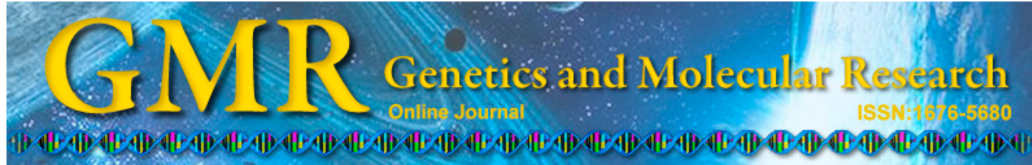

Expression differences in *TEL-AML1* fusion gene in leukemia glucocorticoid-sensitive and -resistant cell lines

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ABSTRACT. We investigated the expression differences of the *TEL-AML1* fusion gene in a leukemia glucocorticoid (GC)-sensitive cell line (CEM) and a GC-resistant cell line (Jurkat). Changes in *TEL-AML1* expression before and after GC exposure were analyzed. Expression of GC-sensitive and GC-resistant leukemia cells following initial diagnosis and during treatment was simulated. Leukemia cells were divided into a GC-unexposed or a GC-exposed group. A methyl thiazolyl tetrazolium assay was used to detect cell proliferation inhibition, flow cytometry was used to observe cell apoptosis, reverse transcription-polymerase chain reaction was used to detect the mRNA expression of *TEL-AML1* before and after exposure, and western blotting was used to analyze protein levels of TEL-AML1 before and after exposure. Inhibitory concentrations of 50% of cells in the Jurkat and CEM cells at 24 h were 382 and 9 μM, respectively, and at 48 h they were 216 and 2 μM. The proliferation inhibition effect of dexamethasone sodium phosphate on Jurkat cells was much lower than that on CEM cells. Jurkat cells showed obvious apoptosis after exposure to 100 μM dexamethasone

sodium phosphate for 48 h. In the exposed group, Jurkat cells showed higher *TEL-AML1* expression than did CEM cells (P < 0.05). In the unexposed group, *TEL-AML1* gene expression in Jurkat cells was not affected by GC exposure (P > 0.05), while the CEM cells presented significant differences before and after exposure (P < 0.05). Sustained high expression of *TEL-AML1* participated in and maintained the occurrence of GC resistance. Inhibition of *TEL-AML1* may provide a new therapeutic approach to reverse GC resistance.

Key words: Cell line; GC-resistant; GC-sensitive; Leukemia; *TEL-AML1* fusion gene

INTRODUCTION

Leukemia is a type of blood system tumor that is caused by the malignant clonal proliferation of a series of hematopoietic cells due to arrest at a primitive differentiation stage. It is clinically manifested as corresponding blood system tissue infiltrations, such as anemia, hemorrhage, infection, hepatomegaly, splenomegaly, and lymphadenectasis, among others (Eipel et al., 2013). Currently, the etiology of leukemia is not clear. The etiology may be associated with viral infections such as human T cell leukemia virus, physical or chemical stimulations, and genetic defects (Liu et al., 2013). In China, childhood leukemia is one of the malignant tumors with the highest incidence, and acute lymphoblastic leukemia (ALL) accounts for approximately 70% of all leukemia cases (Chen et al., 2012). With the constant development of modern medical technology and the improvement of chemotherapy regimens, the total cure rate of ALL has reached approximately 80% through individualized chemotherapy of multi-course and multi-medicine (Sarvaiya et al., 2012). Therefore, the current goal of childhood ALL treatment is not short-term complete remission, but long-term disease-free survival or even a cure. One key to realizing this goal is the accurate evaluation of prognosis by risk stratification (Harada et al., 2012). According to the therapeutic responses of drugs, glucocorticoid (GC) shows a particularly significant effect. Leukemia cells are not sensitive to prednisone (namely, GC-resistant), which greatly inhibits the ability to cure leukemia (Spijkers-Hagelstein et al., 2012). Therefore, GC resistance has been a research hotspot in the treatment of leukemia. Some studies found that the anomaly in apoptosis pathway may be a new mechanism of the drug resistance of tumor cells (Lv et al., 2012). The *TEL-AML1* fusion gene is one of the most common genetic alterations observed in ALL and is closely associated with childhood ALL. Moreover, it is the most common chromosome translocation in childhood ALL. This alteration developed from the translocation of t(12; 21); the *TEL-AMI1* fusion gene is formed by the short arm 1 region 2 band of chromosome 12 transformed to and fused with the short arm 2 region 2 band of chromosome 21 (Eberhart et al., 2011). However, whether *TEL-AML1* fusion gene expression is associated with GC resistance and is involved in the occurrence of GC resistance through an anti-apoptotic effect remain unknown (Leclerc et al., 2010).

