Ikaros expression sensitizes leukemic cells to the chemotherapeutic drug doxorubicin

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Abstract. Ikaros is an important transcription factor involved in the development and differentiation of hematopoietic cells. However, its role in the treatment of hematopoietic malignancies such as leukemia is less well understood. In the present study, it was observed by data mining of the Oncomine database that high expression levels of full-length Ikaros (IK1) is correlated with increased sensitivity of cancer cells to treatments with chemotherapeutic drugs, including doxorubicin (DOX). To examine the functional significance of this observation, the expression of IK1 in a leukemia cell line was altered, and the response of leukemic cells to DOX treatment was analyzed. It was observed that overexpression of IK1 could enhance DOX-induced apoptosis, while knockdown of IK1 attenuated DOX-induced apoptosis in leukemic cells. Further experiments demonstrated that IK1 sensitized leukemic cells to DOX-induced apoptosis, probably through upregulation of caspase-9. These data suggest that high expression levels of IK1 may be a potential biomarker to predict responses of leukemia patients to treatment with chemotherapy.

Introduction

Acute myeloid leukemia (AML), the most common type of adult leukemia, is characterized by the accumulation of large numbers of abnormal myeloid cells that do not differentiate into functional granulocytes or monocytes during hematopoiesis (1,2) AML is the most frequent form of leukemia, accounting for ~25% of all leukemia cases in adults in the Western world (3,4).

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Despite these intimidating statistics, successful treatment options are available for patients diagnosed with AML (5-7). One of these effective treatments is chemotherapy, which kills leukemic cells or dividing normal hematopoietic cells (8). Doxorubicin (DOX) is a chemotherapeutic agent that belongs to the anthracycline-possessing family of drugs, and has a broad spectrum of activity (9). Beside being one of the most widely used chemotherapeutic agents, DOX is one of the most effective drugs for solid tumors, multiple myeloma and AML treatment (10). Previous studies have demonstrated that DOX can induce apoptosis in leukemic cells *in vivo* and *in vitro* (11). However, *de novo* and acquired resistances to DOX are also widely observed, the mechanism of which appears to be complex and heterogeneous (12). Better understanding of the mechanism of DOX resistance should aid to treat patients with leukemia more effectively.

The Ikaros protein, which was first identified as a lymphoid-specific zinc finger transcription factor, plays an essential role in the development and differentiation of specific lineages of hematopoietic cells (13,14). The Ikaros gene can be transcribed into a number of isoforms due to alternative splicing in exons 3-5, which encode part of the domain for DNA binding in the N-terminus of the Ikaros protein (15,16). In the present study, overexpression of full-length Ikaros (IK1) increased DOX-induced apoptosis in AML cells, while knockdown of IK1 expression attenuated DOX-induced apoptosis in these cells. Furthermore, the present study provides evidence that high IK1 expression contributes to chemotherapeutic sensitivity, probably by regulating pro-caspase-9 expression.

Materials and methods

Cell culture and reagents. Leukemic NB4 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in a 5% CO_2, 95% air humidified atmosphere at 37˚C. For the experiments, cells were seeded at a concentration of $3x10^5$ cells/ml and treated with dimethyl sulfoxide as vehicle control or with 0.4 μM DOX (Merck Millipore, Darmstadt, Germany) for 12 or 24 h. Prior to treatment with these compounds, cell viability was ≥95% by trypan blue exclusion assay.

