Knockdown of DGKZ induces apoptosis and G2/M phase arrest in human acute myeloid leukemia HL-60 cells

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Abstract
Diacylglycerol kinase zeta (DGKZ) is associated with the pathogenesis of a variety of malignant diseases, but its biological function on acute myeloid leukemia (AML) has not been explored. This study was aimed to analyze apoptosis induced by Knockdown of DGKZ and its mechanism in human acute myeloid leukemia HL-60 cells. In the present study qRT-PCR was used to detect the expression of DGKZ in HL-60, THP-1, K562, H9, Jurkat and CD34 cell lines. DGKZ-shRNA lentiviral vector was established and used to infect acute myeloid leukemia HL-60 cells. Cell Counting Kit-8 (CCK-8) assay was used to determine the viability of HL-60 cells DGKZ knocked down. Apoptosis and cell cycle phase of HL-60 cells after DGKZ knockdown were evaluated by flow cytometry. Western blot analysis was performed to investigated the protein expression related to apoptosis and cell cycle. Results showed DGKZ expression were stable and higher in Jurkat, HL-60, THP-1, K562 leukemia cells than those of H9 and CD34 cells. Compared with cells of the shCtrl group, DGKZ was markedly knocked down in cells which were transfected with lentivirus encoding shRNA of DGKZ in HL-60 cells. DGKZ knockdown significantly inhibited the proliferation and induced cycle arrest at the G2/M phase in HL-60 cells. Western blot results indicated the expressions of caspase-3, caspase-8, and survivin markedly increased in HL-60 cells after knockdown of DGKZ. The results suggest Knockdown of DGKZ can inhibit proliferation of acute myeloid leukemia HL-60 cell caused cell cycle arrest at the G2/M phase through caspases pathway.

Keywords: DGKZ; apoptosis; G2/M phase arrest; HL-60
Introduction

Acute myeloid leukemia (AML) is a malignant disease group with an idiosyncratic nature and its incidence tends to increase year by year. Leukemia poses a major threat to the survival of patients, and researchers have been working on understanding the pathogenesis of the disease \(^1\)-\(^3\). However, the mechanism remains unclear and a number of studies have suggested that there is a significant correlation between disease and multiple genes \(^4\)-\(^6\).

Several studies have suggested that diacylglycerol kinase zeta (DGKZ) is associated with the pathogenesis of a variety of malignant diseases, such as acquired aplastic anemia, gastric cancer, and neuroblastoma \(^7\)-\(^9\). However, its biological function in AML has not been studied.

DGKZ, which is encoded by *DGKZ*, is a subtype of DGK-IV \(^10\). It is thought to act in cell cycle regulation, where diacylglycerol (DGA) acts as an intracellular second messenger \(^11\). It can specifically activate protein kinase C and protein tyrosine kinase, which further activate signal transduction pathways through protein phosphorylation and ultimately affect cell proliferation as well as differentiation \(^12\). DGKZ is involved in transient ischemic attack, liver function, and myocardial pathological damage repair, showing its protective effects in different cells. Research shows that DGKZ has a role in the regulation of glioma, gastric cancer, liver cancer, and the pathological process of colon cancer \(^13\)-\(^16\).

The purpose of this study is to evaluate the effect of DGKZ knockdown on cell proliferation and apoptosis in leukemia HL-60 cells, which is helpful for exploring therapeutic strategies targeting leukemia.

Materials and Methods

Materials

Human leukemia cell lines HL-60, THP-1, K562, H9, Jurkat and CD34 cells were purchased from the Institute of Hematology at the China Academy of Chinese Medical Sciences (Beijing, China). RPMI-1640 media, fetal bovine serum (FBS) were purchased from Gibco Co., Ltd (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT), propidium iodide (PI), Propidium Iodide, dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich China, Inc. (Shanghai, China). Mouse flag antibody (catalogue No.F1804) was purchased from Merck Life Science (Shanghai) Co., Ltd. (Shanghai, China). Mouse anti-human GAPDH (catalogue No.sc-32233) were purchased from Mouse anti-human Caspase-3 (catalogue No.sc-65496), caspase-8 (catalogue No.sc-70502) and survivin (catalogue No.sc-73082) Santa Cruz Biotechnology, Inc. (CA, USA).

