Upregulation of AIOLOS induces apoptosis and enhances etoposide chemosensitivity in Jurkat leukemia cells

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Received September 24, 2014; Accepted November 26, 2014

DOI: 10.3892/or.2014.3677

Abstract. T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplastic disorder of immature hematopoietic precursors committed to T-cell lineage. T-ALL accounts for ~15% of pediatric ALL cases and is prone to early relapse. With new and improved treatment protocols, the prognosis of T-ALL has improved particularly in children; however, the outcome of relapsed T-ALL cases remains poor. The AIOLOS gene is necessary to control lymphocyte differentiation and may be a potential target of T-ALL therapy. In the present study, Jurkat cells were divided into three groups: untransfected (UT) control, lentiviral vector control (Lenti-Mock) and AIOLOS-overexpressing (Lenti-AIOLOS) groups. Lenti-AIOLOS Jurkat cells were constructed by lentiviral transduction; cell cycle analysis, apoptosis and cytotoxicity assays were then performed to evaluate the effects of AIOLOS on cell cycle distribution, apoptosis and cell chemosensitivity to etoposide of Jurkat cells in vitro. Moreover, the expression levels of genes associated with apoptosis and cell cycle were investigated by quantitative reverse transcription-polymerase chain reaction. Results showed that the percentage of Jurkat cells in the G0/G1 phase increased from 71.5 (UT) to 85.4% (Lenti-AIOLOS; P<0.05), yet the percentage of cells in the S-phase decreased from 15.1 (UT) to 11.6% (Lenti-AIOLOS; P<0.05). The percentage of total apoptotic cells was significantly increased in the AIOLOS-transfected Jurkat cells (21.93%) compared with this percentage in the Lenti-Mock (13.35%) or the UT group (13.30%; P<0.05). Consistent with these results, AIOLOS overexpression induced P21 and P27 upregulation and CCND3 and SKP2 downregulation. Furthermore, AIOLOS overexpression synergistically increased the cytotoxic effects of etoposide and downregulated NF-κB expression. Our findings revealed that lentivirus-mediated AIOLOS overexpression in Jurkat cells induced cell apoptosis, arrested the cell cycle at the G0/G1 phase, and synergistically increased the sensitivity of Jurkat cells to etoposide by inhibiting NF-κB activity.

Introduction

Acute lymphoblastic leukemia (ALL), which can be divided into B-lineage ALL (B-ALL) and T-lineage ALL (T-ALL) (1), is one of the most common forms of pediatric malignancies originating from lymphoid precursors (2). T-ALL accounts for 10 to 15% of pediatric cases and 25% of adult ALL cases (3). With current intensified multi-agent chemotherapy protocols, the 5-year event-free survival (EFS) of children with T-ALL has reached 70-75% (4). However, these therapies are highly toxic. Moreover, relapsed patients often develop resistance to chemotherapy and experience very poor prognosis (5). Therefore, the mechanisms that cause relapses and chemoresistance in T-ALL should be understood to identify novel molecular targets and design effective therapies.

T-ALL is an aggressive blood malignancy originating from T-cell progenitors in the thymus. Genes encoding transcriptional regulators of T-cell development and maturation are potential targets of T-ALL therapy. The IKAROS family, an important group of transcription factors in hematopoietic lineages, encodes a group of zinc-finger DNA-binding proteins essential for normal lymphocyte development (6-8). AIOLOS is an IKAROS family member that was first described in committed lymphoid progenitors and was strongly upregulated as these progenitors become restricted into T- and B-lymphoid pathways (7). Previous studies have shown that AIOLOS controls T and B lymphocyte apoptosis by regulating Bcl-xL (9,10) and also regulates cell death in T cells by controlling Bcl-2 expression and cellular localization (11). Deregulated AIOLOS expression has been associated with leukemia and lymphoma in human patients (12-15).