Plasmids and small hairpin RNA (shRNA) constructs. Human IK1 complementary DNA (cDNA) was amplified from the leukemic NB4 cell line by reverse transcription-polymerase chain reaction under the following conditions: 95˚C for 5 min followed by 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 1.5 min and a final extension at 72˚C for 10 min (forward primer, 5'-CGACTCGAGATGGACTAC AAAGACGATGAC-3'; reverse primer, 5'-ACCGAATTC TTAGCTCATGTGGAAGCGGTG-3'. Amplified DNA was then subcloned into the pMSCVpuro retroviral vector (Clontech Laboratories, Inc., Mountainview, CA, USA), thus generating the pMSCV-puro-FLAG-IK1 plasmid. The sequence of the IK1 cDNA fragment was verified by DNA sequencing. Four different shRNA oligonucleotides (SH1, SH2, SH3 and SH4) against human IK1 were synthesized by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The target sequences of the shRNAs are as follows: SH1, 5'-AATCACAGTGAAATGGCAGAA-3'; SH2, 5'-AAG ACCTGTGCAAGATAGGAT-3'; SH3, 5'-AAGCCAAAC GTAAGAGCTCTA-3'; and SH4, 5'-AACTGCCACAAC TACTTGGAA-3'. Each sequence was checked against the human genome database by Basic Local Alignment Search Tool search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which revealed minimal sequence homology to other genes. For each target sequence, complementary 59 or 60-mer oligonucleotides with 5' single-stranded overhangs for ligation into the pSIREN-RetroQ vector (Clontech Laboratories, Inc.) were designed. These oligos were annealed and ligated into the pSIREN-RetroQ vector, and a non-targeting shRNA was also cloned into the above vector as a negative control (NC).

Transfection. The pMSCV-puro, pMSCV-puro-FLAG-IK1, pSIREN-RetroQ-NC-shRNA or pSIREN-RetroQ-IK1-shRNA vectors (provided by Professor Yingli Wu) were co-transfected with the packaging plasmids VSV-G and Gag-Pol into 293T cells to produce retroviruses using transfection reagent polybrene (Sigma-Aldrich). Supernatants containing retroviruses were collected 48 h after transfection and were used to infect NB4 cells. At 48 h post-infection, 0.5 μg/ml puromycin (Calbiochem; Merck Millipore) was added to the medium to select puromycin-resistant clones. The expression of IK1 was verified by western blot analysis.

Apoptosis assay. Apoptosis was measured using the Annexin-V-FLUOS apoptosis detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Annexin V-positive and propidium iodide (PI)-negative cells are considered to be in the early phase of apoptosis; while cells exhibiting positive staining both for Annexin V and PI are deemed to be at the late stage of apoptosis or necrotic (17).

Western blotting. Protein extracts from lysed cells were prepared as described previously (18) and were equally loaded onto 10-15% sodium dodecyl sulfate-polyacrylamide gels, and electrophoretically transferred to Immobilon polyvinylidene fluoride membranes (Merck Millipore). After blocking with 5% non-fat milk in Tris-buffered saline, the membranes were incubated overnight at 4˚C with polyclonal antibodies against Ikaros (ab26083; Abcam, Cambridge, MA, USA), caspase-8 (#4790; Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-9 (#9508; Cell Signaling Technology, Inc.), p53 upregulated modulator of apoptosis (PUMA; #12450; Cell Signaling Technology, Inc.), B-cell lymphoma-extra large (Bcl-xL; #2762; Cell Signaling Technology, Inc.), Bcl-2-interacting killer (Bik; #4592; Cell Signaling Technology, Inc.), Bcl-2 associated X protein (Bax; #2772; Cell Signaling Technology, Inc.) and BH3 interacting-domain death agonist (Bid; (#11423; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at a dilution of 1:1,000. β-actin (Calbiochem; Merck Millipore) was used as a loading control. Next, the blots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (#7074 and #7076; Cell Signaling Technology, Inc.) at room temperature for 2 h. Detection was performed using the Phototope®-HRP Western Blot Detection system (Cell Signaling Technology, Inc.) according to the manufacturer's protocol.

Measurement of caspase-9 activity. Caspase-9 activity was determined using a commercial assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, cells were lysed with Cell Lysis Buffer from the caspase-9 activity assay kit and the supernatant obtained by centrifuging the lysed (Beyotime Institute of Biotechnology) cells at 16,000 x g for 15 min at 4˚C was added to the reaction mixture, which contained Ac-LEHD-pNA, a colorimetric caspase-9 substrate. The absorbance at 405 nm was determined 2 h after the initiation of the reaction. The activity was expressed as fold-change over the control once corrected for the baseline (protein and buffer without colorimetric substrate).

Statistical analysis. All statistical analyses were performed using SPSS software (version 16; SPSS, Inc., Chicago, IL, USA). Student's *t* test was used to evaluate the differences between two groups. All experiments were repeated three times with similar results. $P<0.05$ was considered to indicate a statistically significant difference.