Cell culture

HL-60, THP-1, K562, H9, Jurkat and CD34 cells were obtained from the Institute of Hematology of China Academy of Chinese Medical Sciences (Beijing, China). All cell lines were cultured with RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Lentivirus (Lv) packaging and infection

For DGKZ knockdown, lentivirus expressing short hairpin RNA (shRNA) targeting the sequence of DGKZ was constructed by Genechem Co. Ltd (Shanghai, China). The interference sequence against DGKZ gene were as follows: 5′-CTCTGAAAGCAAGCAAGAA-3′. The primers for identification positive cloning were as follows: 5′-CCTATTTCCCATGATTCCTTCATA-3′ (Forward) and 5′-GTAATACGGTTATCCACGCG-3′ (Reverse). The sequence control shRNA (Negative Control, NC) was as follow: 5′-TTCTCCGAACGTGTCACGT-3′. The recombinant plasmid containing the DGKZ-shRNA sequence was transformed in E. coli to amplify the plasmid. PCR identification and sequence comparison were performed on positive clones.

Lentiviruses were generated by triple transfection of 80% confluent 293T cells with GV115 plasmid vector, Helper 1.0 and Helper 1.0 helper plasmids using Lipofectamine 2000 according to the manufacturer’s procedure. Then, lentiviral particles were harvested by ultracentrifugation for 10 min, filtered through a 0.45 mm filter, and centrifuged again for 15 min. HL-60 cells were collected for immediate transfection and plating in 6-well plates at 1×10⁶, 2×10⁶, and 3×10⁶ cells per well. 200μl transfection reagent was added dropwise to the suspension cells and gently plated with 1mL pipettes. Six-well plates
were then placed in an incubator for 12 hs and replaced with fresh medium. The infection efficiency was observed after 96 hs through a fluorescence microscope for the green fluorescence protein (GFP) expression.

**Identification of DGKZ expression by qRT-PCR**

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA). Samples were collected for Trizol cleavage by centrifuging the samples for 5 min at 2,000 rpm and adding 1 ml of Trizol to the cell supernatant. Samples precipitated after being allowed to mix for 5 min at room temperature, subsequently, samples were transferred to a new 1.5 mL tube. Then, cDNA was obtained by reverse transcription using the Promega M-MLV Kit. RNA reverse transcription was performed and quantitative real-time PCR (qRT-PCR) was used to detect expression. The primer sequences for DGKZ detection were as follows:

5′- AGCAAGCAAGAAGAAGAGG -3′(Forward) and
5′-GGATTGAGATACCAGAGGAAAGAC-3′(Reverse).

The primer sequences for reference gene (GAPDH) were as follows:

5′-TGACTTCACAGCGACACCCA-3′(Forward)
and 5′- CACCTGTGTGCTGTAGCCAAA -3′(Reverse).

**Western blot**

HL-60 cells were lysed after lentivirus infection and centrifuged 12,000 ×g for 15 min at 4 °C. Then, total protein was extracted from the resulting supernatant and the concentration was quantified by the bicinchoninic acid assay (BCA assay). Equal amounts (30 μg) of protein were separated by 10% SDS-polyacrylamide gel, followed by transfer onto PVDF membranes. After blocking, the membranes were treated overnight at 4 °C with rabbit monoclonal anti-human GAPDH, and Flag primary antibodies(1:2000 dilution). This was followed by incubation with appropriate horseradish Peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1h and detection was achieved with an enhanced chemiluminescence (ECL) kit (GE Healthcare).

**Detection of CCK-8 cell viability**

Cell Counting Kit-8 (CCK-8) assay was used to detect cell viability following DGKZ knockdown. The CCK-8 assay was performed with 1×10⁴ cells /well in 96-well plates. After culture for 1, 2, 3, 4, and 5 days, 10 μl CCK-8 was added and cells were cultured
for another 4 h. The cell suspensions were vortexed for 5 min, after which the absorbance was read at 450 nm and cell proliferation rate was determined.