In this study, we examined a GC-unexposed group and a GC-exposed group to investigate whether GC-sensitive and GC-resistant leukemia cells showed different expression in initial diagnosis and treatment. Moreover, we explored the expression differences of the *TEL-AML1* fusion gene in GC-sensitive and GC-resistant leukemia cell lines and the effects

of GC exposure on the expression of the *TEL-AML1* fusion gene. The aim of this study was to understand the relationship between the *TEL-AML1* fusion gene and GC resistance and the significance of this relationship.

MATERIAL AND METHODS

Materials

The human acute T lymphoblastic leukemia GC-sensitive cell lines (CEM) and GC-resistant cell lines (Jurkat) were provided by Department of Immunology, Inner Mongolia Medical University, China.

The optical microscope was purchased from Hach Company (Loveland, CO, USA). The desktop refrigerated centrifuge was purchased from Beijing Jingli Centrifuge Co., Ltd. (Beijing, China). The polymerase chain reaction (PCR) amplifier and flow cytometer were purchased from Thermo Scientific (Waltham, MA, USA). The electrophoresis apparatus was purchased from Medica Corp. (Bedford, MA, USA). The enzyme mark instrument was purchased from Labnet International, Inc. (Edison, NJ, USA).

Methyl thiazolyl tetrazolium (MTT) powder and dimethyl sulfoxide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Bradford protein assay kit, Trizol, and reverse transcription kit were purchased from Invitrogen (Carlsbad, CA, USA). Diethylpyrocarbonate water was purchased from Shanghai Schreck Biological Technology Co., Ltd. (Shanghai, China). RMPI1640 was purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China). The annexin V-fluorescein isothiocyanate (FITC) cell apoptosis detection kit was purchased from Fermentas (Vilnius, Lithuania). β-actin mouse monoclonal, mouse anti-human TEL-AML1, and rabbit anti-mouse polyclonal antibodies were purchased from Sigma (St. Louis, MO, USA). DNA Marker D2000 and PCR MasterMix were purchased from Shanghai Baoman Biotechnology Co., Ltd. (Shanghai, China). Primers were synthesized by the Biomedical Engineering Institute of Chinese Academy of Medical Science.

The primers had the following sequences: *TEL-AML1*: upstream 5'-AGGGCCTTTCGA GCTTATC-3', downstream 5'-CGCCAGACAGCCAAGTATAT-3', amplified product of 378 bp; β-actin: upstream 5'-CAAAGCAGTTAGCGATCCCC-3', downstream 5'-CGAAA CTTGGGCACATGGCT-3', amplified product of 241 bp (Husak et al., 2010).

Experimental groups

According to the expression of GC-sensitive and GC-resistant leukemia cells in initial diagnosis and treatment, cell lines were divided into GC-unexposed and GC-exposed groups. Negative control and blank control groups were also included. The exposed group was exposed using 1, 10, 100, 1000 μM dexamethasone sodium phosphate (DEX) for 48 h.

Cell culturing

The GC-sensitive cell line CEM and GC-resistant cell line Jurkat were cultured in RPMI1640 culture medium containing 10% fetal bovine serum at 37°C and 5% CO_2. Cells were stained using Trypan blue. A phase showing an exclusion rate of more than 90% was considered the cell logarithmic growth phase.