Results

High IK1/2 expression is associated with drug sensitivity in cancer cells. IK1 and IK2 are the two functional isoforms of Ikaros. By searching the publicly available database Oncomine (http://www.oncomine.com), a significant increase ($P<0.05$) was observed in the expression of IK1/2 in DOX-sensitive cell lines compared with DOX-resistant cell lines (Fig. 1A). In addition, IK1/2 expression was correlated with sensitivity to topotecan (Fig. 1B), another drug clinically used in cancer therapy. These data suggest that high expression levels of IK1 increase the sensitivity of certain types of cancer cells to DOX treatment.

Overexpression of IK1 accelerates DOX-induced apoptosis in leukemic cells. To test whether IK1 expression plays a role in conferring sensitivity to DOX in leukemic cells, NB4 cells were infected with retroviruses carrying control empty vector ($NB4^{EV}$) and vector overexpressing IK1 ($NB4^{FLAG\text{-}IK1}$) (Fig. 2A). Stable pools of $NB4^{EV}$ and $NB4^{FLAG\text{-}IK1}$ cells were

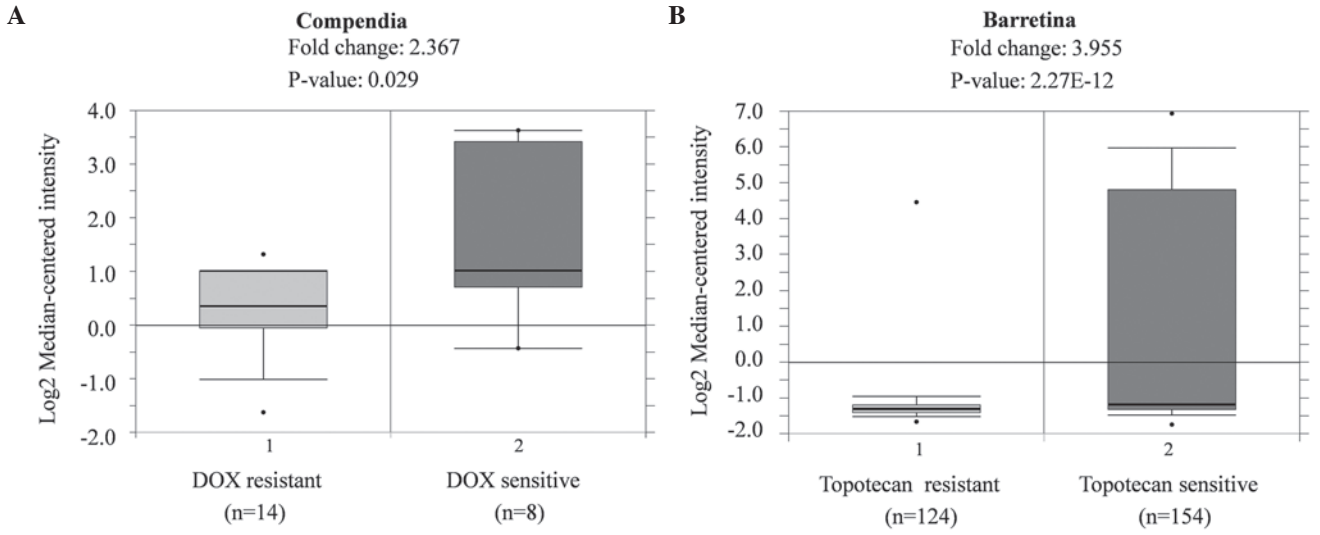

Figure 1. IK1/2 expression is associated with chemotherapy drug sensitivity. (A) Increased expression of IK1/2 in DOX-sensitive cell lines compared with DOX-resistant cells in the Compendia cell line (The Oncomine™ Platform; Life Technologies; Thermo Fisher Scientific, Inc.). (B) Increased expression of IK1/2 in topotecan-sensitive cell lines compared with topotecan-resisitant cells in the Barretina cell line (The Oncomine™ Platform). DOX, doxorubicin; IK, Ikaros.