In the present study, a lentiviral system was used to stably overexpress the AIOLOS gene in Jurkat cells, a T-ALL cell line, and to examine apoptosis, cell cycle distribution and cell chemosensitivity to etoposide in vitro. Our results demonstrated that AIOLOS overexpression in Jurkat cells induced cell apoptosis, arrested the cell cycle at the G0/G1 phase, and synergistically increased the sensitivity of Jurkat cells to etoposide by inhibiting NF-κB activity.

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Key words: AIOLOS, acute lymphocytic leukemia, cell apoptosis, cell cycle, overexpression, chemosensitivity
Materials and methods

Cell lines and cell culture. Two T-ALL cell lines (Jurkat and Molt-4) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in standard culture medium [RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco, Grand Island, NY, USA)] at 37°C in 5% CO₂ in air. The cells were subcultured after 24-48 h with an initial concentration of 4x10⁴ cells/ml and were used at the logarithmic phase in all of the experiments. Peripheral blood lymphocytes collected from consenting normal healthy children were used as control cells. The experimental design and protocols were approved by the Ethics Committee of Qilu Hospital. Informed consent was obtained for all participants prior to enrollment.

Lentiviral vector construction, virus production and transfection. The lentiviral vectors pWPT-PURO-GFP-AIOLOS (Lenti-AIOLOS) and pWPT-PURO-GFP (Lenti-Mock) were constructed and identified as previously described (16). Viral concentrate was diluted in Polybrene (5 µg/ml; Sigma, St. Louis, MO, USA) to infect Jurkat cells at a multiplicity of infection (MOI) of 100. Successful transduction was confirmed by visualizing enhanced green fluorescent protein (EGFP; included in the pWPT-PURO-GFP vector) after 4 days. The cells were maintained and allowed to grow for another 3-5 days; the AIOLOS expression level was confirmed by qRT-PCR and western blot analysis. Virus-infected cells were selected with 8 µg/ml puromycin (Invitrogen, Carlsbad, CA, USA) with SYBR-Green I dye (Toyobo, Osaka, Japan). Primers (Table I) were obtained from Bioasi Co., Ltd., Shanghai, China. Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystems). Results are expressed as fold change relative to the cDNA of the UT group. Data were also analyzed using Sequence Detection Software 1.4 (Applied Biosystems). Reported data are representative of at least three independent experiments.

Protein extraction and western blot analysis. Jurkat cells were harvested and washed twice with cold phosphate-buffered saline (PBS). Total and nuclear protein fractions were extracted using RIPA lysis buffer and nuclear and cytoplasmic protein extraction kit (both from Beyotime Institute of Biotechnology, Jiangsu, China), respectively, according to the manufacturer's protocols. Total AIOLOS and nuclear NF-κB expression levels were analyzed. The proteins were quantified using the Bradford protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of proteins were loaded in each well of 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride microporous membranes (Millipore, Bedford, MA, USA). Membranes containing the transferred proteins were blocked with PBS containing 0.1% Tween-20 (PBS-T) and 5% skim milk for 1 h at room temperature. After three washes with PBS-T, the membranes were incubated with antibodies against AIOLOS (1:1,000), NF-κB (1:1,000), GAPDH (1:1,000) or lamin B (1:1,000) (all from Abcam Inc., Cambridge, MA, USA). Membranes containing the transferred proteins were blocked with PBS containing 0.1% Tween-20 (PBS-T) and 5% skim milk for 1 h at room temperature. After three washes with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. After three final washes in PBS-T and two in PBS, chemiluminescence was detected using an ECL Plus immunoblotting detection system (Beyotime Institute of Biotechnology).

Cell cycle and apoptosis assay. Jurkat cells were obtained 9 days after transfection; cell cycle and apoptosis were detected using a Muse™ cell cycle reagent, Muse™ Annexin V and a dead cell kit (all from Millipore) according to the manufacturer's instructions. Assay results were obtained using a Muse™ cell analyzer (Millipore). Cell cycle results are expressed as the percentage of cells in each cell cycle phase. Cell apoptosis results are expressed as the percentage of apoptotic cells. Error bars represent standard errors of the means (SEM).