Cell proliferation detection by MTT

The cell density of cells in the logarithmic growth phase was adjusted to 5 x 10^4/mL. The cell suspensions obtained were transferred to a 96-well plate with 90 µL per well. A blank control well (without cells) and negative control well (without drugs) were used. Each cell line was tested in 5 wells. To each test well, we added 10 µL DEX solution of corresponding gradient concentration and exposed the cells for various times. To each well, we added 20 µL 5 mg/mL MTT solution. After culturing for 4 h, the cell suspensions in the wells were centrifuged at 1000 *g* for 10 min. Supernatants were removed and discarded. Next, 100 µL dimethyl sulfoxide was added, and the cell suspensions obtained were oscillated for 10 min to fully dissolve crystals. The optical density of each well was measured at a wavelength of 570 nm using an enzyme-linked immunosorbent assay. We calculated the cell inhibition rate of each well. Inhibition rate (%) = (mean absorbance value of control group - mean absorbance value of test group) / mean absorbance value of control group x 100%. The concentration values of drugs at which 50% of the cells were inhibited (IC_{50}) were calculated using regression analysis.

Cell apoptosis detection by annexin V-FITC/propidium iodide (PI) double-staining method

After DEX exposure, cells were centrifuged at 1000 rpm for 5 min. Supernatants were discarded and cells were collected. Cells collected were then suspended gently using phosphate-buffered saline. Cell numbers were counted. Negative control (without DEX) and blank control (without annexin V-FITC) were included. Next, 1 x 10^6/mL suspended cells were removed and centrifuged at 1000 rpm for 5 min. Supernatants were discarded. The cells obtained were gently resuspended using 195 µL annexin V-FITC binding buffer. The cell suspensions were gently mixed with 5 µL annexin V-FITC and then incubated for 10 min in the dark using aluminum foil at room temperature (20°-25°C). The samples were centrifuged at 1000 rpm for 5 min and the supernatants were discarded. The cells obtained were suspended using 190 µL annexin V-FITC binding buffer. The cell suspensions obtained were mixed gently with 10 µL PI staining solution and then placed in the dark for 10 min. Finally, the samples were evaluated by flow cytometry.

mRNA level detection by reverse transcription-PCR

The cell density of cells in the logarithmic growth phase was adjusted to 1 x 10^6/mL. The cell suspensions were transferred to a 6-well plate with 3 mL in each well. Wells were divided into an exposed group, unexposed group, blank group (only containing culture medium), and negative control group (bone marrow mononuclear cell of a child with non-malignant hematologic disease extracted by lymphocyte separation liquid). Forty-eight hours later, the cell suspension in each well was transferred to a 15-mL centrifuge tube and centrifuged at 1500 rpm for 15 min. Supernatants were discarded. RNA was extracted using the Trizol method. The RNA obtained was reverse-transcribed using Toyobo (Osaka, Japan) reverse transcription kit to obtain the cDNA. PCR was conducted.

Protein level detection by western blotting

Cells were collected and centrifuged at 1500 rpm for 15 min and the supernatants

were discarded. The cells obtained were suspended using pre-cooled phosphate-buffered saline and then centrifuged. This process was repeated twice. The cells obtained were lysed for 30 min using 200 µL lysis buffer on ice. The cell suspensions were centrifuged at 12,000 rpm for 20 min. Supernatants, which included suspended protein, were collected. Next, polyacrylamide gel with 10% sodium dodecyl sulfonate was prepared. Protein samples were prepared by mixing 50 µg protein with sample buffer. After instant centrifugation and sampling, the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, gel strips were cut into proper size. Next, transfer buffer was used for 5 min 3 times. Nitrocellulose membranes and filter papers were cut into the same sizes as the gel strips. Membranes were immersed in the transfer buffer for 10 min, transfered from the gel strips to the membrane and then the membranes were blocked by adding mouse anti-human TEL-AML1 monoclonal antibody to the nitrocellulose membranes in a 1:1000 ratio. The nitrocellulose membrane and the primary antibody were placed on shaking table at 4°C overnight. The membranes were washed 3 times with Tris-buffered saline containing Tween 20 for 5 min each time. The secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse polyclonal antibody) at 1:500 was added to the membranes and incubated for 1 h. The membranes were washed 3 times with Tris-buffered saline containing Tween 20 for 5 min each time. An electrochemiluminescence reaction system was employed for exposure and development. Protein bands were analyzed using software. The ratio of average optical density of target band and internal reference was used to determine the relative expression level of the target protein band.

