Original Article
Small interfering RNAs interacting with DJ-1 affect leukemia cell proliferation, apoptosis, and adriamycin resistance by regulating PTEN-PI3K/AKT pathways

Donghu Wen¹, Jia Liu¹, Dongji Cui², Xiaoxin Wang³

¹Department of Hematology, The First Affiliated Hospital of Qiqihar Medical School, Qiqihar City, Heilongjiang, China; ²Department of Infectious Disease, The First Affiliated Hospital of Qiqihar Medical School, Qiqihar City, Heilongjiang, China; ³Department of Nuclear Medicine, The First Affiliated Hospital of Qiqihar Medical School, Qiqihar City, Heilongjiang, China

Received November 18, 2018; Accepted January 6, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: Phosphatase and tensin homologues deleted on chromosome ten (PTEN)-phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathways widely participate in regulating cell proliferation, cycle, apoptosis, and invasion. DJ-1/Parkinson gene 7 (PARK7) is a negative regulator of PTEN involved in affecting tumorigenesis, progression, and drug resistance. However, it is unclear whether DJ-1 abnormalities play a role in mediating leukemia drug resistance. The current study investigated the roles of DJ-1 in mediating doxorubicin (ADR) resistance in leukemia cells. An ADR resistant HL-60/ADR cell line was established. DJ-1 and PTEN mRNA and protein expression was tested and compared. Cell viability was determined by CCK-8 assay. HL-60/ADR cells were divided into the siRNA-NC group and siRNA-DJ-1 group. DJ-1, PTEN, and p-AKT expression was detected. Cell apoptosis was evaluated via flow cytometry. Cell proliferation was assessed using EdU staining. Caspase-3 activity was detected using the appropriate kit. Compared with HL-60 cells, DJ-1 expression was significantly increased and PTEN levels were obviously reduced in HL-60/ADR cells. Under the same dose of ADR treatment, the proliferation activity of HL-60/ADR cells was markedly higher than in HL-60 cells. Compared with the siRNA-NC group, DJ-1 and p-AKT expression was detected. Cell apoptosis was evaluated via flow cytometry. Cell proliferation was assessed using EdU staining. Caspase-3 activity was detected using the appropriate kit. Compared with HL-60 cells, DJ-1 expression was significantly increased and PTEN levels were obviously reduced in HL-60/ADR cells. Under the same dose of ADR treatment, the proliferation activity of HL-60/ADR cells was markedly higher than in HL-60 cells. Compared with the siRNA-NC group, DJ-1 and p-AKT levels apparently declined, while PTEN expression was significantly upregulated in the siRNA-DJ-1 group. Caspase-3 activity was enhanced, cell apoptosis was increased, and cell proliferation was attenuated when treated by ADR. Increased expression of DJ-1 plays a role in regulating PTEN-PI3K/AKT pathway activity and mediating ADR resistance in leukemia HL-60 cells, while interference with DJ-1 expression reduces ADR resistance in leukemia HL-60 cells.

Keywords: Leukemia, ADR, resistance, DJ-1, PTEN-PI3K/AKT

Introduction
Leukemia is a group of heterogeneous hematopoietic stem cell malignant clonal diseases caused by differentiation blockage, apoptosis disorders, and malignant proliferation at different stages of hematopoietic stem/progenitor cell differentiation [1-3]. Chemotherapy is the first choice for treatment of leukemia. However, leukemia has the characteristics of easy recurrence and drug resistance, reducing the effects of chemotherapy and seriously affecting patient lives [4-6].

