

Decreased expression of the augments of liver regeneration results in growth inhibition and increased chemosensitivity of acute T lymphoblastic leukemia cells

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Abstract. Augmenter of liver regeneration (ALR) plays crucial roles in cell survival and growth. Previous studies have demonstrated that ALR exerts a protective effect on toxic agent-induced cell death in acute T lymphoblastic leukemia cells and ALR knockdown can sensitize cancer cells to radiation. However, the biological functions of ALR against drug resistance in T-cell acute lymphoblastic leukemia are mostly unknown. In the present study, we investigated the effect of small interfering RNA (siRNA)-induced ALR silencing on cell proliferation and sensitivity to vincristine (VCR) of Jurkat cells. We found that ALR siRNA effectively decreased the ALR expression, then inhibited cell growth and increased sensitivity to VCR in Jurkat cells. Flow cytometry assay revealed that the downregulation of ALR expression promoted cell apoptosis and regulated cell cycle distribution. Following incubation with VCR, apoptosis-related proteins, such as pro-PARP, pro-caspase 8, pro-caspase 3 and Bcl-2 were downregulated in the siRNA/ALR group. Pretreatment with siRNA/ALR in combination with VCR resulted in prolonged G2/M arrest, accompanied by downregulation of cdc25c and cdc2 expression and dissociation of cyclin B1. In conclusion, the results of this study demonstrated that targeted inhibition of the ALR expression in Jurkat cells triggered cell growth inhibition and sensitized cells to VCR via promoting apoptosis and regulating the cell cycle.

Introduction

It is known that T-cell acute lymphoblastic leukemia (T-ALL) is an invasive hematological malignancy derived from normal

immature T cells, causing accumulation of leukemia cells in the bone marrow and suppression of normal hematopoiesis. T-ALL accounts for ~15% of pediatric and 25% of adult ALL cases (1). Intensive combination chemotherapy has improved the prognosis of patients with acute leukemia, however the outcome of patients who fail to respond to conventional chemotherapy is usually poor and the mortality rate is ~20% in children and up to 50% in adults with T-ALL (2-4). The emergence of resistance to chemotherapy is one of the main causes of treatment failure in acute leukemia (5,6). Therefore, the development of effective, new therapeutic methods, such as molecular-targeted therapy and enhancement of sensitivity to chemotherapy, in order to overcome drug resistance in leukemia cells may potentially improve the effects of chemotherapy (4,5).

Apoptosis, the natural process of programmed cell death, is essential for maintaining homeostasis (7,8). Apoptosis defects results in resistance to chemotherapeutic medicines (9-11). As cytotoxic agents exert their anticancer effects by inducing apoptosis, new approaches for antitumor treatment have focused on targeting mediators of the apoptosis pathways (12-14).

As a flavin-dependent sulfhydryl oxidase, augmenter of liver regeneration (ALR) is encoded by the growth factor *ervl*-like (GFER) gene (15). ALR is expressed in a variety of organs and tissues, such as hepatocellular carcinoma (16), tubular epithelial cells (17), human-derived glioma cells (18) and muscle tissue (19), and plays a central role in the synthesis of hepatocyte DNA, immune adjustment, cell cycle regulation and anti-apoptosis (20-24). Our previous studies revealed that exogenous ALR protected acute lymphocytic leukemia cells from vincristine (VCR)-induced cell death, via decreasing activated caspase 8, increasing the ratio of Bcl-2/Bax, reducing apoptotic cells and attenuating G2/M arrest (25). It has been reported that downregulation of ALR expression through antisense oligonucleotides and small interfering RNA (siRNA) led to cell proliferation inhibition and increased the sensitivity of cancer cells to radiation (18,26,27). To date, there are few studies on whether the suppression of ALR expression by siRNA could sensitize T-ALL cells to chemotherapeutic agents.

In the present study, we investigated the effect of siRNA-induced ALR silencing on apoptosis and cell cycle distribution and evaluated whether the suppression of ALR

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could sensitize the human T-ALL cell line Jurkat to the anti-leukemic agent VCR.