Statistical analyses

All data are reported as means ± standard deviation of three independent tests. Statistical analysis was conducted using the SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Comparison between groups used the *t*-test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Proliferation of CEM and Jurkat cell lines detected by MTT

CEM and Jurkat cell lines were treated by 1, 10, 100, and 1000 µM DEX exposure for 24 and 48 h. The MTT results showed that the concentrations at which 50% of Jurkat and CEM cell lines were inhibited were 382 and 9 µM at 24 h and 216 and 2 µM at 48 h, respectively. The proliferation inhibition effect of DEX on Jurkat was far lower than that on CEM. Jurkat cell lines were observed with notable inhibition after exposure to 100 µM DEX for 48 h, as shown in Tables 1 and 2.

Table 1. Cell inhibition rates at 24 h.

Group	1 µM	10 µM	100 µM	1000 µM
CEM	13.5 ± 2.4	34.6 ± 3.7	57.1 ± 4.1	66.5 ± 4.2
Jurkat	1.1 ± 0.3*	4.8 ± 0.9*	10.5 ± 1.2*	59.1 ± 3.7

Compared with CEM group, *P < 0.05.

Table 2. Cell inhibition rates at 48 h.

Group	1 μM	10 μM	100 μM	1000 μM
CEM	19.3 ± 2.2	41.6 ± 2.4	64.3 ± 2.5	76.4 ± 2.8
Jurkat	1.0 ± 0.2*	5.1 ± 0.6*	23.7 ± 1.7*	68.2 ± 2.4

Compared with CEM group, *$P < 0.05$.

Apoptosis of CEM and Jurkat cell lines detected by flow cytometry

Annexin V-FITC/PI double staining was employed to detect the apoptosis of CEM and Jurkat cell lines at 48 h. In Figure 1, the x-axis refers to the annexin V-FITC pathway and the y-axis refers to the PI pathway. The left lower quadrant (annexin V-FITC$^-$/PI$^-$) denotes living cells, while the left upper quadrant (annexin V-FITC$^-$/PI$^+$) represents mechanically damaged cells. The right upper quadrant (annexin V-FITC$^+$/PI$^+$) represents dead cells and the right lower quadrant (annexin V-FITC$^+$/PI$^-$) refers to apoptotic cells.

