

Original Article

EDAG-1 promotes the proliferation of human T-acute lymphoblastic leukemia cells by activating MAPK/Erk and Akt signaling pathways

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Abstract: Embryonic development associated gene 1 (EDAG-1) is a novel gene identified from the EST bank of differentially expressed genes between the 4 months fetal liver and adult liver. Previous studies found that EDAG-1 differentially expressed in hematopoietic tissues, fetal liver and adult liver tissues. High EDAG-1 expression is also found in human T-cell acute lymphoblastic leukemia (T-ALL) cells and peripheral blood of patients with T-ALL, but its functional significance in T-ALL was unclear. This present study aimed to investigate how EDAG-1 is involved in T-ALL. Our results suggested that EDAG-1 was highly expressed in T-ALL cells and peripheral blood of patients with T-ALL. We found that knockdown of EDAG-1 could inhibit the proliferation of T-ALL cells, whereas overexpression of EDAG-1 reversed this change. Furthermore, we found that overexpression of EDAG-1 could activate the MAPK/Erk and AKT signal pathways. Our findings demonstrated that EDAG-1 should play an oncogenic role in T-ALL progression and silencing EDAG-1 might be a potential therapeutic approach for T-ALL.

Keywords: EDAG-1, T-acute lymphoblastic leukemia cells, MAPK/Erk, Akt

Introduction

T-ALL is the most commonly diagnosed leukemia, together with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) around the world [2]. T-ALL is an aggressive type of blood cancer that accounts for about 15% of pediatric and 25% of adult acute lymphoblastic leukemia (ALL) cases [3-5]. It is characterized clinically by low red blood cell counts, and abnormal white blood cells in the blood and bone marrow, which interrupt the immune response. Although the disease develops mostly in the thymus, it tends to spread throughout the body, including the central nervous system, complicating the therapeutic treatment of the disease. Today's multiagent chemotherapy protocols achieve an overall survival rate of 70% for children and 30-40% for adults below 60 years of age, and 10% above this age [6]. Historically, T-ALL was associated with a poor prognosis because of resistance to therapy and relapse, in particular bone marrow (BM) relapse. Therefore it is

important to understand the molecular mechanisms of T-ALL to identify novel molecular targets and design more specific therapies.

EDAG-1 was originally cloned by Yang et al [6]. This gene encodes a protein of 484 amino acids, and is highly homologous with mice Hemogen and rats RP59 [7]. Previous studies have shown that the expression of EDAG-1 was different in hematopoietic tissues, fetal liver and adult liver tissues [8]. Chen et al [9] confirmed that the expression level of EDAG-1 is extremely low in most differentiated tissues, such as the normal brain, heart, kidney, liver, lung, muscle, tonsil, mature white blood cells. In contrast, Li et al [10] found that the expression of EDAG-1 was high in adult bone marrow, most embryonic tissues and thyroid tumor tissue.

An, L.L. et al detected EDAG-1 mRNA expression in a panel of leukemia cell lines and the bone marrow mononuclear cells (BMMC) of 75 acute myeloid leukemia (AML) patients. They suggested that abnormal high expression of

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EDAG-1 was associated with leukemia and EDAG-1 should play an important role in the hematopoietic development and differentiation by regulating cell's proliferation and differentiation [11]. EDAG was thought to enhance the protein stability of NPM1 via binding to NPM1, which played a critical role in the anti-apoptosis of leukemia cells. [12, 13] Li, C.Y. et al [14] found that EDAG-1 had transcriptional activation activity, and abnormal activity of EDAG-1 could disrupt intracellular microenvironment [13]. However, the functions of EDAG-1 in T-ALL are currently unknown.

In this study, we confirmed that EDAG-1 was highly expressed in T-ALL cell line Jurkat and peripheral blood cells of patients with T-ALL compared with the peripheral blood cells of normal human. Overexpression of EDAG-1 could promote the proliferation of T-ALL cells. On the contrary, silencing EDAG-1 would inhibit the proliferation T-ALL cells. Furthermore, we studied the mechanism of EDAG-1 regulated the progression of T-ALL cells. We found that knockdown of EDAG-1 arrested cells in G0/G1 phase and overexpression of EDAG-1 could increase the cell percentage of T-ALL cells in S phase. By performing Western blotting, we confirmed that EDAG-1 promoted proliferation of the T-ALL cells by activating the MAPK/Erk and AKT signal pathways. Thus, our results might provide a new therapeutic target for the treatment of T-ALL.