Materials and methods

Cell culture. Jurkat T leukemia cells (Nanjing KeyGen Biotech, Nanjing, China) were maintained in suspension in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml penicillin and streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

RNA interference. The target sequence for ALR was 5'-GTGTGCTGAAGACCTAAGA-3'. Sense siRNA was 5'-GAGTGTGCTGAAGACCTAAGA-3' and antisense siRNA was 5'-TCTTAGGTCTTCAGCACACTC-3'. Then, the siRNA was cloned into a CMV promoter-driven lentiviral expression vector GV118 (GeneChem, Shanghai, China). The GV118-mock vector was set as a negative control. For infection 5.5x10⁴ Jurkat cells were collected and resuspended in 1 ml complete RPMI-1640 medium. The cells were infected with GV118 or GV118-mock at a multiplicity of infection (MOI) of 1. Twenty-four hours after the infection, the medium was replaced with 1 ml of fresh culture medium. Then the cells were grown for another 48 h and fluorescence microscopy analysis of the GFP expression was performed. The expression of ALR mRNA was further confirmed by fluorescence quantitative polymerase chain reaction (FQ-PCR) and the ALR protein was determined by western blotting and confocal laser scanning microscope.

Real-time reverse transcription PCR. Total RNA was extracted from Jurkat cells using the RNeasy Micro kit (Nanjing KeyGen Biotech, Nanjing, China) according to the manufacturer's instructions. Total RNA (1 µg) from each sample was used to generate first-strand cDNA synthesis using PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China). Reverse transcription reaction started at 37°C for 15 min, followed by 5 sec at 85°C. Real-time PCR was conducted using the SYBR Premix Ex Taq™ II kit (Takara) according to the manufacturer's instructions. cDNA product (1 µl) was used for the real-time PCR in a final volume of 25 µl containing 12.5 µl of 2X SYBR Premix Ex Taq™ II and 0.75 µl of the forward and reverse primers (Table I). The thermal cycling conditions were as follows: initial denaturation step at 95°C for 1 min, followed by 40 cycles of denaturation for 10 sec at 95°C and annealing for 30 sec at 60°C. The CFX96 Manager software (Bio-Rad, Hercules, CA, USA) was used to calculate the relative concentrations of the PCR products. All the samples were tested in triplicate and the data were analyzed using the 2^{-ΔΔCt} method.

Western blotting. Briefly, Jurkat cells were collected and then washed twice with PBS. RIPA (500 µl) was added to the cell pellet to extract the total protein of the cells. Protein samples were resolved on 12% SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in a Tris-buffered saline/Tween-20 (TBST) solution containing 2% nonfat dry milk at room temperature for 1 h and subsequently incubated with the respective primary antibodies (Epitomics, Burlingame,

Table I. Primers of human ALR and β-actin.

Target gene	Primer sequence
ALR	F: (5'-3') AAGGTGAGGCTGGGAATTT R: (5'-3') GTCTTCATGTCGCGCTTCT
β-actin	F: (5'-3') TCAGGTCATCACTATCGGCAAT R: (5'-3') AAAGAAAGGGTGTAACGCA

ALR, augments of liver regeneration; F, forward; R, reverse.

CA, USA) diluted in PBS for 2 h at room temperature. Following washing with TBST for three times, the membranes were incubated with HRP-conjugated secondary antibodies. The expression of GAPDH was used as an internal loading control. Immunoblot quantification was carried out by densitometry using Image Lab statistical software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell proliferation assay. To investigate the effects of ALR silencing on Jurkat leukemia cell growth and sensitivity to VCR, cells were seeded at 1x10⁵/well in 96-well microtiter plates. After culturing for 1, 2, 3 and 4 days, the quantity of viable cells was detected. Afterwards, 20 µl of MTS solution was added into each well and the cells were incubated for 3 h. Complete solubilization of the dye was achieved by vortexing the plate, and then absorbance was read at 490 nm using SpectraMax M2 plate reader. Wells containing medium but no cells, served as blank controls. Meanwhile, after 48 h of infection, cells were incubated with various concentrations of VCR (0.0625-10 µg/ml) for another 24 h at 37°C. The viable cell count in each well was assessed by the OD value. The percentage of surviving cells was calculated and the IC₅₀ value was determined by nonlinear regression analysis using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Cell cycle analysis. After 72 h of infection, cell samples were harvested and washed once in cold PBS, then fixed and stained with PI solution. The cell cycle was detected using the cellular DNA flow cytometric analysis kit (Nanjing KeyGen Biotech) according to the manufacturer's protocol. The percentages of cells within the G1/G0, S and G2/M phases were determined by flow cytometry. The results were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA). For the chemotherapeutic drug tests, after 48 h of infection, the cells were cultured with VCR 1 µg/ml for an additional 24 h and then analyzed as aforementioned.