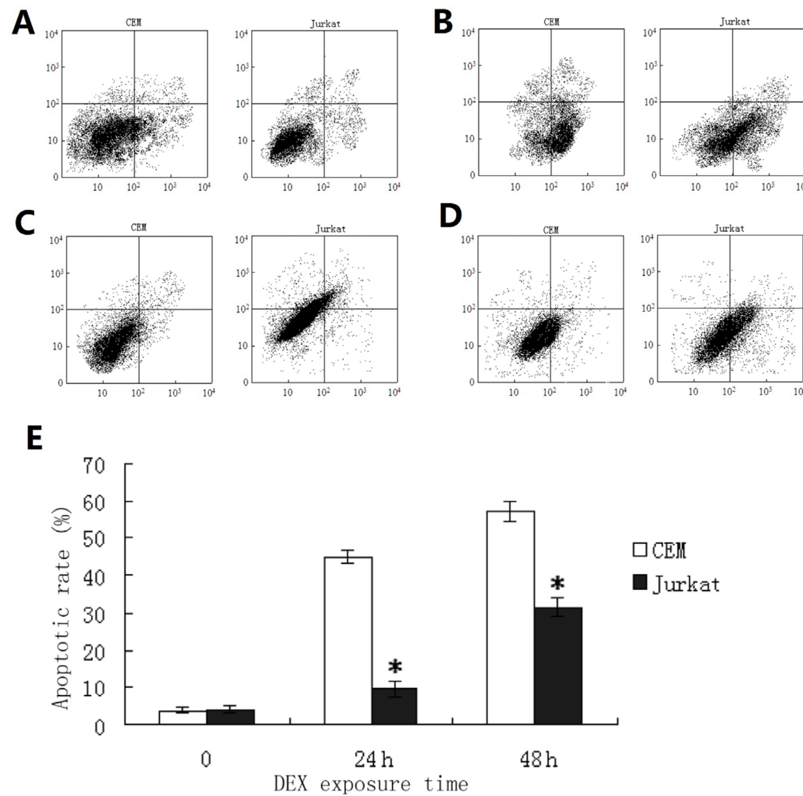

Figure 1. Apoptosis of CEM and Jurkat cell lines. **A.** After exposure to 0 μM DEX (without annexin V-FITC, blank control). **B.** After exposure to 0 μM DEX (negative blank control). **C.** After exposure to 100 μM DEX for 24 h. **D.** After exposure to 100 μM DEX for 48 h. **E.** Apoptosis of CEM and Jurkat cell lines. Compared with CEM group, *$P < 0.05$.

Flow cytometry showed that the 2 cell lines had clear differences in the apoptosis induced by DEX ($P < 0.01$); Jurkat cell lines exhibited poor sensitivity to DEX. Consistent with the MTT results, Jurkat cells showed clear apoptosis after exposure to 100 μM DEX for 48 h. Table 3 shows the expression of *TEL-AML1* gene in 100 μM DEX exposure for 48 h.

Table 3. Apoptosis rates of CEM and Jurkat cell lines induced by DEX.

Exposure time	DEX concentration (μM)	CEM	Jurkat
0 h	0	3.7 ± 0.8	4.1 ± 0.9
24 h	100	44.8 ± 2.7	9.7 ± 1.1*
48 h	100	57.3 ± 3.6	31.6 ± 2.9*

Compared with CEM group, *$P < 0.05$.

mRNA expression level of *TEL-AML1* fusion gene detected by reverse transcription-PCR

In this study, we examined a blank control (phosphate-buffered saline without cells), negative control (bone marrow mononuclear cells from a child with non-malignant hematologic disease), unexposed group (CEM and Jurkat cell lines), and exposed group (2 cell lines were exposed to 100 μM DEX for 48 h). Electrophoresis of samples from blank control wells showed no DNA bands, while negative controls only showed internal reference DNA bands. The unexposed and exposed groups showed inference DNA bands and target DNA bands. Software analysis results suggested that in the exposed group, the *TEL-AML1* expression in Jurkat cells was higher than that in CEM cells ($P < 0.05$). In the unexposed group, *TEL-AML1* gene expression in Jurkat cells was not affected by GC exposure ($P > 0.05$), while that of CEM cells showed significant differences before and after exposure ($P < 0.05$), as shown in Figure 2 and Table 4.

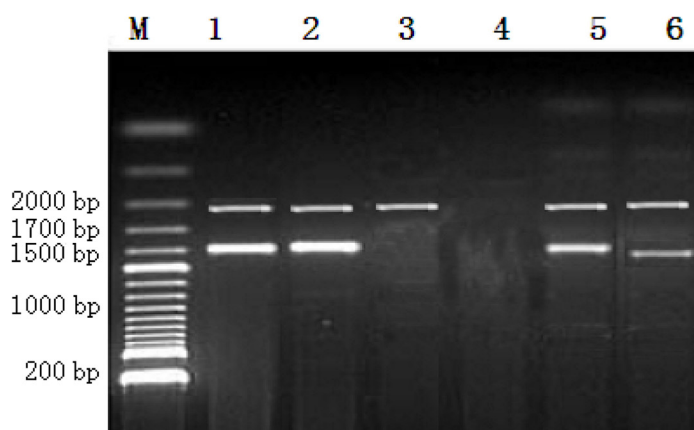

Figure 2. Reverse transcription-PCR results. *Lane M* = molecular marker; *lane 1* = unexposed group (Jurkat cell line); *lane 2* = unexposed group (CEM cell line); *lane 3* = negative control; *lane 4* = blank control; *lane 5* = exposed group (Jurkat cell line); *lane 6* = exposed group (CEM cell line).

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Table 4. Differences in mRNA expression levels.

Group	CEM	Jurkat
Unexposed group	9.14 ± 0.87	8.42 ± 0.88
Exposed group	9.03 ± 0.86	1.77 ± 0.24*#

mRNA expression comparison of the two cell lines in exposed condition, *P < 0.05; mRNA expression comparison of the CEM cell line before and after exposure, #P < 0.05.