Materials and methods

Patient samples and cell culture

Jurkat cells were obtained from Cell Bank of Shanghai Institute of Biochemistry Cell Biology, Chinese Academy of Sciences. T-ALL cells Jurkat, peripheral blood cells of patients with T-ALL and peripheral blood cells of normal human were cultured in complete medium with 10% fetal bovine serum purchased from GIBCO and incubated at 37°C in a humidified air atmosphere containing 5% CO₂.

Western blotting analysis

The expression of EDAG-1 protein was detected by Western blotting. Western blot analysis was performed as described before [15]. Briefly, cells were lysed in 200 µl lysis buffer (0.5 M Tris-HCl, pH6.8, 2 mM EDTA, 10% glycerol, 2%

SDS, and 5% β-mercaptoethanol). Protein extracts (20 µg) were electrophoresed on 10% SDS polyacrylamide gels (30% Acrylamide, 1.5 M Tris pH8.8, 10% SDS, 10% Ammonium Persulfate, TEMED) and then transferred to polyvinylidene fluoride membranes (PVDF, Millipore, Billerica, MA, USA). Primary antibodies used in this study were at the following concentrations: EDAG-1 (Santa Cruz) 1:2000, β-actin (Santa Cruz) 1:2000, Erk (CST) 1:2000, p-Erk (CST) 1:2000, AKT (CST) 1:2000, p-AKT (CST) 1:2000. Blots were quantified by densitometry, and normalized by β-actin.

Cell transfection

The retroviral vector RNAi-Ready pSIREN-RetroQ was purchased from BD. EDAG-1/siRNA was synthesized by Gemma Shanghai. The sense strand was 5'-AUAAGGAUGGCCU-AAAGATT-3'; the anti-sense strand was 5'-UCUUUAGGCACAUCCUUUUUUT-3. The Negative control sequences were 5'-UUCUCCGAA-CGUGUCACGUTT-3; 5'-ACGUGACACGUUCGGAGATT-3' [9]. siRNA was transfected into Jurkat cells according to manufacturer's instruction. The overexpression plasmid vector of EDAG-1 (pcDNA3.1 carrying the sequences of EDAG-1) was synthesized in Life Technology (Invitrogen, USA). The empty vector, pcDNA3.1, was used as a negative control. After transfection, total protein was extracted from 1×10⁶ cells to test the effectiveness of transfection.

RNA extraction and real-time PCR

Total RNA was isolated from the tissue samples or the transfected cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. PCR products were analyzed on 1% agarose gels. The primers used for PCR amplification were: EDAG-1 sense, 5'-AC-ACCTCATTCTGAAGAC-3'; EDAG -1 antisense, 5'-GGGGTACCTAAAACAAAACATAACTATAG-3'; β-actin sense, 5'-GTGGGGCGCCCCAGGCACCA-3'; β-actin antisense, 5'-CTCCTTAATGTCA-CGCACGATTT-3' [15].

Cell cycle analysis

Cell cycle analysis was performed with PI/RNase staining solution (Tian-jin Sungene Biotech Co., Ltd., China). 1×10⁶ Jurkat cells without IL-3 were collected and then fixed in ethanolat (70%) at 4°C overnight. After washing

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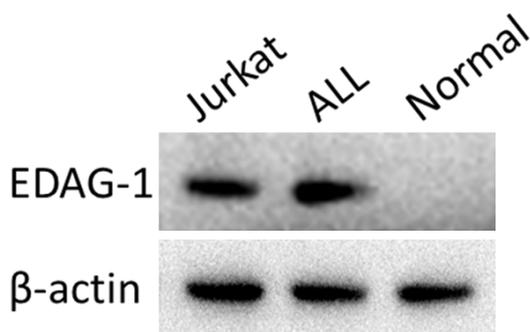


Figure 1. Basal expression of EDAG-1 in Jurkat cells, patients with T-ALL and healthy people. There was an original high expression of EDAG-1 in T-ALL cells Jurkat and peripheral blood of patients with T-ALL, and there was almost no expression of EDAG-1 in the peripheral blood from normal people.

with PBS, cells were permeabilized with 100 μ l RNAase in PBS for 30 min at 37°C in the absence of light, and then cells were stained with 400 μ l of propidium iodide (Sigma, US) for 30 min. The cell-cycle phases were analyzed by flow cytometry system (Beckman Coulter, Indianapolis, IN) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data were obtained in three independent experiments and analyzed by FlowJo 7.6 software.