Analysis of apoptosis. Apoptosis was assessed using the Annexin-V-PE/7-AAD Apoptosis Detection kit (Nanjing KeyGen Biotech). After 48 h of infection, the cells were incubated with 1 µg/ml VCR or alone, for an additional 24 h. Then cell samples were collected and washed twice with cold PBS. Subsequently, the cells were stained with 1 µl Annexin V-PE and 5 µl 7-AAD. Flow cytometric analysis was performed on a FACScan flow cytometer (BD Biosciences). Results were analyzed using CellQuest PRO software (BD Biosciences).

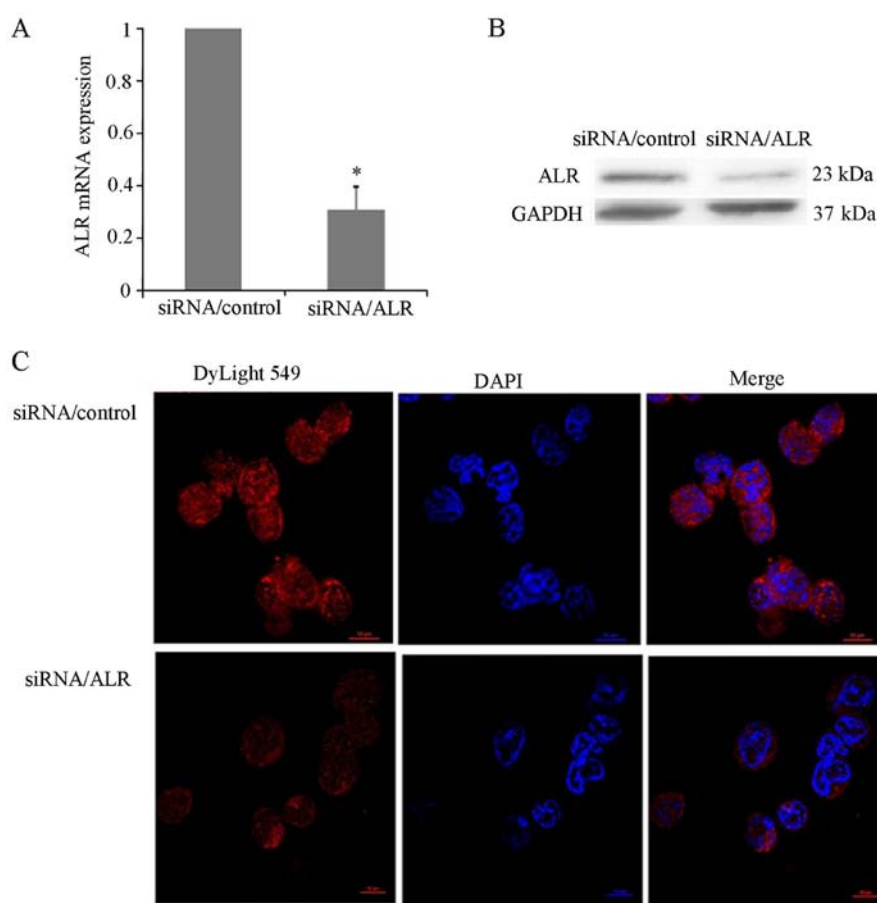


Figure 1. ALR expression in ALR siRNA-infected group and control group. Data represent the mean \pm SEM from three independent experiments. (A) ALR mRNA expression was detected by real-time PCR (* $p=0.0003$, $n=3$). (B) The protein levels of ALR were determined by western blot analysis. (C) The presence of ALR was revealed by confocal microscopy immunodetection. The nuclei were stained with DAPI, the specific ALR immunodetection was revealed by DyLight 549, and the 'merge' indicates colocalization. ALR, augmentor of liver regeneration.

Statistical analysis. All data are reported as the means \pm SEM. Comparisons between pairs of groups were made by Student's t-test or one way ANOVA for multiple group comparisons. A P-value <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc.).

