placenta growth factor fragment; Ang-1, angiogenin-1; Ang-2, angiogenin-2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9.

VEGF, Flt-1, PlGF, Ang1, Ang2, MMP-2 and MMP-9 between Nalm-6/Ik6 cells and the control ( $P > 0.05$ ) (Fig. 7A). In the migration and invasion assays, cells from both groups transmi-

grated from the upper to the lower chamber, but the quantity of cells in the lower chamber was not statistically different ( $P > 0.05$ ) (Fig. 7B).

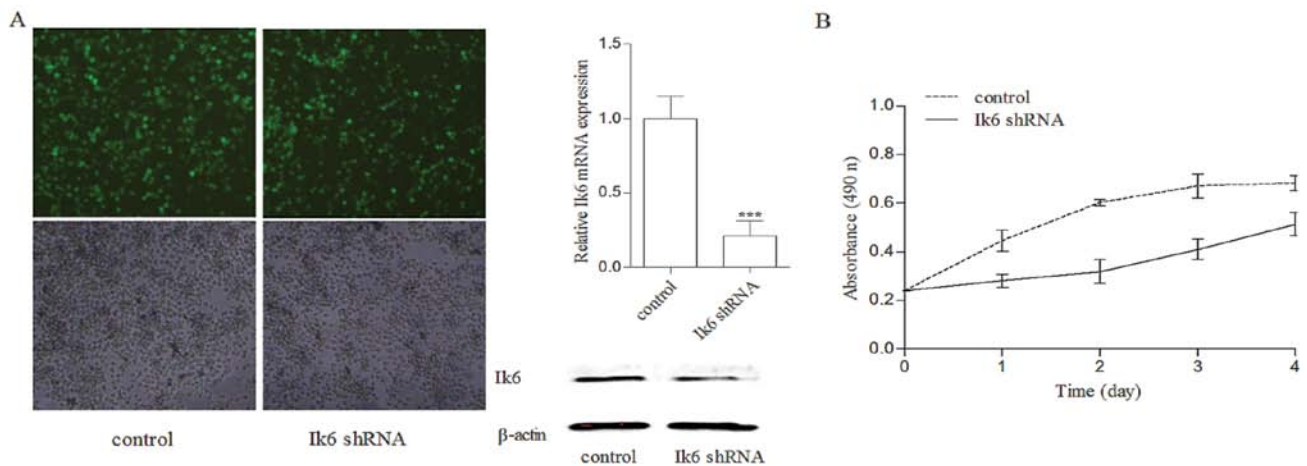


Figure 8. Silencing of Ik6 decreases the proliferation of Sup-B15 cells. (A) Lentiviral-mediated shRNA targeting Ik6 in Sup-B15 cells. The expression of Ik6 mRNA and protein was detected by real-time RT-PCR and western blotting, respectively. (\*\*\*P<0.001, n=5). (B) Silencing of Ik6 in Sup-B15 cells inhibited cell proliferation (\*P<0.05, n=5). Ik6, Ikaros isoform 6; shRNA, small hairpin RNA.

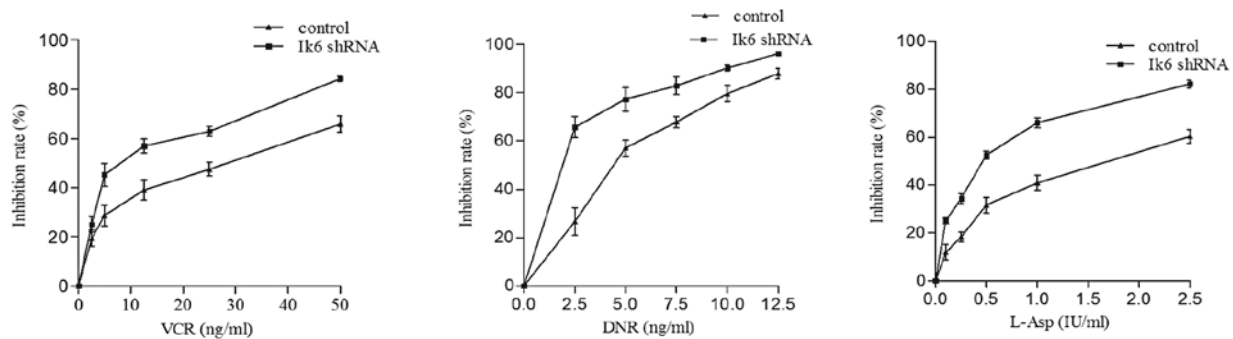


Figure 9. Silencing of Ik6 enhances the chemosensitivity of Sup-B15 cells to VCR, DNR and L-Asp (P<0.05, n=5). Ik6, Ikaros isoform 6; VCR, vincristine; DNR, daunorubicin; L-Asp, L-asparaginase.