MTT assay

The proliferation assay of the T-ALL Jurkat cells was performed using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, USA). Cells were grown in a 96-well plate and the cell density was adjusted to 1×10^4 /well. The plates were incubated for 24 h in a humidified atmosphere containing 5% CO₂ at 37°C. Then the cells were incubated in 0.1 mg/ml MTT for 4 h and lysed in dimethyl sulfoxide (DMSO) in a humidified atmosphere containing 5% CO₂ at 37°C for 10 min. The absorbance in each well was calculated at 490 nm by using a microplate reader (Bio-Rad, CA). Each experiment was performed in triplicate wells and repeated three times.

Statistical analysis

Each experiment was repeated at least three times. All data were summarized and represented as mean \pm standard deviation (SD). Comparison between different groups were

determined using Student's t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni t-tests. Statistical analyses were performed by SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered as statistically significant. *indicates statistical difference with $P < 0.05$, **indicates statistical difference with $P < 0.01$ and ***indicates statistical difference with $P < 0.001$.

Results

EDAG-1 is highly expressed in T-ALL cells and peripheral blood cells of patients with T-ALL

The expression of EDAG-1 in T-ALL cells Jurkat, peripheral blood cells of patients with T-ALL and peripheral blood cells of normal human was confirmed by Western blot. As shown in **Figure 1**, the expression of EDAG-1 was at a high level in T-ALL cells Jurkat and peripheral blood of patients with T-ALL compared with the level of peripheral blood cells of normal human. This result indicated that EDAG-1 should play critical roles in T-ALL progression.

Overexpression of EDAG-1 promotes the proliferation of T-ALL cells

Overexpression experiment and MTT assay were performed to investigate the role of EDAG-1 in the proliferation of T-ALL cell Jurkat. Infection was observed to be efficient at 96 h, and over 80% Jurkat cells were presented to be GFP positive in both pcDNA3.1-EDAG-1 and empty vector groups (**Figure 2A**). We found that the mRNA expression, protein level of EDAG-1 and the proliferation rate of T-ALL cells transfected with EDAG-1 overexpression plasmid (pcDNA3.1- EDAG-1) were all significantly higher than the negative control group (empty vector) (**Figure 2B**, *** $P < 0.001$; **Figures 2C, 4A**, *** $P < 0.001$). These results indicated that overexpression of EDAG-1 could promote the proliferation of T-ALL cells.

Knockdown of EDAG-1 inhibits the proliferation of T-ALL cells

Meanwhile, knockdown experiment and MTT assay were also performed to investigate the role of EDAG-1 in T-ALL cell Jurkat proliferation. Infection was observed to be efficient at 96 h, and over 80% Jurkat cells were presented to be GFP positive in both Lv-shNC and Lv-shEDAG-1

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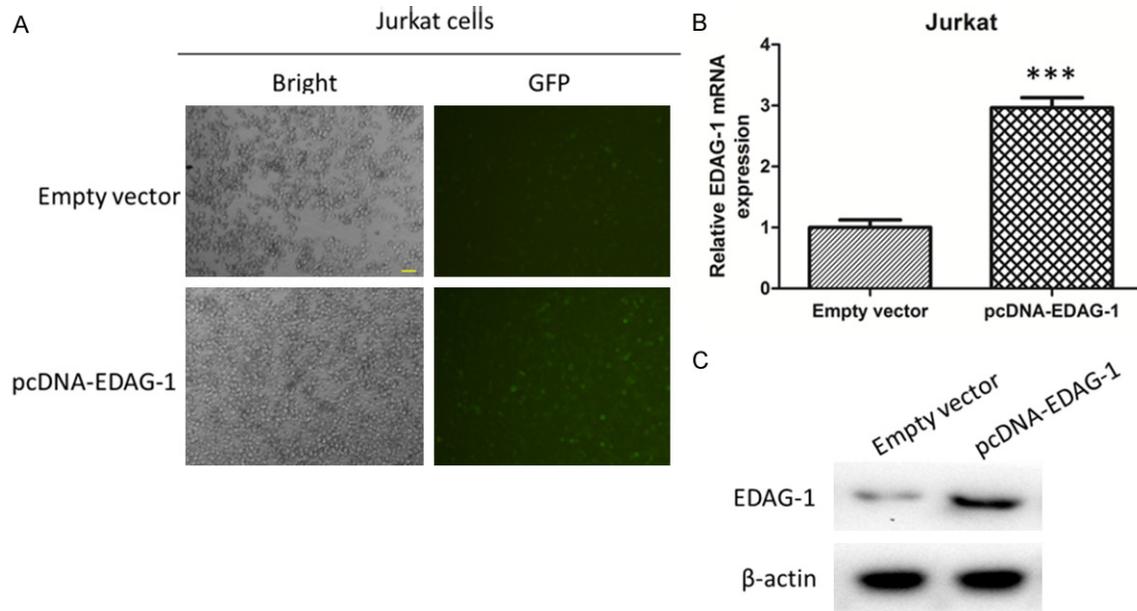