**Apoptosis assessment**

Apoptosis of HL-60 cells with DGKZ knockdown and control were evaluated with the AnnexinV-FITC kit (BD Co. Ltd, USA) according to the manufacturer’s instructions. Analysis was performed by flow cytometry (BD Bioscience, USA) with the CellQuest software (BDIS).

**Cell cycle detection**

HL-60 cells with DGKZ knockdown (1×10⁶/well) in 6-well plates were incubated in a humidified incubator at 37 °C with 30% humidity and 5% CO₂ for 24 hr. Then, the cells were fixed with 70% ethanol overnight, and incubated with 1 ml of Propidium (PI) solution (20 μg/ml in PBS with 1% Triton X-100) containing RNaseA at 37°C in the dark for 30 min. Samples were assessed by flow cytometry (BD Bioscience, U.S.A.) with the CellQuest software (BDIS).

**Relative caspase-3 activity detection**

Caspase-Glo®-3/7 Assay (Invitrogen, USA) was used to detect apoptosis by measuring the activity of caspase-3. HL-60 cells with DGKZ knockdown (1×10⁴/well) in 6-well plates were incubated in a humidified incubator at 37 °C with 30% humidity and 5% CO₂ for 72 hr. Then, 100 μl Caspase-Glo3/7 was added and vortexed for 30 min at 500 rpm. The cell suspension was incubated at room temperature for 2 hs, absorbance was obtained, and activity of caspase 3 was determined.

**Intracellular signaling array**

Cell lysates were prepared as mentioned above and total proteins were isolated. Intracellular signaling molecules were detected using a PathScan intracellular signaling array kit (Cell Signaling Technology) according to the manufacturer’s procedure.

**Statistical analysis**

Data were presented as mean ± standard deviations (SD) from at least three independent experiments. Statistical analysis was performed by Student’s t-test. \( p < 0.05 \) was considered statistically significant.
Results

Expression of DGKZ in different leukemia cell lines
The expression of DGKZ was detected in four pairs of human myeloid leukemia cells by qRT-PCR. The abundance of DGKZ gene expression in Jurkat, HL-60, and K562 cell lines were higher and their ΔCt values were 9.86±0.270, 7.05±0.055 and 8.88±0.070, respectively. The abundance of DGKZ gene expression in THP-1 cell line was medium level and the ΔCt value was 15.65±0.035. The abundance of DGKZ gene expression in H-9 cell line and CD34 cell were lower and their ΔCt values were 19.40±0.058 and 21.34±0.034.(Fig. 1)

Expression of DGKZ was suppressed by Lv-shDGKZ in HL-60 cells
HL-60 cells were infected with lentivirus vector LV-shDGKZ and green fluorescence signals were observed by a fluorescent microscope after 72 hs. The result showed that more than 72.9% HL-60 exhibited GFP expression after shRNA lentivirus infection, indicating the high infection efficiency (Fig. 2a) (p<0.05). In order to determine the knockdown effect of DGKZ with lentivirus, the mRNA and protein levels of DGKZ in HL-60 infected with LV-shDGKZ and LV-shCtrl were detected through qRT-PCR and western blot. The results showed that mRNA and protein levels of DGKZ in HL-60 infected with LV-shDGKZ were significantly suppressed as compared with LV-shCtrl group (Fig. 2 b,c,d). The data confirmed that DGKZ expression was successfully knocked down in HL-60 cells by LV-shDGKZ infection.

DGKZ knockdown inhibited HL-60 cell proliferation
After shRNA-lentivirus infection, 2,000 cells were plated on 96-well plates and cultured in a humidified incubator at 37 °C with 30% humidity and 5% CO₂ for 5 days. The absorbance of the wavelength of 450nm of HL-60 cell shIARS2 group and control group (shCtrl) were evaluated with the microplate reader every day and proliferation rates were calculated. Results showed that the proliferation rate of HL-60 cell infected with LV-shDGKZ was significantly lower than control since the 3th day. The result suggested that DGKZ was significantly associated with the proliferation of HL-60 cells (Fig. 3).