Results

ALR siRNA effectively decreases ALR expression in Jurkat cells. The expression level of ALR in the T-ALL Jurkat cell line was detected by real-time PCR, western blot analysis and confocal laser scanning microscopy. ALR-specific siRNA induced a marked decrease of ALR mRNA expression in Jurkat cells (Fig. 1A). The protein level of ALR was greatly downregulated in the ALR siRNA-infected group compared with the level in the mock control group (Fig. 1B). After 72 h of infection, the relative expression of ALR mRNA was $28.17 \pm 2.63\%$, while the relative expression of ALR protein was $21.45 \pm 1.98\%$, respectively. Compared with the control group, a significant reduction of the ALR protein in the ALR siRNA-infected group was determined by confocal microscopic immunodetection (Fig. 1C).

Downregulation of ALR expression inhibits proliferation of Jurkat cells. As extraneous ALR is associated with the

survival of Jurkat T leukemia cells, the effect of ALR siRNA on cell proliferation was detected. Twenty-four, 48, 72 and 96 h after infection with siRNA, cell growth was determined using MTS assay. The cell proliferation curve demonstrated that compared to the control group, ALR siRNA significantly reduced cell growth in a time-dependent manner (Fig. 2). At 24 h post-infection, the cell growth was $96.84 \pm 5.76\%$ and rapidly dropped to $49.38 \pm 7.21\%$ at 96 h in the ALR siRNA group. The results revealed that suppression of the ALR expression by siRNA significantly inhibited cell proliferation in Jurkat cells.

Downregulation of ALR expression induces apoptosis in Jurkat cells. We next investigated whether the ALR siRNA-induced proliferation suppression was related to apoptosis. The FCM analysis revealed that the apoptosis rate was $19.85 \pm 1.74\%$ in the ALR siRNA-infected group, compared with $7.48 \pm 0.83\%$ in the control group (Fig. 3). Western blot analysis revealed that after ALR siRNA infection, pro-PARP, pro-caspase 8, pro-caspase 3 and Bcl-2 expression were downregulated in the Jurkat cells (Fig. 4). These results revealed that the increased apoptosis induced by ALR siRNA was partially responsible for the inhibition of cell proliferation in the Jurkat cells.

Downregulation of ALR expression reduces the G2/M ratio in Jurkat cells. It is known that cell cycle progression influences

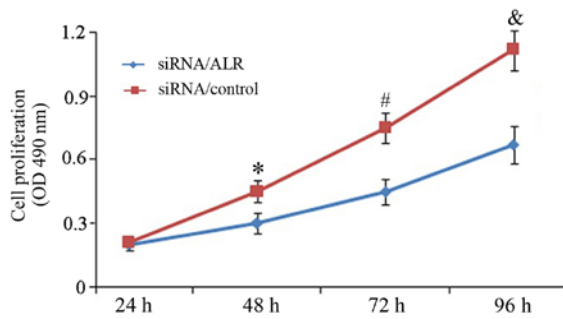


Figure 2. Effect of ALR siRNA on cell proliferation. Jurkat cells were infected with siRNA and cultured for 96 h and the cell growth was detected using MTS assay. Significance: * $p=0.006$, # $p=0.0001$ and & $p=0.0003$ vs. siRNA/control ($n=3$). ALR, augmentin of liver regeneration.

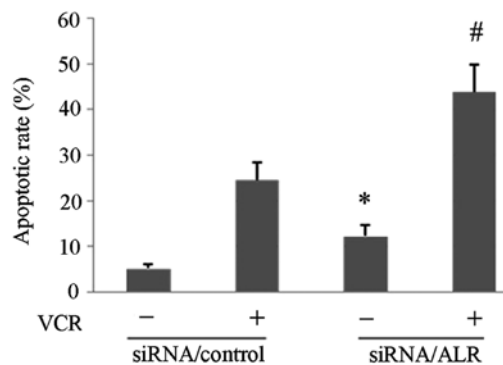


Figure 3. Suppression of the ALR expression by siRNA promotes apoptosis in Jurkat cells. After 48 h of infection, Jurkat cells were cultured for an additional 24 h alone, or with VCR. Then apoptotic cells were detected by AnnexinV/PI staining using FCM. Data represent the mean \pm SEM from three independent experiments. The average percentages of Annexin V-positive Jurkat cells from at least three independent experiments. Significance: * $p=0.001$ vs. siRNA/control ($n=3$), # $p=0.003$, vs. siRNA/control + VCR ($n=3$). ALR, augmentin of liver regeneration; VCR, vincristine.