Table I. Primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
</tr>
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<tbody>
<tr>
<td>AIOLOS</td>
<td>F: 5'-GCCCTTCAAGTGGTTCACCAA-3'</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCCCTTCAACGCAGCAAATAT-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-GGACATCCGCCAGAACCTTGTA-3'</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCATCTGCTTCGCGCAAATTC-3'</td>
<td></td>
</tr>
<tr>
<td>BCL-2</td>
<td>F: 5'-GCTGAGAAGACGAGTACGA-3'</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCTCTGCGACAGCTTTATATGGA-3'</td>
<td></td>
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<tr>
<td>BAX</td>
<td>F: 5'-CTTGTGCTGCCAGGGTGAGT-3'</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCAGAGAATGCTGTTGAACCT-3'</td>
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<tr>
<td>CCND3</td>
<td>F: 5'-GAGGTGCAATCTCCTCTCCG-3'</td>
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<td></td>
<td>R: 5'-TCACATACTCCTCTGTCGGAAT-3'</td>
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<tr>
<td>P21</td>
<td>F: 5'-TGGGGAAGTCGATTCCTTG-3'</td>
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<td></td>
<td>R: 5'-GTTCTGACATGGCGCCTCCT-3'</td>
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</tr>
<tr>
<td>P27</td>
<td>F: 5'-TCCGCTAATCTCGAGGACA-3'</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAAGGAATGCTCGTTGACGAG-3'</td>
<td></td>
</tr>
<tr>
<td>SKP2</td>
<td>F: 5'-AGCTCCTGCAAGTTAATGCAAC-3'</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTGTGCTGGAATCCATCCCATCCC-3'</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
Cytotoxicity assay. The effect of AIOLOS overexpression on the sensitivity of the Jurkat cell line to etoposide (Sigma, St. Louis, MO, USA) was evaluated using the CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) assay. The experiment was divided into six groups: etoposide, Lenti-AIOLOS, Lenti-Mock, Lenti-AIOLOS and etoposide, Lenti-Mock and etoposide, and etoposide blank control. The cells treated with only 0.9% NS were used as etoposide blank controls. In brief, the cells were cultivated at a density of 2x10^4 cells/well in 96-well culture plates. At 96 h after transfection, the cells were treated with various concentrations of etoposide (0, 2.5, 10 and 40 µM). After 48 h of culture, the cytotoxicity of the treatments was determined using WST-8 dye (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. The generated formazan was determined using a Model 450 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA) at an optical density of 570 nm (OD570) to determine cell viability. Survival rate (SR) was calculated using the following equation: SR (%) = (A Test/A Control) x 100%, where A is the absorbance value.

Statistical analysis. All of the experiments were performed at least thrice. Prism 5.0 (GraphPad Software) was used for statistical analysis. P-values were obtained from two-tailed tests and were considered to indicate a statistically significant result at P<0.05.

Results

AIOLOS protein expression in the Jurkat cells is lower than that in normal child peripheral blood lymphocytes. To evaluate whether or not aberrant AIOLOS expression is observed in T-ALL, we quantified AIOLOS expression levels by qRT-PCR and western blot analysis in the T-ALL cell lines and normal child peripheral blood lymphocytes. The mRNA levels of AIOLOS were lower in both T-ALL cell lines than the levels in the normal control (P<0.05; Fig. 1A). Western blot analysis results were consistent with those of qRT-PCR (Fig. 1B). The Jurkat cell line was chosen for a series of functional experiments.

AIOLOS is overexpressed by stable transfection in Jurkat cells. The Jurkat cells were infected with the lentiviral vector pWPT-PURO-GFP-AIOLOS. As a control sample, the Jurkat cells were either infected with a lentiviral vector expressing GFP or not. At 96 h after the Jurkat cells were infected, infection efficiency was detected using a fluorescence microscope. More than 90% of the cells emitted bright green fluorescence, which represented high infection efficiency. (Fig. 2A-F). Cells were maintained and allowed to grow for 3-5 days. The mRNA and protein expression levels of AIOLOS in the Jurkat cells of the three groups were determined by qRT-PCR and western blot analysis at 7 days after infection. qRT-PCR results demonstrated that the mRNA expression level of AIOLOS in the Jurkat cells of the Lenti-AIOLOS group was markedly increased compared with that of the Lenti-Mock and the UT group. This finding was consistent with the increase in AIOLOS protein expression (Fig. 2G and H). No significant difference between the cells of the Lenti-Mock and the UT group was observed.