DGKZ knockdown induced apoptosis of HL-60 cells
Apoptosis of HL-60 cells infected with LV-shDGKZ and LV-shCtrl was evaluated with the Annexin V-FITC kit and flow cytometry. As shown in Fig. 4, apoptotic rates were 3.37±0.14 in control group and 14.86±0.52 in HL-60 cells after DGKZ knockdown respectively. These findings indicated DGKZ knockdown can induce apoptosis in HL-60 cells.

**DGKZ knockdown arrests HL-60 cells at the G2/M phase**

To reveal the mechanism of growth suppression effect following DGKZ knockdown, the cell cycle distributions of HL-60 cells were performed using flow cytometry. The results showed percentage of G2/M phases in DGKZ knockdown cells (30.58±0.586) was markedly higher compared to control (22.74±0.345) (p < 0.05). While the percentage of S phases in DGKZ knockdown cells (34.79.58±0.561) was much lower than control (40.15±0.262)(p < 0.05). (Fig. 5)

**DGKZ knockdown promotion the activities of Caspase3 in HL-60 cells**

To investigate the apoptotic effect of DGKZ knockdown, relative caspase-3 activity in HL-60 infected with LV-shDGKZ and LV-shCtrl was measured. The results showed the activity of caspase3 in HL-60 infected with LV-shDGKZ was significantly higher than that of LV-shCtrl group (p<0.05) (Fig. 6).

**Regulation of signaling molecules in DGKZ knockdown cells**

To further illuminate the molecular mechanisms by which DGKZ affects HL-60 cells, a PathScan® Antibody Array Kit was used to detect changes in signaling molecules in HL-60 cells before and after DGKZ knockdown. The results showed that expression of ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK/JNK were significantly down-regulated in HL-60 cells after RNAi against DGKZ. Meanwhile, expression levels of PARP, caspase-3, and Survivin were up-regulated in HL-60 cells after RNAi against DGKZ. The data indicated that DGKZ knockdown could significantly inhibit the growth of HL-60 cells via blockade of anti-apoptotic genes and promotion of apoptosis (Fig.7).

**Expression of Caspase-3, Caspase-8 and Survivin**

To further unveil the mechanism of DGKZ knockdown-induced apoptosis, various key effector of programmed cell death were quantified by western blot. The results showed caspase-3, caspase-8, and survivin protein levels in HL-60 infected with
LV-shDGKZ were much higher than those of LV-shCtrl group.

Discussions

The pathogenesis of AML has been unclear and every effort to increase our understanding of AML pathogenesis is of great significance\textsuperscript{17-18}. DGKZ is associated with a variety of malignant diseases in humans\textsuperscript{19-20} and is also related to proliferation and apoptosis of T cells in the blood system\textsuperscript{21-23}. With the popularity of precision medical advancement, stem cell technology, cell therapy technology, and the progress of new drugs, survival will greatly enhance in cancer patients. Furthermore, certain cancers may be avoided through preventive treatment, while some may even be completely cured. Whether cancer can be completely overcome remains a question that can only be answered by time. However, technological breakthroughs remain a strategic turning point.

Recently, several studies have shown that DGKZ plays a certain role in multiple system diseases suggesting that the gene is relevant to the pathogenesis and prognosis of a variety of diseases. Studying this emerging gene may bring new hope to those seriously ill. We conducted a series of related experiments to investigate the expression level of DGKZ gene in various cell strains of the leukemia and cytologically the effect of DGKZ knockdown on proliferation, apoptosis and cell cycle. Existing research has showed that expressions of DGKZ in Jurkat, HL-60, and K562 cell lines were higher than those of H-9 and CD34 cell lines, suggesting that DGKZ could be a bio-marker of leukemia. After DGKZ knockdown, the proliferation rate of HL-60 cell infected with LV-shDGKZ was significantly lower than control since the 3th day and the number of apoptotic leukemic cells increased along with a decline in the total number of cells. These results indicated DGKZ is associated to proliferation and DGKZ knockdown can induce apoptosis of HL-60 cells.