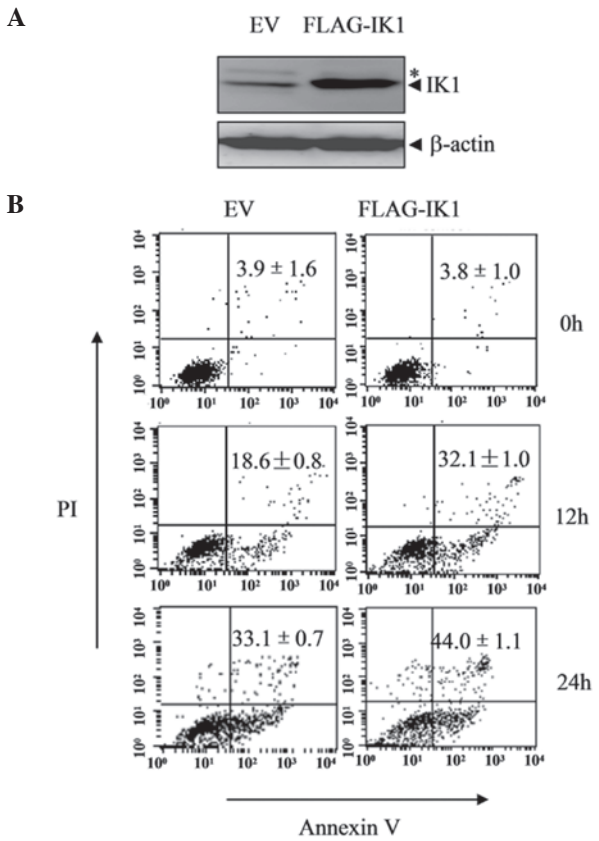

Figure 2. Overexpression of IK1 accelerates DOX-induced apoptosis. (A) NB4$^{FLAG\text{-}IK1}$ and NB4^{EV} cells were established as described in Materials and methods. Cell lysates from NB4$^{FLAG\text{-}IK1}$ and NB4^{EV} cells were immunoblotted with antibodies against IK1 and β-actin. *Non-specific band. (B) NB4^{EV} and NB4$^{FLAG\text{-}IK1}$ cells were treated with 0.4 μM DOX for 12 or 24 h, and the % of apoptotic cells was determined by Annexin V/propidium iodide staining. IK1, full-length Ikaros; DOX, doxorubicin; PI, propidium iodide; EV, empty vector.

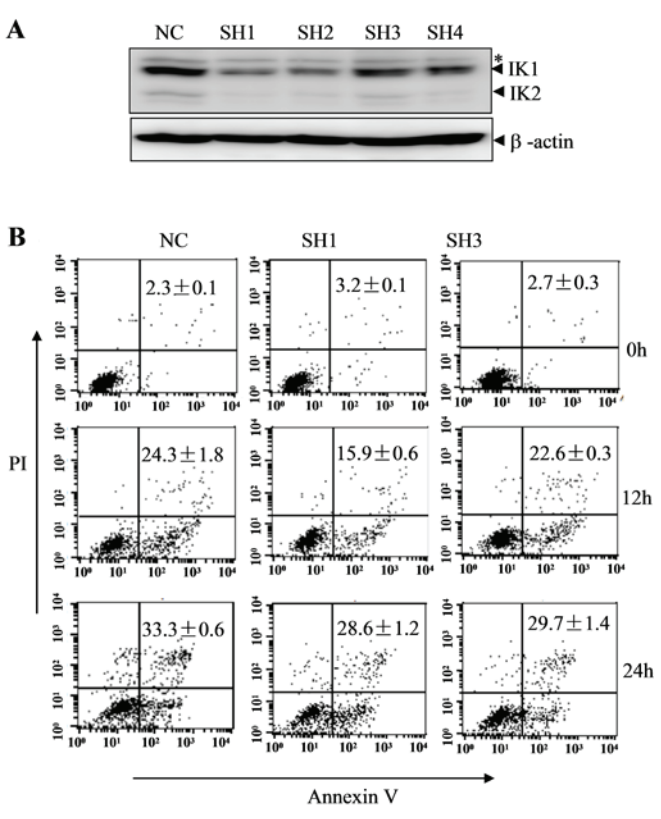

Figure 3. Knockdown of IK1 attenuates DOX-induced apoptosis. (A) NB4 cells were infected with pSIREN-RetroQ viruses expressing NC-shRNA and IK1-shRNAs (SH1, SH2, SH3 and SH4), lysed and subjected to western blot analysis using antibodies against IK1 and β-actin. *Non-specific band. (B) NB4^{NC}, NB4^{SH1} and NB4^{SH3} cells were treated with 0.4 μM DOX for 12 or 24 h, and the percentage of apoptotic cells was determined by Annexin V/propidium iodide staining. IK1, full-length Ikaros; DOX, doxorubicin; PI, propidium iodide; NC, negative control; shRNA, small hairpin RNA.