Protein level of *TEL-AML1* fusion gene detected by western blotting

In this study, we investigated the protein expression of TEL-AML1 in the 2 cell lines and the effect of DEX exposure on expression. Reverse transcription-PCR results showed that the negative control and the blank control did not express the *TEL-AML1* gene. Therefore, at the protein level, we examined TEL-AML1 expression in the exposed and unexposed groups. Western blotting results suggested that the unexposed and exposed groups showed TEL-AML1 protein expression; in the exposed group, TEL-AML1 protein expression in Jurkat cells was higher than that in CEM cells (P < 0.05). In the unexposed group, TEL-AML1 protein expression in Jurkat cells was not affected by GC exposure (P > 0.05), while CEM cells showed significant differences before and after exposure (P < 0.05), as shown in Figure 3 and Table 5.

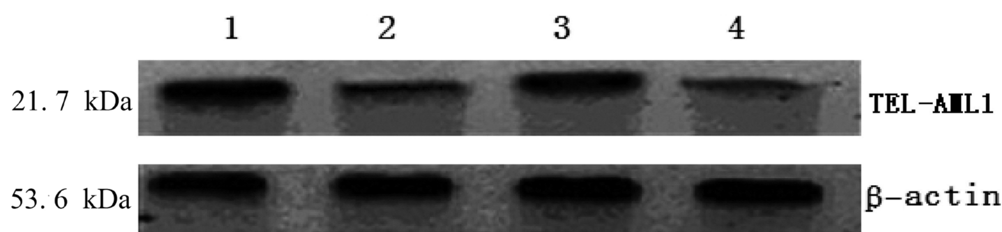

Figure 3. Western blot results. *Lane 1* = Unexposed group (Jurkat cell line). *Lane 2* = Unexposed group (CEM cell line). *Lane 3* = Exposed group (Jurkat cell line). *Lane 4* = Exposure group (CEM cell line).

Table 5. Differences in protein levels.

Group	CEM	Jurkat
Unexposed group	7.36 ± 0.73	6.37 ± 0.68
Exposed group	7.14 ± 0.76	0.98 ± 0.24*#

Protein expression comparison of the two cell lines in exposed condition, *P < 0.05. Protein expression comparison of the CEM cell line before and after exposure, #P < 0.05.

DISCUSSION

GC is a steroid hormone secreted by the adrenal cortical zona fasciculata. Since its regulatory function in carbohydrate synthesis and metabolism was first identified, this steroid hormone was named “GC” (Schwartz et al., 2012). GC shows effects in immuno-suppression, anti-inflammation, and anti-shock, among others. As a very effective therapeutic drug, it is

widely used to treat variety of diseases (Mansha et al., 2012). Bhadri et al. (2011) found that GC had a dissolving effect on lymphocytes. Thereafter, researchers used GC to treat childhood leukemia. Other studies revealed that GC was partially or completely effective in more than 80% of newly diagnosed ALL children as monotherapy; however, GC showed poor efficacy for recurrent ALL and acute myeloid leukemia (Namazi et al., 2011). Because it can effectively induce the apoptosis of ALL cells, GC is described as a core drug throughout the chemotherapy of ALL treatment in various major guides (Heidari et al., 2010); for example, the Berlin-Frankfurt-Muster plan employs 7 days of prednisone monotherapy induction test as an independent risk factor to evaluate the prognosis of newly diagnosed children (Carlet et al., 2010). Children with leukemia are insensitive to GC treatment (GC-resistant). This situation is a sign of poor efficacy, bad prognosis, and requirement of highly intensive chemotherapy (Beesley et al., 2010). Currently, the GC-resistance mechanism is not well understood. Therefore, exploration of the GC-resistant mechanism has been a hotspot in studies of leukemia therapy.