These results revealed that the stable transfection of pWPT-PURO-GFP-AIOLOS upregulated AIOLOS expression in the Jurkat cells.

AIOLOS overexpression arrests the cell cycle in the G1 phase in Jurkat cells. The cell cycle distribution of the Jurkat cells in the Lenti-AIOLOS, Lenti-Mock and UT groups was characterized by fluorescence-activated cell sorting (FACS) analysis 9 days after transfection. The percentage of Jurkat cells in the G0/G1 phase increased from 71.5 (UT) to 85.4% (Lenti-AIOLOS; P<0.05; Fig. 3), and the percentage of S-phase cells was decreased from 15.1 (UT) to 11.6% (Lenti-AIOLOS; P<0.05). The difference between Jurkat cells of the Lenti-AIOLOS and the UT group in the G2/M phase was significant (2.0 vs. 6.8%; P<0.05). No significant difference between the Lenti-Mock and the UT Jurkat cells was observed (P>0.05). These data revealed that the upregulation of AIOLOS expression arrested Jurkat cells at the G0/G1 phase.

AIOLOS overexpression induces apoptosis in Jurkat cells. To determine whether or not AIOLOS overexpression results in apoptosis in the Jurkat cells, we used the Muse™ Annexin V and the dead cell kit and determined the changes in cell apoptosis on day 9. The percentage of total apoptotic cells was significantly increased in the AIOLOS-transfected Jurkat cells (21.93%) compared with the percentage in the Lenti-Mock (13.35%) or UT group (13.30%; P<0.05; Fig. 4). In particular, the difference between AIOLOS-transfected Jurkat and UT Jurkat cells in regards to the percentage of early apoptotic cells was minimal (6.46 vs. 4.81%; P>0.05). The difference between the cell groups in regards to the percentage of late apoptotic cells was significant (14.55 vs. 9.05%; P<0.05). These data revealed that AIOLOS overexpression suppressed cell apoptosis in the Jurkat cells.

AIOLOS affects the expression levels of apoptosis- and cell cycle-related genes in Jurkat cells. To investigate the mechanism by which AIOLOS blocks the cell cycle and promotes the apoptosis of Jurkat cells, we examined the expression of genes associated with apoptosis and the cell cycle in response to AIOLOS overexpression by performing
Figure 2. Determination of lentiviral transduction efficiency and the AIOLOS expression level in Jurkat cells of the Lenti-AIOLOS group. (A-F) Lentiviral transduction efficiency in the Jurkat cells. Transduction efficiency was estimated 4 days after infection at an MOI of 100. GFP expression was observed by (A, C and E) fluorescence microscopy or (B, D and F) light microscopy. (A and B) Untransfected (UT) group. (C and D) Lentiviral vector (Lenti-Mock) group. (E and F) AIOLOS-transfected (Lenti-AIOLOS) group. Scale bar, 200 µm. (G) qRT-PCR results showed relative AIOLOS transcript levels in the Lenti-AIOLOS, Lenti-Mock and UT groups. β-actin was used as the internal control. (H) Western blot analysis showed that AIOLOS was expressed in Jurkat cells of the three groups. GAPDH served as the loading control. Error bars indicate SEM (n=3 experiments). *P<0.05 Lenti-AIOLOS vs. Lenti-Mock. MOI, multiplicity of infection.