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB, protein kinase B) signaling pathways are widely expressed in a variety of cells, participating in the regulation of biological processes, including cell growth, survival, and apoptosis [7]. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a negative regulator of PI3K/AKT signaling pathways. Thus, it participates in cell proliferation, apoptosis, metastasis, and drug resistance in lung cancer [8], breast cancer [9], and gastric cancer [10]. DJ-1 is a negative regulator of PTEN that activates PI3K/AKT signaling pathways through inhibiting PTEN. It is, therefore, involved in reducing cell apoptosis, promoting cell proliferation, and regulating survival and progression [11]. Abnormally elevated expression of DJ-1 plays a role in mediating drug resistance in various tumor cells, such as lung cancer [12, 13], cervical cancer [14], and pancreatic cancer
DJ-1 affects leukemia [15]. However, whether DJ-1 abnormalities mediate leukemia drug resistance remains unclear. The current study established a leukemia Adriamycin (ADR)-resistant cell line and compared expression of DJ-1 in the parental leukemia cells, exploring the roles of DJ-1 in leukemia ADR resistance.

Materials and methods

Main reagents and materials

Human normal peripheral blood mononuclear cells (PBMCs) were purchased from Beijing Beina Biological Co. Leukemia HL-60 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. RPMI 1640 was purchased from Hyclone. Opti-MEM and fetal bovine serum (FBS) were purchased from Gibco. TRIzol Reagent and Lipo2000 were purchased from Invitrogen. QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen. siRNA-NC and siRNA-DJ-1 were purchased from Gene Pharma. Rabbit anti-human polyclonal DJ-1 and PTEN antibodies were purchased from Abcam. Rabbit anti-human p-AKT and β-actin antibodies were purchased from CST. Goat anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson ImmunoResearch. The CCK-8 kit was purchased from Solarbio. Annexin V-FITC/PI apoptosis kit was purchased from Bestbio. EdU Flow Cytometry kit was purchased from Sigma. ADR was purchased from Sellceck and the BCA protein quantification kit was purchased from Beyotime.

Cell culture

PBMC and HL-60 cells were cultured in RPMI 1640 medium containing 10% FBS at 5% CO2 and 37°C. Cells in the log phase were subjected to experimentation.

ADR resistant cell line modeling

HL-60 cells in the logarithmic phase were treated with ADR from 0.01 μM for 2 weeks. The cells continued growing and were treated by increasing concentrations of ADR, up to 1.6 μM when the cells could stably grow in ADR. Finally, the cells were stably passaged in ADR to obtain ADR resistant leukemia cell line HL-60/ADR.

CCK-8 assay

HL-60 and HL-60/ADR cells were seeded in 96-well plates at 10000/well and treated with different concentrations (0, 0.01, 0.1, 1, 10, 100 μM) of ADR for 48 hours. Next, the cells were added with CCK-8 to measure absorbance values (A450). Relative viability = (A450 in drug group - A450 in blank group)/(A450 in control - A450 in blank group) × 100%.

Cell transfection and EdU staining

HL-60/ADR cells were divided into the siRNA-NC group and siRNA-DJ-1 group. After incubating in 10 μM EdU at 37°C for 2 hours, the cells were seeded in 6-well plates and treated with 1.6 μM ADR. After incubation for 48 hours, the cells were fixed in paraformaldehyde. Next, the cells were incubated in 100 μL TritonX-100 at room temperature and in 500 μL reaction fluid at room temperature, void of light, for 30 minutes. Lastly, the cells were tested using FC-500MCL flow cytometry.

qRT-PCR

Total RNA was extracted using TRIzol. QuantiTect SYBR Green RT-PCR Kit was used for qRT-PCR. The PCR reaction system was composed of 10.0 μL 2 × QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL forward primer (0.5 μmol/L), 1.0 μL reverse primer (0.5 μmol/L), 2.0 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH2O. The reaction was performed on Bio-Rad CFX96 at 45°C for 5 minutes and 94°C for 30 seconds. This was followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds.

Western blotting

Total protein was extracted from cells using RIPA. After quantification using the BCA method, a total of 40 μg protein was separated by SDS-PAGE and transferred to PVDF membranes at 300 mA for 90 minutes. After blocking with 5% skim milk at room temperature, the membranes were incubated in primary antibodies at 4°C overnight (DJ-1, PTEN, p-AKT, and β-actin at 1:2000, 1:2000, 1:1000, and 1:8000, respectively). After washing with PBST, the membranes were further incubated in HRP conjugated secondary antibodies at room temperature for 60 minutes (1:10000). Finally, the membranes were treated with the ECL chemiluminescence reagent and developed.