cell proliferation, thus we detected the effects of ALR siRNA infection on cell cycle distribution. Data revealed that suppression of ALR expression induced a decreased percentage of G2/M-phase cells (Fig. 5). The percentage of G0/G1-phase cells was 51%, of S-phase cells was 39.23% and of G2/M-phase cells was 8.53% in typical ALR siRNA-infected cells, compared with 50.19%, 28.59% and 20.5% in the control cells, respectively. Then we detected the G2/M-phase regulatory factors, including cyclin B1, P53, cdc2 and cdc25c proteins, for their molecular mechanisms. Western blot analysis revealed that cyclin B1 and P53 expression was downregulated, while cdc25c and cdc2 expression was upregulated (Fig. 4).

Downregulation of ALR expression increases sensitivity to VCR in Jurkat cells. To investigate the effect of ALR downregulation on the response of Jurkat cells to a chemotherapeutic agent, we suppressed the ALR expression by siRNA in Jurkat cells and then detected cell proliferation and apoptosis and cell cycle change induced by VCR. Data revealed that the IC_{50} value for VCR in the control siRNA group was 4.87 ± 0.52 μ g/ml. Jurkat cells infected with siRNA/ALR were more sensitive and the IC_{50} value decreased to 1.32 ± 0.17 μ g/ml (Fig. 6). Subsequently, we

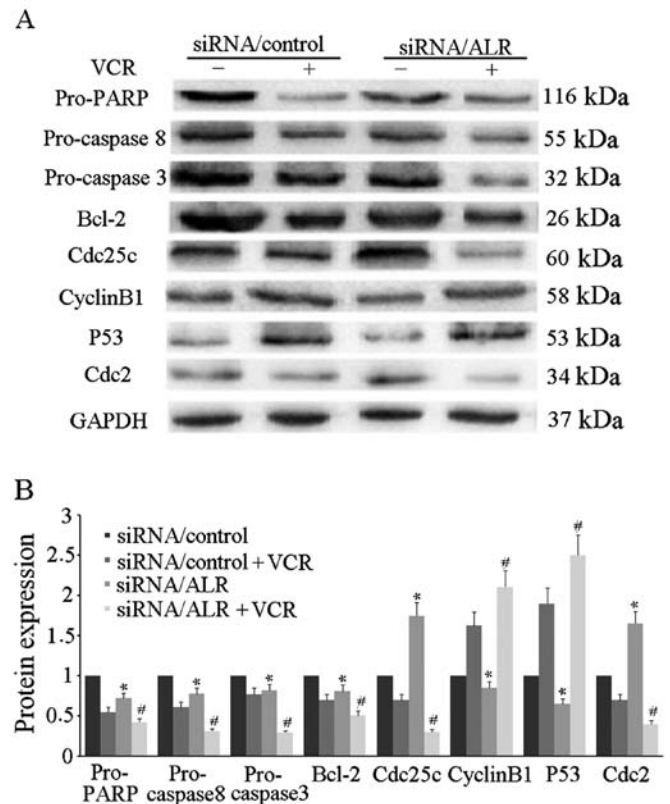


Figure 4. Effect of ALR siRNA on the expression level of apoptosis and cell cycle-related proteins. After 48 h of infection, Jurkat cells were incubated for an additional 24 h alone, or with VCR. Western blot analyses were performed to detect the protein expression. (A) Representative western blot analysis of proteins from cells transfected with siRNA. (B) The expression level of each band was measured by densitometry and normalized to the respective GAPDH. Data represent the mean \pm SEM ($n=3$). * $p<0.05$ vs. siRNA/control, # $p<0.05$ vs. siRNA/control + VCR. ALR, augmentin of liver regeneration, VCR, vincristine.

examined the apoptotic rate induced by VCR after the siRNA/ALR infection. FCM analysis revealed that after the siRNA infection, VCR induced a higher apoptotic rate in the siRNA/ALR-infected group ($43.75 \pm 5.96\%$) than that in the control siRNA infected group ($24.59 \pm 3.76\%$) (Fig. 3). The western blot analysis revealed that the process of VCR-induced apoptosis in the siRNA/ALR-infected cells was accompanied by the decreased expression of the pro-PARP, pro-caspase 8, pro-caspase 3 and Bcl-2 proteins (Fig. 4). Furthermore, cell cycle stage analysis revealed that in comparison with the control siRNA group, VCR prolonged G2/M arrest in the siRNA/ALR group (Fig. 5). Then we detected the expression of several G2/M-phase cell cycle regulatory factors to explore the molecular mechanisms. Western blot analysis revealed that the expression of cyclin B1 and P53 was increased while that of cdc25c and cdc2 was decreased (Fig. 4).