*Silencing of Ik6 in Sup-B15 cells inhibits proliferation and increases chemosensitivity.* To further confirm the effect of Ik6 on proliferation and chemosensitivity of leukemia cells, Sup-B15 cells were modified to block Ik6 expression via a lentiviral-mediated shRNA vector. As shown in Fig. 8A, Ik6 mRNA and protein expression was downregulated in the Sup-B15/Ik6 shRNA cells when compared with the control. Ik6 shRNA significantly inhibited the proliferative activity of Sup-B15 cells (Fig. 8B, P<0.05) and enhanced the chemosensitivity to VCR, DNR and L-Asp. The IC<sub>50</sub> values of control cells to VCR, DNR and L-Asp were 2.6-, 2.9- and 3.4-fold higher than these values in the Sup-B15/Ik6 shRNA cells (P<0.05, Fig. 9).

## Discussion

In an effort to understand the phenomenon of leukemia relapse, several predictors of the ultimate outcome have been identified in the hopes of providing clues that may lead to more effective treatment (17,18). The Ik6 variant of the IKZF1 gene, an unfavorable prognostic marker in the outcome analysis of ALL, was independently associated with both overall survival and relapse-free survival (16). Ph/BCR-ABL was also known as a high-risk prognostic factor, but the emergence of tyrosine kinase inhibitors has significantly improved complete remission rates and the outcome of Ph-positive ALL patients (19,20).

Therefore, just as Ph not only indicates risk but is also a therapeutic target, Ik6 may not only provide insight into leukemogenesis but may also lead to the establishment of new treatment strategies targeting ALL.

We previously reported that Ik6 expression in the bone marrow cells of newly diagnosed ALL patients is associated with a higher level of 33-day minimal residual disease, which indicated excessive proliferation and primary chemoresistance of leukemia cells (16). However, there have been few published data concerning the dynamic expression of Ik6 during chemotherapy. The present study demonstrated that Ik6 expression is downregulated by chemotherapeutic agents *in vivo* and *in vitro*. A high level of Ik6 mRNA expression was detected in relapsed patients. Thus, Ik6 is not only a predictor of poor prognosis at initial diagnosis but is also a marker for monitoring chemotherapeutic efficacy and relapse during treatment. Certainly, more data from Ik6-positive patients are needed to provide the relationship between the exact level of Ik6 mRNA expression and response to treatment and relapse.

To explore the potential role of Ik6 in the treatment of ALL, we evaluated the effect of Ik6 on leukemia cell growth and found that overexpression of Ik6 increased cell proliferation. The results were in accordance with these of studies on *in vitro* systems, which demonstrated that Ik6 transfection stimulated the proliferation of pituitary cells (21) and human CD34<sup>+</sup> cord

blood cells (22). Furthermore, Ik6-expressing cells progressed more rapidly through the cell cycle than the control cells, in as much as they peaked in the S phase earlier.

Additionally, we analyzed the role of Ik6 in leukemia cell chemosensitivity. Our study demonstrated that the overexpression of Ik6 increased the chemoresistance of leukemia cells *in vitro*. The Ik6-expressing Nalm-6 cells were 1.7 times more resistant to VCR, 6.5 times more resistant to DNR and 6.6 times more resistant to L-Asp. The following clinical studies support the above-mentioned results. Tonnelles *et al* (14) reported that the response of patients with positive expression of Ik6 to induction treatment was not favorable; 7 of 8 patients did not reach complete remission and 1 achieved remission at the end of the induction therapy. A large sample of clinical data showed that 16.07% of the patients with Ik6 did not achieve remission and 48.44% suffered from relapse (16). VCR, DNR and L-Asp are currently the first-line chemotherapeutic drugs for the treatment of pediatric ALL. All of these drugs can induce apoptosis of leukemic cells. In the present study, we found that when Ik6 expression was increased in Nalm-6 cells, following treatment with the three drugs, significantly enhanced proliferative activity of Nalm-6 cells and a decreased level of apoptosis were noted with upregulation of bcl-xl. To further confirm the role of Ik6 in therapy, we found that silencing of Ik6 significantly inhibited proliferation and sensitizes Sup-B15 cells to the chemotherapeutic agents.

As well as acquired drug resistance, extramedullary tissue infiltration of leukemic cells is a major obstacle to leukemia treatment (23). Excessive egress of leukemia cell blasts results in invasion into various organs or tissues, such as the central nervous system (CNS) and testis (24,25). The results of our studies on leukemia cell invasion indicated that there was no effect of Ik6 on the invasive ability of leukemia cells *in vitro*.

In the present study, we found that Ik6 may be utilized as a gene marker to predict the clinical efficacy of chemotherapy. The patients with Ik6 overexpression should receive more intensive therapy, and detection for multidrug resistance should be carried out. More importantly, Ik6 regulates the proliferation and chemosensitivity of leukemia cells, and anti-apoptosis may be the mechanism of action. Regulatory apoptosis pathways that are associated with Ik6 are a potential target for a novel strategy for the chemotherapy of ALL.

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