then treated with 0.4 μM DOX for 12 and 24 h. Apoptosis was evaluated by Annexin V/PI staining. Upon DOX treatment, a significant increase in apoptosis was observed in IK1-overexpressing NB4$^{FLAG\text{-}IK1}$ cells compared with NB4^{EV} cells at 12 h (32.1±1.0% vs. 18.6±0.8%, P<0.05) and 24 h (44.0±1.1% vs. 33.1±0.7%, P<0.05) (Fig. 2B).

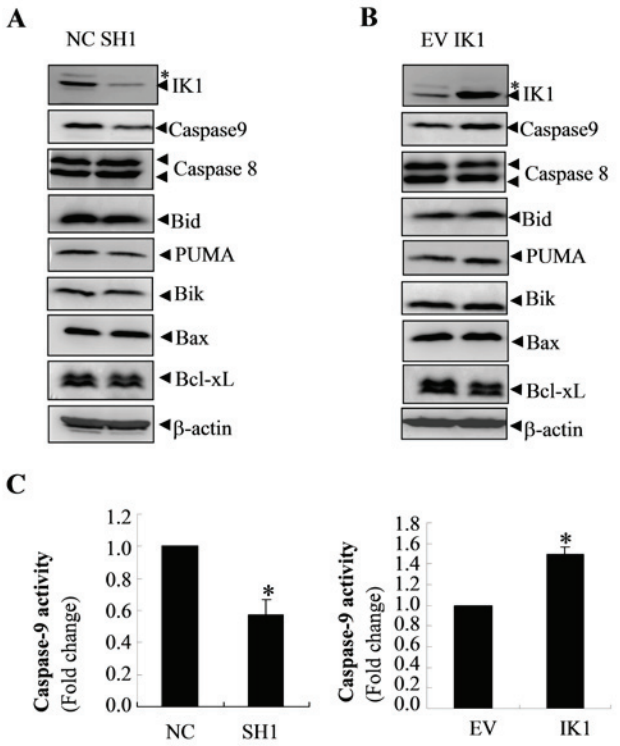

Figure 4. IK1 expression regulates the expression of caspase-9. (A) Effects of knocking down IK1 expression on the expression of apoptosis-associated proteins. Equal amounts of cell lysates from $NB4^{NC}$ or $NB4^{SH1}$ cells were immunoblotted with the indicated antibodies. *Non specific band. (B) Effects of IK1 overexpression on the expression of apoptosis-associated proteins. Equal amounts of cell lysates from $NB4^{EV}$ and $NB4^{FLAG\text{-}IK1}$ cells were immunoblotted with the indicated antibodies. The upper caspase-8 and Bcl-xL bands are predicted to be modified proteins (i.e., phosphorylated or acetylated). *Non-specific band. (C) Cells were treated with 0.4 μM doxorubicin for 12 h, and caspase-9 activity was measured using Ac-LEHD-pNA substrate. The enzyme-catalyzed release of pNA was measured at 405 nm, and fold-changes were calculated. *P<0.05 vs. control. EV, empty vector; IK1, full-length Ikaros; NC, negative control; SH, small hairpin; PUMA, p53 upregulated modulator of apoptosis; Bcl-xL, B-cell lymphoma-extra large; Bik, Bcl-2-interacting killer; Bax, Bcl-2 associated X protein; Bid, BH3 interacting-domain.