Caspase-3 activity detection

According to manufacturer instructions, the pNA standard was diluted by concentration gradients to prepare standards at 200 μM, 100
μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, and 0 μM. Absorbance at 405 nm was measured as A405 to make a standard curve. The cell pellet was added with 100 μL Caspase Lysis buffer and lysed on ice for 20 minutes. After being centrifuged at 12,000 g and 4°C for 10-15 minutes, the supernatant was moved to a new 1.5 mL centrifuge tube and quantified using the BCA kit. Next, 65 μL Assay buffer, 25 μL lysate supernatant, and 10 μL c-DEVD-pNA (2 mM) were added to 96-well plates at 37°C for 2 hours. The A405 value was detected to calculate relative enzyme activity as experimental group A405/control group A405 × 100%.

Cell apoptosis detection

The cells were resuspended in 100 μl binding buffer and incubated in 5 μl Annexin V-FITC and 5 μl PI, void of light, for 15 minutes. Next, the cells were added with 5 μl PI and tested with flow cytometry, evaluating cell apoptosis.

Statistical analysis

Data analyses were performed with SPSS 18.0 software. Measurement data are depicted as mean ± standard deviation and were compared by t-test or one-way ANOVA. Bonferroni’s method was selected for post hoc testing. P < 0.05 indicates statistical significance.

Results

HL-60/ADR cell model establishment

HL-60/ADR cell model resistant to ADR was established by gradually increasing concentrations of ADR treatment. CCK-8 assay showed that, under the same concentrations of ADR treatment, the proliferation activity of HL-60 cells was significantly lower than that of HL-60/ADR cells. The IC50 of HL-60 cells was 1.13 ± 0.15 μM, the IC50 of 60/ADR cells was 14.29 ± 1.336 μM, and the resistance index of HL-60/ADR cells was 12.65 (Figure 1A). HL-60 and HL-60/ADR cells were treated by 1.13 μM ADR. Flow cytometry revealed that 1.13 μM ADR treatment resulted in an apoptotic rate of 23.73% in HL-60 cells, while only 3.21% in HL-60/ADR cells (Figure 1B).

DJ-1 upregulated in HL-60/ADR cells

qRT-PCR demonstrated that, compared with human normal PBMC, DJ-1 mRNA expression was significantly increased (Figure 2A) and PTEN mRNA was obviously reduced (Figure 2B) in leukemia HL-60 cells. Compared with HL-60 cells, DJ-1 mRNA expression was markedly upregulated, while PTEN mRNA levels apparently declined in HL-60/ADR cells. Western blotting exhibited that, compared with human normal PBMC, DJ-1 protein levels were significantly enhanced, but PTEN protein expression was obviously attenuated in leukemia HL-60 cells. Compared with HL-60 cells, DJ-1 protein expression was markedly elevated, while PTEN protein levels were apparently reduced in HL-60/ADR cells (Figure 2C).

siRNA-DJ-1 transfection obviously reduced DJ-1 expression in HL-60/ADR cells

qRT-PCR demonstrated that DJ-1 mRNA was significantly decreased in the siRNA-DJ-1 group, compared with the siRNA-NC group (Figure 3A).
DJ-1 affects leukemia

Western blotting indicated that DJ-1 protein expression was obviously declined in the siRNA-DJ-1 group, compared with the siRNA-NC group (Figure 3B).

Interference with DJ-1 significantly attenuated HL-60/ADR cell proliferation and drug resistance

Western blotting demonstrated that, compared with the siRNA-NC group, PTEN protein expression was apparently upregulated, while p-AKT protein levels were markedly decreased in the siRNA-DJ-1 group (Figure 4A). Results showed that the siRNA-DJ-1 group exhibited significantly enhanced caspase-3 activity under ADR treatment, compared with the siRNA-NC group (Figure 4B). Flow cytometry demonstrated that transfection of siRNA-DJ-1 obviously attenuated HL-60/ADR cell proliferation (Figure 4D) and promoted apoptosis (Figure 4C).