Figure 2. Overexpression of EDAG-1 in Jurkat cells by pcDNA3.1-EDAG-1. A. Fluorescence microscopy examination of lentivirus infection efficiency in Jurkat cells (Scale bar: 200 μ m). B. RT-qPCR analysis of EDAG-1 mRNA level in Jurkat cells after pcDNA3.1-EDAG-1 infection. C. Western blot analysis of EDAG-1 protein level in Jurkat cells after pcDNA3.1-EDAG-1 infection. Data represent as means \pm SD from three independent experiments, ***P < 0.001.

groups (**Figure 3A**). The knockdown of EDAG-1 in the Jurkat cells was confirmed by qRT-PCR that the mRNA expression level of EDAG-1 was significantly decreased (**Figure 3B**, ***P < 0.001), which was further confirmed by western blot that the protein level was also reduced in line with mRNA (**Figure 3C**). Then we explored the effect of EDAG-1 on T-ALL cell proliferation by MTT assay. According to our experimental results, the proliferation rate of T-ALL cells transfected with shEDAG-1 was significantly decreased compared with the negative control group (shNC) (**Figure 4B**, ***P < 0.001). These results indicated that knockdown of EDAG-1 could inhibit the proliferation of T-ALL cells.

Overexpression of EDAG-1 increases the cell percentage of T-ALL cells in S phase

Aberrance of cell cycle progression could lead to tumor-specific phenotype, such as unlimited cell proliferation, abnormal cell division, and chromosomal instability [16]. In order to examine the effects of EDAG-1 overexpression in Jurkat cells on the cell cycle progression, the cell cycle distribution of Jurkat cells after pcDNA3.1-EDAG-1 transfection was analyzed through FACS assay. As shown in **Figure 5A**, 43.86% pcDNA3.1-EDAG-1-transfected cells

were in G0/G1 phase compared to 56.61% of the control cells (**P < 0.01), whereas 31.55% of pcDNA3.1-EDAG-1 cells were in S phase compared to 23.35% of negative control (**P < 0.01), which indicated that overexpression of EDAG-1 could speed up cell division of Jurkat cells. No significant difference of the cell percentage of pcDNA3.1-EDAG-1 cells in G2/M phase compared with the control cells (24.60% vs 20.05%) was found. These results suggested overexpression of EDAG-1 promoted cell proliferation by speeding up the cell division during phase S of cell cycle.

Knockdown of EDAG-1 arrests cells in G0/G1 phase

We further investigated the cell cycle progression for the mechanisms underlying the promotion of cell proliferation by depletion of EDAG-1. The cell cycle distribution of Jurkat cells after knockdown of EDAG-1 was analyzed through FACS assay. As shown in **Figure 5B**, 65.8% shEDAG-1 cells were in G0/G1 phase compared to 56% of the control cells (*P < 0.05), whereas 15.81% of shEDAG-1 cells were in S phase compared to 20.88% of control cells (**P < 0.01), which indicated that cell cycle was arrested in G0/G1 phase. However, the cell percentage of