Knockdown of IK1 attenuates DOX-induced apoptosis in leukemic cells. The effects of knocking down the expression of endogenous IK1 in NB4 cells were also examined. NB4 cells were infected with pSIREN-RetroQ retrovirus expressing NC-shRNA or four different shRNAs against IK1 (SH1, SH2, SH3 and SH4) (Fig. 3A). While the expression of IK1-SH1 and IK1-SH2 shRNAs significantly inhibited IK1 expression, the expression of IK1-SH3 and IK1-SH4 shRNAs had minimal effects on IK1 expression in NB4 cells (Fig. 3A). Stable pools of $NB4^{NC}$, $NB4^{SH1}$ and $NB4^{SH3}$ cells were treated with 0.4 μM DOX and subjected to apoptosis analysis using Annexin-V/PI staining. Upon DOX treatment, a significant decrease in apoptosis was observed in $NB4^{SH1}$ cells compared with $NB4^{NC}$ cells at 12 h (15.9±0.6% vs. 24.3±1.8%, P<0.05) and 24 h (28.6±1.2% vs. 33.3±0.6%, P<0.05) (Fig. 3B). However, no significant difference in the rates of apoptosis was observed between $NB4^{SH3}$ and $NB4^{NC}$ cells, which is consistent with the fact that IK1-SH3 shRNA did not inhibit IK1 expression in NB4 cells (Fig. 3A).

IK1 expression specifically regulates the expression of the pro-apoptotic protein caspase-9 in leukemic cells. To investigate how IK1 expression increases DOX-induced apoptosis, the expression of various apoptosis-related proteins in $NB4^{EV}$, $NB4^{FLAG\text{-}IK1}$, $NB4^{NC}$ and $NB4^{SH1}$ cells was examined by western blot analysis. Although the expression of almost all the proteins examined, including caspase-8, Bid, PUMA, Bik, Bax and Bcl-xL, was not affected, the expression of pro-caspase-9 was inhibited when IK1 was knocked down, and enhanced when IK1 was overexpressed (Fig. 4A and B). Following treatment with 0.4 μM DOX for 12 h, caspase-9 activity increased in $NB4^{FLAG\text{-}IK1}$ cells and decreased in $NB4^{SH1}$ cells compared with control cells (Fig. 4C).

Discussion

In the present study, it was demonstrated that IK1 expression accelerates DOX-induced apoptosis and increases the expression of the caspase-9 protein in AML cells. Besides their roles in the differentiation of specific lineages of hematopoietic cells, the Ikaros family members were recently reported to be involved in the apoptosis of hematopoietic cells (19). For example, bone marrow erythroid cells derived from Ikaros-null mice are more resistant to apoptosis than those from wild-type mice (19). Aiolos, another member of the Ikaros family, has also been reported to control T-cell death by upregulating the expression of Bcl-2 (20), which is consistent with the present observation that IK1 regulates the expression of the apoptosis-associated protein caspase-9.

Apoptosis is controlled by a balanced expression of pro-apoptotic and anti-apoptotic proteins, including the pro-apoptotic proteins PUMA, Bax, Bid, Bik, caspase-8 and caspase-9 (21-23) and the anti-apoptotic protein Bcl-xL (24,25). To investigate how IK1 contributes to apoptosis, the present study examined the effect of IK1 on the expression of these proteins. Notably, only pro-caspase-9, an initiator caspase (26), was significantly upregulated with the expression of IK1 (Fig. 4).

The aspartic acid specific protease caspase-9 plays a central role in the mitochondrial or intrinsic apoptotic pathway, which is engaged in response to numerous apoptotic stimuli (27). Activation of stress signaling pathways, including Jun amino-terminal kinase/stress-activated protein kinase, causes the release of cytochrome *c* from the mitochondria and the activation of the apoptosome via apoptotic protease activating factor 1 activation, which in turn cleaves the pro-enzyme form of caspase-9 into its active form (26). Once activated, caspase-9 cleaves and activates the effector caspases-3 and -7 to execute apoptosis (26,28). Ikaros could increase the protein levels of pro-caspase-9, which may in turn increase the sensitivity of cells to DOX-induced cell death. It is worth noting that the messenger RNA level of caspase-9 was not changed with Ikaros expression (data not shown), suggesting that Ikaros may interfere with the translation or stability of caspase-9. Further studies are required to uncover the mechanism by which IK1 regulates the protein expression of caspase-9.

In summary, the present study revealed that IK1 expression sensitizes leukemic cells to DOX-induced apoptosis and upregulates caspase-9 protein. IK1 may be a potential biomarker to predict the response to DOX treatment in a subset of leukemia patients.

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