The GC-resistance mechanism is complex; however, it is closely related to the GC-induced apoptosis pathway (Geng and Vedeckis, 2010). In general, the GC-induced apoptosis pathway is generated by the process in which GC enters into the cytoplasm and forms a polymer by combining with a cytoplasm receptor (glucocorticoid receptor, GR). When the heat shock protein is isolated from the polymer, the polymer becomes activated. By activating the glucocorticoid response element in the nucleus, apoptosis genes are activated by transcription or regulatory factors (nuclear factor-κB or activator protein-1) and transcription is activated. Expression of growth genes is thereby inhibited, resulting in cell apoptosis (Zhao et al., 2011). Studies confirmed that an anomaly at any link in this pathway can lead to GC-resistance (Bachmann et al., 2010). Based on relationship between this pathway and GR, researchers divided the pathway into an upstream pathway (anomaly before GR, abnormal expression of GR gene, and anomaly of related protein) and a downstream pathway (target gene anomaly affected by GC, and other signal pathways that can interfere GC reaction) (Wasim et al., 2010). Anomalies in the apoptosis pathway can interfere in the transfer of the leukemia cell apoptosis signal. Thus, leukemia cells can escape apoptosis induced by a gene or drug to generate GC resistance (van Galen et al., 2010).

In this study, GC-sensitive (CEM cell line) and GC-resistant (Jurkat cell line) T-series leukemia cell lines were examined. The GC unexposed group and the GC exposed group were established to investigate whether GC-sensitive and GC-resistant leukemia cells showed different expression levels in initial diagnosis and treatment. The relationship between TEL-AML1 and GC resistance is also discussed. In the exposed group, *TEL-AML1* fusion gene expression in the GC-resistant ALL cell line was higher than that in the GC-sensitive cell line. This implies that the *TEL-AML1* fusion gene expression level was associated with the sensitivity of ALL cells in the initial state and initial diagnosis. The *TEL-AML1* fusion gene expression level may be helpful for the hierarchical diagnosis of leukemia at initial diagnosis. In addition, we examined whether GC exposure could influence *TEL-AML1* fusion gene expression. Because the currently published *TEL-AML1* fusion gene sequence does not contain the glucocorticoid response element, the *TEL-AML1* fusion gene is not directly regulated by GC-GR complexes in theory. von Goessel et al. (2009) showed that DEX could downregulate the expression of the *TEL-AML1* fusion gene and thereby induce the apoptosis of CEM cells. It is likely that the apoptosis of CEM cells was induced by downregulation of *TEL-AML1* expression through indirect or alternative pathways.

The overexpression of the *TEL-AML1* fusion gene in leukemia may overcome the down-regulation of DEX and enable leukemia cells to resist apoptosis and drug treatment (Wiemels et al., 2008). Thus, we compared *TEL-AML1* fusion gene expression levels in the GC-sensitive cell line and GC-resistant cell line in the exposed group. By studying changes in *TEL-AML1* fusion gene expression, we found that *TEL-AML1* fusion gene expression was higher in the GC-resistant cell line than in the GC-sensitive cell line. After GC exposure, *TEL-AML1* fusion gene expression in the GC-sensitive cell line significantly decreased with increased GC exposure concentration. Gandemer et al. (2010) reported that *TEL-AML1* fusion gene expression in GC-resistant cell lines was not affected by GC exposure and was sustained at a high level.

Chemotherapy is the main therapy used to treat childhood ALL. Using individualized chemotherapy based on risk stratification, the cure rate of childhood ALL has increased to approximately 80% (Bonapace et al., 2010). GC-resistance is an important stratification index for the poor efficacy of childhood ALL (Schwartz et al., 2010). TEL-AML1, as an anti-apoptotic protein, is closely related to the drug resistance of tumors (Nicholson et al., 2010). Our results revealed and association between GC-resistance and *TEL-AML1* fusion gene expression. We found that the sustained high expression of the *TEL-AML1* fusion gene in ALL cells may be one of the mechanisms of GC-resistance. Following additional studies, TEL-AML1 is expected to become a new marker or a new target that can be used to reverse GC-resistance in the leukemia hierarchical diagnosis.

The expression differences in the *TEL-AML1* fusion gene in GC-sensitive and GC-resistant leukemia cell lines may be related to the sensitivity of leukemia cells to GC in the initial state or at initial diagnosis. The level of *TEL-AML1* fusion gene expression may contribute to the risk stratification of leukemia in initial diagnosis; inhibition of *TEL-AML1* expression provides a new therapeutic approach to reverse GC-resistance.

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