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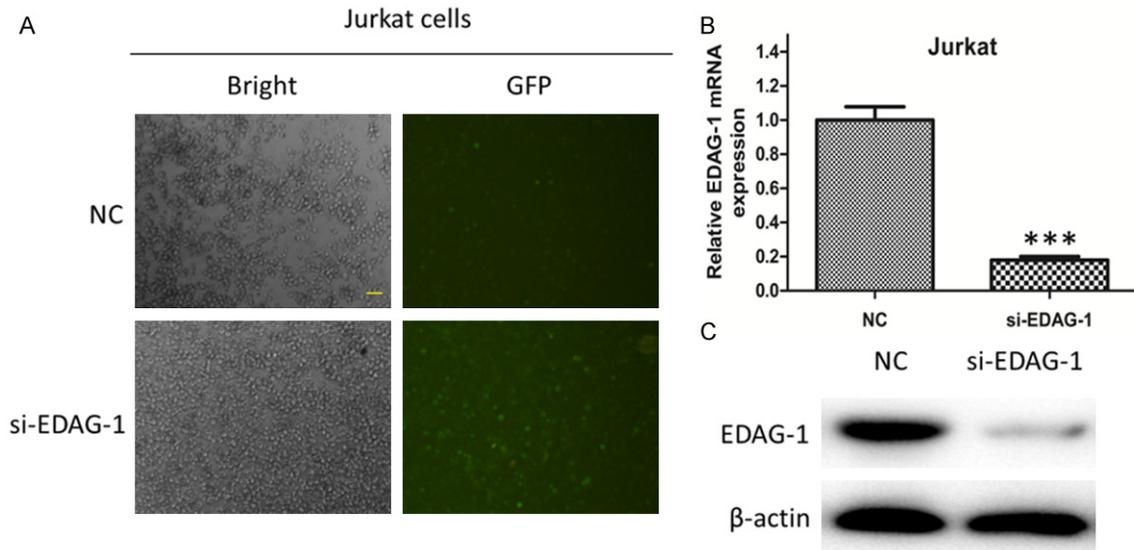


Figure 3. Knockdown of EDAG-1 in Jurkat cells by Lv-shEDAG-1. A. Fluorescence microscopy examination of lentivirus infection efficiency in Jurkat cells (Scale bar: 200 μ m). B. RT-qPCR analysis of EDAG-1 mRNA level in Jurkat cells after Lv-shEDAG-1 infection. C. Western blot analysis of EDAG-1 protein level in Jurkat cells after knockdown of EDAG-1. Data represent as means \pm SD from three independent experiments, ***P < 0.001.

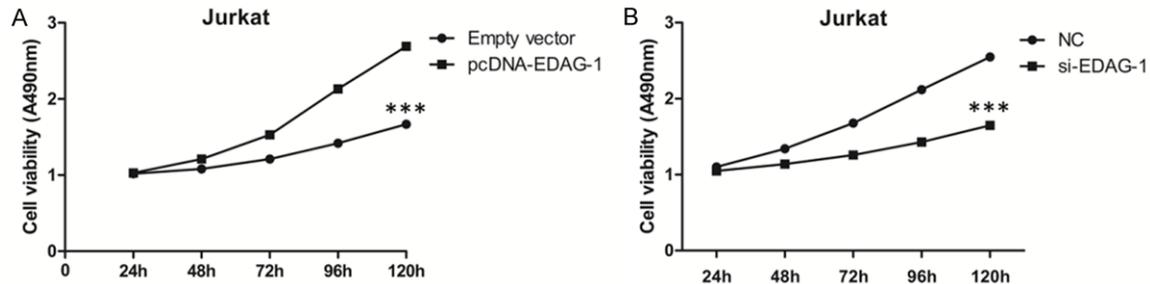


Figure 4. Overexpression of EDAG-1 promoted the viability and proliferation of Jurkat cells, while knockdown of EDAG-1 suppressed the proliferation ability of Jurkat cells. A. Growth curves of Jurkat cells in two groups (Empty vectors and pcDNA3.1-EDAG-1) measured by MTT assay. B. Growth curves of Jurkat cells in another two groups (Lv-NC and Lv-shEDAG-1) measured by MTT assay. Data represented as means \pm SD from three independent experiments, ***P < 0.001.

shEDAG-1 in G2/M phase was not very different from the control cells. These results suggested EDAG-1 knockdown inhibited T-ALL cell growth possibly by inducing cell cycle G0/G1 phase arrest and apoptosis.