Discussion

ALR was first isolated from neonatal mouse hepatocytes in 1994 (28). ALR exhibits numerous biological activities, such as anti-apoptosis, immunoregulation, antioxidant and preserving mitochondrial function (20-24,29). Our

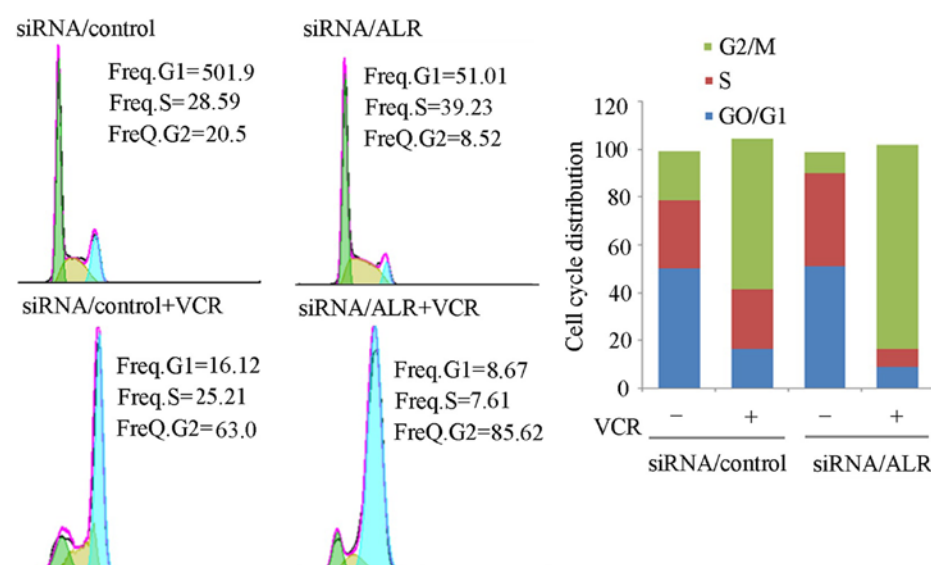


Figure 5. Suppression of ALR expression by siRNA alters cell cycle distributions in Jurkat cells. After 48 h of infection, Jurkat cells were incubated for an additional 24 h alone or with VCR and stained with PI. FCM analyses were performed to determine the cell cycle distribution using the cell cycle detection kit. ALR, augmentin of liver regeneration; VCR, vincristine.

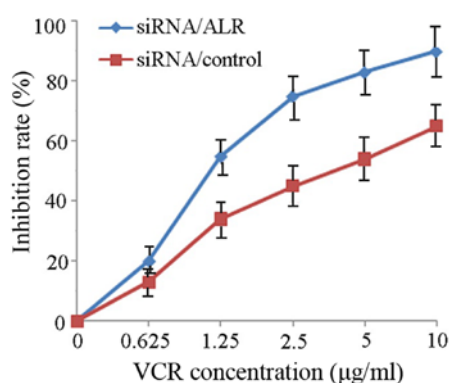


Figure 6. Suppression of ALR expression by siRNA enhances the chemosensitivity of Jurkat cells. The MTS assay revealed a dose-dependent inhibition of cell proliferation. The inhibition rates of the siRNA/ALR group were higher than that of the control siRNA group after incubation with the same VCR concentrations ($p < 0.05$, $n = 3$). ALR, augmentin of liver regeneration.

previous study revealed that exogenous ALR could protect Jurkat T leukemia cells from VCR-induced cell death, by decreasing apoptotic cells and attenuating G2/M arrest (25). The present study revealed that ALR may play an important role in Jurkat cell resistance to chemotherapeutic drugs. Cao *et al* found that ALR could enhance radiation sensitivity in hepatoma cells (26). Thus we further investigated whether or not the suppression of ALR expression could enhance the sensitivity of Jurkat cells to an anti-leukemic agent. In the current study, we investigated the effect of ALR silencing by siRNA on the sensitivity of Jurkat cells to VCR and explored the related mechanism.