To reveal the mechanism of growth suppression effect following DGKZ knockdown, the cell cycle distributions of HL-60 cells were assayed by flow cytometry. Results showed percentage of G2/M phases markedly increased while the percentage of S phases decreased in DGKZ knockdown cells. These results suggest DGKZ knockdown could
induce G2/M phase arrest of HL-60 cells. Following DGKZ knockdown, the expression of ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK /JNK in the stress and apoptosis pathways were significantly up-regulated in HL-60 cells after DGKZ knockdown. Meanwhile, expression levels of PARP, caspase-3, and survivin were up-regulated in HL-60 cells knocked down DGKZ.

In conclusion our experimental results indicated DGKZ knockdown could inhibit proliferation of acute myeloid leukemia HL-60 cells and caused cell cycle arrest at the G2/M phase arrest by regulating Caspases pathways. It suggest that DGKZ be a novel target gene for the treatment of leukemia.

Footnote
Conflicts of Interest: The authors have no conflicts of interest to declare.

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Figure legend

Figure 1. Expression of DGKZ in different leukemia cell lines. Jurkat, HL-60, THP-1, K562, and H9 cell lines were cultured in complete growth medium and mRNA expression of DGKZ was detected by qRT-PCR. *p<0.05, **p<0.01 and ***p<0.001 versus shDGKZ.

Figure 2. Expression of DGKZ was suppressed by Lv-shDGKZ in HL-60 cells. (a) HL-60 cells exhibited green fluorescence after lentivirus infection. (b) Lv-shDGKZ significantly decreased mRNA expression of DGKZ compared to Lv-Ctrl group in HL-60 cells. (c) Lv-shDGKZ significantly decreased protein expression of DGKZ compared to Lv-Ctrl group in HL-60 cells. (d) The histograms of protein expression of DGKZ after DGKZ knockdown and control. Lv-shDGKZ was constructed and HL-60 cells were infected. Green fluorescence was observed under microscope after 24hr. qRT-PCR and western blot were performed to detected mRNA and protein expression of DGKZ in HL-60 cells after DGKZ knockdown and control. **p<0.01 versus shDGKZ.

Figure 3. DGKZ knockdown inhibited the proliferation of HL-60 cells. HL-60 cells that were infected with lentivirus vectors were cultured and CCK-8 assay was used to detect proliferation rate of HL-60 cells. *p<0.05 and **p<0.01 and ***p<0.001 versus shDGKZ.

Figure 4. DGKZ knockdown induced apoptosis of HL-60 cells. (a) Annexin V-FITC/PI staining was performed on HL-60 cells after lentivirus infection and samples were analyzed by flow cytometry. (b) The histograms of apoptotic cell percentages of HL-60 cells after lentivirus infection and control. *p<0.05 versus shDGKZ.

Figure 5. DGKZ knockdown induced cell cycle arrest in HL-60 cells. (a) Propidium staining was performed on HL-60 cells after lentivirus infection and samples were analyzed by flow cytometry. (b) The histograms of cell number percentages of HL-60 cells at different phases after lentivirus infection and control. *p < 0.05 versus shCtrl.
Figure 6. DGKZ knockdown activated caspase-3 in HL-60 cells. HL-60 cells after DGKZ knockdown were cultured and relative caspase-3 activity of was determined by Caspase-Glo®-3/7 Assay. *p < 0.05 versus shCtrl.

Figure 7. The apoptotic signaling pathway involved in DGKZ. HL-60 cells were cultured after lentivirus infection and PathScan intracellular signaling array kit was used to detect the changes of signaling molecules. (a) The original picture of the PathScan Intracellular Signaling Array results. (b) The histograms of relative protein level of HL-60 cells after lentivirus infection and control. *p < 0.05 versus shCtrl.

Figure 8. Expression of caspase-3, caspase-8 and survivin in HL-60 cells. (a) The images of protein expression in HL-60 cells after DGKZ knockdown and control. (b) The histograms of protein level of HL-60 cells after lentivirus infection and control. *p < 0.05 versus shCtrl.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(a) Comparison of YEL-B-ALin fluorescence area between shCtrl and shDGKZ groups. The histograms show the distribution of fluorescence intensity across different cell populations.

(b) Percentage distribution in G1, S, and G2/M phases between shDGKZ and shCtrl groups. The bars indicate a significant difference (*) in the G2/M phase, suggesting an alteration in cell cycle progression.
Figure 6
Figure 7
Figure 8