Figure 3. Effects of AIOLOS on the cell cycle distribution in Jurkat cells. (A-C) Flow cytometric analysis results demonstrated that the fraction of G0/G1 phase cells was increased and the proportion of S and G2/M phase cells was decreased in the Jurkat cells with increased AIOLOS expression. (D) The proportion of G0/G1 cells was increased and the proportions of S and G2/M phase cells were decreased in Jurkat cells of the Lenti-AIOLOS group compared with those of the Lenti-Mock group (*P<0.05, Lenti-AIOLOS vs. Lenti-Mock). Error bars represent SEM (n=3 experiments).
The mRNA expression levels of *P21* and *P27* were significantly increased in the AIOLOS-transfected Jurkat cells compared with these levels in the UT and Lenti-Mock Jurkat cells (Fig. 5); this result was consistent with that of the cell cycle assay. In addition, *CCND3*, one of the known cell cycle–related genes and *SKP2*, a typical representative of cell cycle negative regulators, were downregulated in the AIOLOS-transfected Jurkat cells. Furthermore, *BCL-2* expression in the Lenti-AIOLOS group was significantly decreased in Jurkat cells of the Lenti-AIOLOS group compared with those of the control groups; *P21* (*P<0.05) and *P27* (*P<0.05) expression levels were downregulated. Minimal change was observed in *BAX* expression (*P>0.05). β-actin was used as the internal control. Error bars represent SEM (n=3 experiments).

Figure 5. Effects of AIOLOS on the expression levels of apoptosis- and cell cycle–related genes in Jurkat cells. qRT-PCR results showed the relative transcript levels of *BCL-2*, *BAX*, *CCND3*, *P21*, *P27* and *SKP2* in Jurkat cells of the three groups. *BCL-2* (*P<0.05), *CCND3* (*P<0.05) and *SKP2* (*P<0.05) were markedly reduced in Jurkat cells of the Lenti-AIOLOS group compared with those of the control groups; *P21* (*P<0.05) and *P27* (*P<0.05) expression levels were downregulated. Minimal change was observed in *BAX* expression (*P>0.05). β-actin was used as the internal control. Error bars represent SEM (n=3 experiments).

qRT-PCR (Fig. 5). The mRNA expression levels of *P21* and *P27* were significantly increased in the AIOLOS-transfected Jurkat cells compared with these levels in the UT and Lenti-Mock Jurkat cells (Fig. 5); this result was consistent with that of the cell cycle assay. In addition, *CCND3*, one of the known cell cycle–related genes and *SKP2*, a typical representative of cell cycle negative regulators, were downregulated in the AIOLOS-transfected Jurkat cells. Furthermore, *BCL-2* expression in the Lenti-AIOLOS group was significantly decreased in Jurkat cells of the Lenti-AIOLOS group compared with those of the control groups; *P21* (*P<0.05) and *P27* (*P<0.05) expression levels were downregulated. Minimal change was observed in *BAX* expression (*P>0.05). β-actin was used as the internal control. Error bars represent SEM (n=3 experiments).

Figure 6. Effects of Lenti-AIOLOS on the sensitivity of the Jurkat cell line to etoposide. (A) Single etoposide treatment induced cytotoxicity in a dose-dependent manner (0, 2.5, 10 and 40 µM). (B) The cytotoxic effects of Lenti-AIOLOS, Lenti-Mock, etoposide and their combined effects were determined by CCK-8 assay, as described in Materials and methods. Combined therapy further reduced the cell survival rate compared with Lenti-AIOLOS or etoposide monotherapy. (C) Western blot analysis showed the decreased NF-κB expression in Jurkat cells of the Lenti-AIOLOS group. Data represent means ± S eM (n=4); *P<0.05.
AIOLOS was successfully overexpressed in Jurkat cells. Cells were successfully transduced with the lentivirus, and a constant increase in AIOLOS expression at the mRNA and protein levels was observed. Our findings showed that Lenti-AIOLOS pretreatment synergistically increased the sensitivity of Jurkat cells to etoposide. This finding revealed that AIOLOS overexpression could sensitize leukemia cells in response to etoposide. Cell cycle and apoptosis assay results indicated that AIOLOS overexpression may play a critical role in drug resistance of leukemia cells. CCK-8 assay showed that Lenti-AIOLOS pretreatment synergistically increased the cytotoxic effect of etoposide. This finding revealed that AIOLOS overexpression could sensitize leukemia cells in response to etoposide. The exposure of leukemia cells to etoposide initiates both signaling pathways of apoptosis by activating multiple caspases (25, 26). T-ALL is associated with NF-κB activity. To test this hypothesis, we performed immunoblotting experiments for nuclear NF-κB activity. As expected, the results of such experiments clearly indicated that AIOLOS overexpression indeed inhibited NF-κB activity in Jurkat cells. Therefore, AIOLOS overexpression may sensitize leukemia cells to etoposide by inhibiting NF-κB activity. Further studies should be conducted to verify this conclusion.