EDAG-1 activates MAPK/Erk and Akt signal pathways

To further investigate the possible molecular mechanism associated with the process, we evaluated the protein expression of Erk/p-Erk and Akt/p-Akt by Western blotting. After Jurkat cells were transfected with pcDNA3.1-EDAG-1 or EDAG-1-siRNA, the expression levels of the proteins were detected respectively. As shown in **Figure 6**, overexpression of EDAG-1 signifi-

cantly increased the expression of p-Erk and p-Akt (**Figure 6A**, ***P < 0.001), while silencing of EDAG-1 significantly decreased the expression of p-Erk and p-Akt (**Figure 6B**, ***P < 0.001). These results suggested that overexpression of EDAG-1 could activate both MAPK/Erk and Akt signal pathways.

Discussion

EDAG -1 is a novel gene abundantly expressed in human fetal liver tissues. EDAG-1 was isolated by screening a human fetal liver cDNA library and the 5' RACE. The full length of EDAG-1 mRNA is 2 166 bp, with an open reading frame of 1452 bp nucleotides, encoding a 484 amino acid protein [6]. Previous studies have shown

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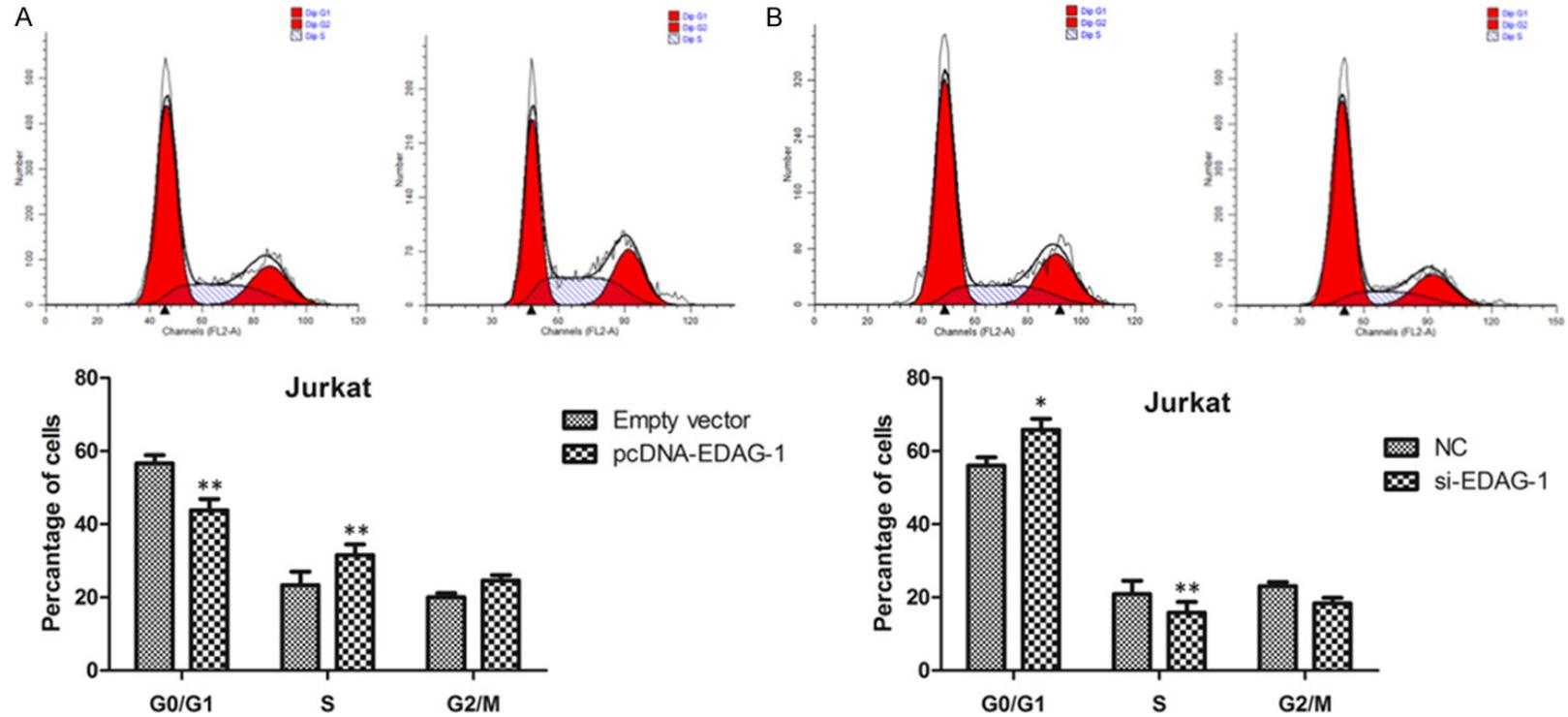