RT-PCR, western blot analysis and confocal laser scanning microscopy revealed that ALR siRNA infection significantly reduced ALR gene mRNA and protein levels at 72 h post-infection in Jurkat cells. These data revealed that ALR-specific siRNA translationally suppressed the ALR mRNA. The MTS assay for cell proliferation revealed that

the suppression of ALR expression markedly restrained the proliferation of the Jurkat cells, proving that ALR can exert a positive effect on leukemic cell growth. Most prominently, the data from the cell proliferation assay revealed that with ALR siRNA infection, the IC_{50} value of VCR obviously decreased, which led to a significant synergistic anti-leukemia effect. This supports the hypothesis that downregulation of ALR can increase the sensitivity of leukemia cells to VCR and possibly improve chemotherapy efficiency *in vivo*.

To further investigate the influence of ALR on survival of Jurkat leukemia cells *in vitro*, we detected the effect of down-regulation of the ALR expression on cell apoptosis. These experiment findings indicated that siRNA-mediated suppression of ALR expression exerted an obvious effect on cell apoptosis. ALR siRNA pretreatment in combination with the chemotherapeutic agent VCR resulted in obvious apoptosis in Jurkat cells and in enhancement of chemotherapy sensitivity. On the contrary, cells infected with non-targeting siRNA in the control group had no alteration of the ALR gene expression level. Subsequently, cell growth and toxicity of VCR were not changed. These findings illustrated the specific impact of ALR siRNA in Jurkat cells. However, our findings are similar with other research which focused on the biological effects of ALR in a variety of cell lines (17,18,30). Collectively, these results revealed that ALR plays a significant role in cell survival, proliferation and chemotherapy sensitivity of malignant cells. All of the observations above, ascertained that the presence of the multifunctional ALR protein is critical to the survival and chemotherapeutic agent resistance in Jurkat cells. Therefore, ALR gene silencing could induce cell apoptosis and sensitize leukemia cells to cytotoxic drugs.

The findings of the signaling pathway analysis revealed that targeted suppression of ALR expression could promote apoptotic signaling and lead to an increase of chemotherapeutic efficiency. Consequently, Jurkat ALR-knockdown cells exhibited more sensitivity to the chemotherapeutic drug. Enhanced cell death was mediated by a higher percentage

of apoptotic cells, which was accompanied by decreased expressions of the pro-PARP, pro-caspase 8, pro-caspase 3 and Bcl-2.

Previous findings have demonstrated that ALR acts as a regulatory factor for cell cycle regulation (31-33). In the present study, we found that downregulation of ALR prevented Jurkat cell transition from the S to the G2/M phase which resulted in a decrease of the G2/M ratio, while it prolonged VCR-induced G2/M arrest. The targeted suppression of the ALR expression increased the protein levels of cdc25c and cdc2, inhibited the dissociation of cyclin B1 and decreased G2/M ratio. Finally, it led to prevention of cell mitosis and induced Jurkat cell apoptosis. Treatment with siRNA/ALR and VCR significantly downregulated the expression of cdc25c and cdc2, promoted dissociation of cyclin B1, resulted in prolonged G2/M arrest and enhanced apoptosis. The evidence revealed that ALR knockdown may exert a bidirectional regulatory effect on Jurkat cell cycle progression. However, more research is needed to identify the roles of ALR in the regulation of leukemia cell cycle and apoptosis.

In conclusion, our findings ascertained that ALR plays important roles in both cell growth and drug susceptibility of Jurkat cells *in vitro*. Targeted inhibition of the ALR expression by siRNA triggered cell apoptosis and enhanced sensitivity of leukemic cells to VCR in a synergistic manner. Our study focused on the capability of ALR siRNA to sensitize the leukemia cells to chemotherapeutic agent and decrease the occurrence of anti-leukemia drug resistance. Therefore, siRNA silencing of ALR gene expression may offer a new strategy for the treatment of drug-resistant T-ALL.

Acknowledgements

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