Discussion

As a member of the IKAROS family of zinc-finger proteins, the AIOLOS transcription factor, encoded by the IKZF3 gene, is necessary to control lymphocyte differentiation, proliferation and maturation. The AIOLOS transcription factor AIOLOS in the pathogenesis of T-ALL. To mimic the isoforms and cellular localizations of AIOLOS in T-cells, we constructed a plasmid pWPT-PURO-GFP-AIOLOS containing the entire AIOLOS coding sequence and performed lentiviral-mediated transduction in Jurkat cells to create a stable transfection cell line. qRT-PCR and western blot analysis revealed that Lenti-AIOLOS treatment caused a constant increase in AIOLOS expression at the mRNA and protein levels for 4 days. These results revealed that the Jurkat cells were successfully transduced with the lentivirus, and AIOLOS was successfully overexpressed in Jurkat cells.

Cell cycle assay results indicated that AIOLOS overexpression arrested the cell cycle of the Lenti-AIOLOS cells at the G0/G1 phase. To explore the potential mechanisms of AIOLOS in the Jurkat cell cycle, we analyzed the expression of cell cycle-related genes, including p21, p27, CCND3 and SKP2 by qRT-PCR. Skp2 functions as an oncoprotein, participates in many aspects of cancer progression by inducing p27 and p21 degradation (17, 18), and establishes a crosstalk with other major signaling pathways (19-21). A previous study reported that activation of the JAK2/STAT3 pathway enhances leukemogenesis (22). In addition, JAK2/STAT3 pathway inhibition was found to upregulate p27 and p21 expression (23). Consistent with these results, our findings showed that AIOLOS overexpression upregulated p27 and p21 expression and downregulated Skp2. This result revealed that AIOLOS may interact with the Skp2/p27/p21 pathway via JAK2/STAT3 signaling in Jurkat leukemia cells.

AIOLOS reportedly controls T-cell death by regulating the expression and localization of the anti-apoptotic molecule Bcl-2 (11), suggesting the possibility that apoptotic cell death evasion is a common mechanism by which IKAROS family proteins participate in leukemogenesis. In the present study, AIOLOS overexpression in Jurkat cells induced cell apoptosis. Considering that Bcl-2 family proteins play a critical role as promoters or inhibitors in the regulation of apoptosis (24), we investigated whether or not the disruption of apoptosis-related genes BCL-2 and BAX contributes to apoptosis induction of Jurkat cells by AIOLOS overexpression. Although no distinct changes were found in BAX, BCL-2 was downregulated, resulting in a low BCL-2/BAX ratio, which may be a possible reason for the increased apoptosis in the Jurkat cells. However, further research is required to explore the complete mechanism.

In summary, the present study is the first to explore the function of the transcription factor AIOLOS in regards to the biological behaviors of a human T-ALL cell line. The present study provides the basis for further research on the pathogenesis of T-ALL. Our results revealed that the upregulation of AIOLOS expression in Jurkat cells induced cell apoptosis and arrested the cell cycle at the G0/G1 phase. In addition, AIOLOS overexpression synergistically increased the sensitivity of Jurkat cells to etoposide by inhibiting NF-κB activity. However, the mechanism by which AIOLOS interacts with other regulators remains poorly understood. These potential genetic interactions should be characterized in future studies.

Acknowledgements

The present study was supported by Grants of the Shandong Province Natural Science Foundation (ZR2011HM007 and 2013GSF11812), the Innovation Fund Project of Shandong University (2014KY003-11), and the Scientific Research Fund of Shenzhen (JCYJ20140418115449178).
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