Figure 5. pcDNA3.1-EDAG-1 and Lv-shEDAG-1 blocked the cell cycle progression of Jurkat cells in S phase and G0/G1 phase, respectively. A. FACS analysis of cell cycle distribution of Jurkat cells in two groups (empty vector and pcDNA3.1-EDAG-1). Overexpression of EDAG-1 in Jurkat cells led to an increase of cells at S phase and concomitantly a decrease of cells at G0/G1 phase. B. FACS analysis of cell cycle distribution of Jurkat cells in another two groups (Lv-NC, Lv-shEDAG-1). Knock-down of EDAG-1 in Jurkat cells led to a decrease of cells at S phase and concomitantly an increase of cells at G0/G1 phase. Data represent means \pm SD from three independent experiments, * $P < 0.05$, ** $P < 0.01$.

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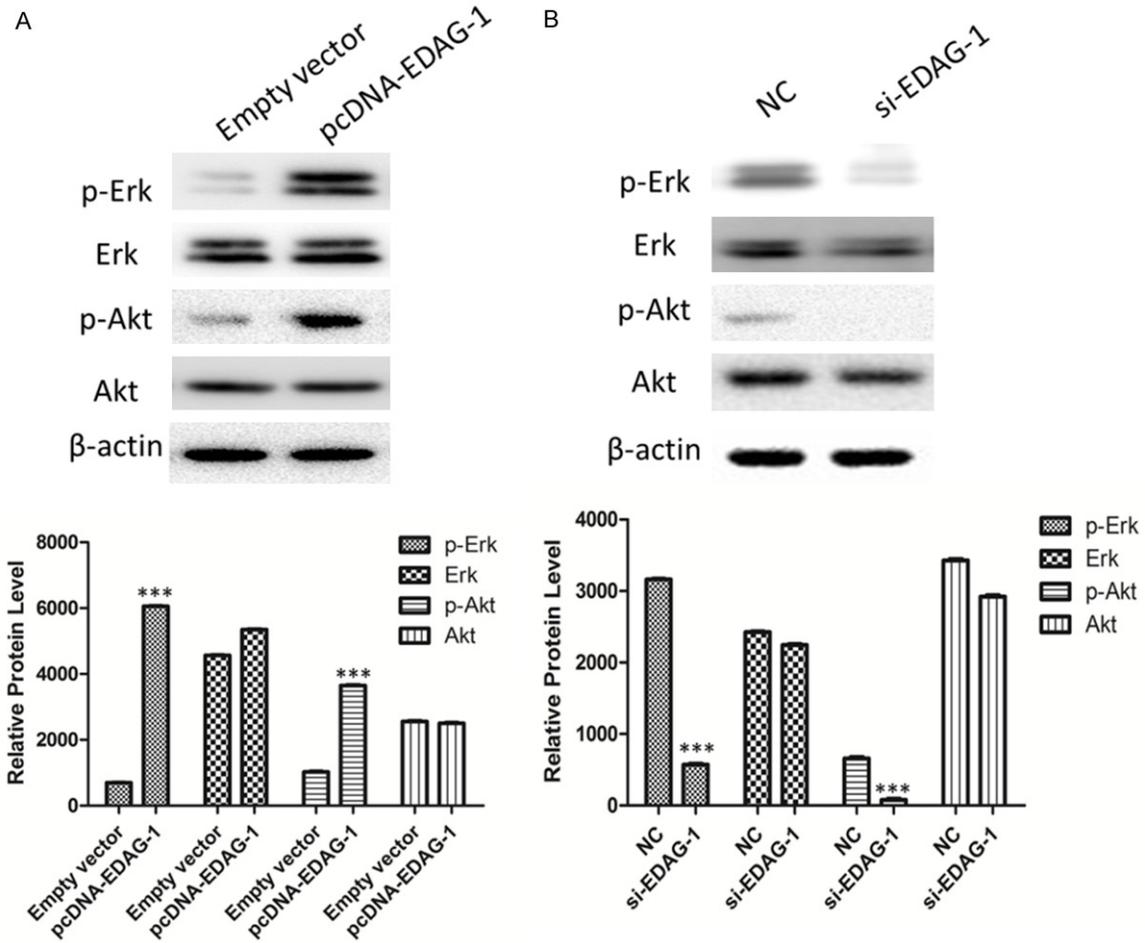


Figure 6. The expression of EDAG-1 caused activation of Akt and Erk in Jurkat cells and Lv-EDAG-1 caused inactivation of Akt and Erk. A. The protein levels of p-Akt and p-Erk were significantly increased by the overexpression of EDAG-1 in Jurkat cells, while there was no obvious difference of Akt and Erk expression between Jurkat cells infected with empty vectors and pcDNA3.1-EDAG-1. B. The protein levels of p-Akt and p-Erk were significantly decreased by the knockdown of EDAG-1 in Jurkat cells, while there was no obvious change of Akt and Erk expression before and after the infection of Lv-EDAG-1. Data represent means \pm SD from three independent experiments, ***P < 0.001.

EDAG-1 was specifically expressed in hematopoietic tissues, and was quickly down-regulated during the differentiation of K562 cells induced by hemin and EPO, which indicated EDAG-1 was related to the regulation in hematopoietic system and the development of leukemia [8]. Lu, J., et al demonstrated that EDAG overexpression in NIH3T3 cells caused anchorage-independent growth, loss of cells' contact inhibition, and tumorigenesis in nude mice, which suggested that EDAG-1 was a transforming gene and might be associated with the development of tumors, especially in hematopoietic system. Through overexpression of EDAG in HL-60 cell, Li, C.Y., et al [10] found that EDAG regulated the proliferation and differentiation of hematopoietic cells and resists cell

apoptosis through the activation of NF-kappa B. Ding, Y.L., et al [17] showed that overexpression of EDAG in a myeloid cell line 32D led to an erythroid phenotype with increased number of benzidine-positive cells and up-regulation of erythroid specific surface marker TER119. They also found silencing of EDAG by in K562 cells resulted in down-regulation of the target genes of GATA-1.

Dysregulated PI3K/AKT and p38 MAPK pathways have been implicated in T-ALL since both pathways are abnormally activated in high percentage of T-ALL [18]. Schubert, S., et al [19] found that T-ALL LICs could be eliminated by cotargeting the deregulated pathways driven by PI3K and Myc, which are altered commonly in

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human T-ALL and are associated with LIC formation. AKT phosphorylation subsequently activates mammalian target of rapamycin and downstream molecules, such as 70 kd S6 protein kinases (P70S6K) and eukaryotic translation initiation factor 4E-binding protein 1, consequently regulating cell growth and gene transcription [20]. However, the exact role of EDAG-1 on MAPK/Erk and AKT signal pathway in T-ALL is still unknown.

In this study, we focused on how overexpression and knockdown of EDAG-1 regulated the proliferation of T-ALL cells. Firstly, we confirmed that EDAG-1 was highly expressed in T-ALL cell line Jurkat and peripheral blood cells of patients with T-ALL compared with the peripheral blood cells of normal human by Western blotting. Then MTT and cell cycle assay were performed to indicate that high level of EDAG-1 was associated with the high level of proliferation. Overexpression and knockdown experiments were performed. Our results showed that overexpression of EDAG-1 could promote the proliferation of T-ALL cells and could significantly increase the cell percentage of T-ALL cells in S phase. On the contrary, silencing EDAG-1 would inhibit the proliferation T-ALL cells and arrest cells in G0/G1 phase. Collectively, these findings suggested EDAG-1 may play key roles in regulating the malignant biological behavior of T-ALL through multiple mechanisms, especially through regulating tumor proliferation. Mechanistically, we provided the evidence that overexpression of EDAG-1 could activate both MAPK/Erk and Akt signal pathways; therefore, targeting EDAG-1 and/or deregulated MAPK/Erk and AKT signal pathways may have direct clinical implication for T-ALL therapy.

In conclusion, EDAG-1 should play an oncogenic role in T-ALL progression. Although more work is needed to confirm the molecular mechanisms of EDAG-1 as a candidate biomarker for T-ALL in the clinic, our results have provided a new therapeutic target for the treatment of T-ALL.

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Disclosure of conflict of interest

None.

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