Discussion

PI3K (PKB, protein kinase B) is an important signaling pathway, widely expressed in various tissues and cells, playing a crucial role in regulating various biological processes, including cell growth, survival, proliferation, apoptosis, glycogen synthesis, and glucose conversion [16, 17]. Under stimulation by growth factors, mitogens, and other factors, PI3K can be activated by conformational changes. At this time, the PI3K/AKT signaling pathways are activated. PI3K can be recruited to the site adjacent to the plasma membrane, catalyzing its substrate phosphatidylinositol 4,5-trisphosphate (PIP2) phosphorylation to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits AKT translocating to the membrane, phosphorylating Ser/Thr protein kinase AKT at the Ser473 and Thr308 loci by phosphoinositide-dependent protein kinase (PDK). The phosphorylated activated AKT continues to transmit signals downstream to regulate the expression and function of proteins that play a key role in cell proliferation, cell cycle, and apoptosis [18, 19]. The PTEN gene, belonging to the protein tyrosine phosphatases (PTP) gene family, is located at 10q23.3 and the transcript is 515 kb mRNA. PTEN can sustain PIP3 at low levels by dephosphorylating PIP3 and antagonizing the phosphorylation of PI3K to PIP2, thus inhibiting AKT and downstream pathway activation [20, 21]. DJ-1/PARK7 (Parkinson gene 7) gene is located at position 1p36.2-36.3 of the human chromosome. The gene is about 24 kb in length and encodes a protein with a molecular weight of 21 kD, consisting of 189 amino acids [11]. DJ-1 is a negative regulator of PTEN. It can enhance the transduction activity of PI3K/AKT signaling pathways by inhibiting the expression...
and function of PTEN, playing a role in antagonizing cell apoptosis and promoting cell proliferation [11]. Abnormally elevated DJ-1 plays a role in mediating drug resistance in various tumor cells, such as lung cancer [12, 13], cervical cancer [14], and pancreatic cancer [15]. However, whether DJ-1 abnormalities mediate leukemia drug resistance remains unclear. The current study established a leukemia Adriamycin (ADR)-resistant cell line and compared expression of DJ-1 in the parental leukemia cells, exploring the roles of DJ-1 in leukemia ADR resistance.

CCK-8 assay and flow cytometry showed that, under the same ADR treatment conditions, the impact of ADR on HL-60/ADR cell proliferation and apoptosis was obviously higher than in parental HL-60 cells, indicating that the HL-60/ADR cell line was successfully established. This study showed that, compared with human normal PBMC, DJ-1 mRNA expression was significantly increased and PTEN mRNA was obviously reduced in leukemia HL-60 cells. Compared with HL-60 cells, DJ-1 mRNA expression was markedly upregulated, while PTEN mRNA levels apparently declined in HL-60/ADR cells. Results indicate that DJ-1 upregulation plays a role in reducing PTEN expression, which may be related to leukemia drug resistance. In one study of the relationship between DJ-1 and leukemia, Li et al. found that diallyl disulfide (DADS) reduced expression of DJ-1 in leukemia HL-60 cells, promoting cell apoptosis, and inhibited cell proliferation [22]. Ling et al. revealed that DADS treatment significantly inhibited the proliferation of HL-60 cells, weakened migration and invasion abilities, induced cell cycle arrest, and downregulated expression of DJ-1 [23]. Liu et al. showed that DADS treatment can attenuate the migration and invasion of HL-60 cells by inhibiting expression of DJ-1 [24]. Zhou et al. observed that the anti-tumor drug Homoharringtonine (HTT) can significantly induce apoptosis of leukemia K562 cells, related to the downregulation of DJ-1 expression [25]. Results indicated that the abnormal elevation of DJ-1 was associated with the malignant biological characteristics of leukemia cells, with DJ-1 as one of the targets of drugs. In the present study, DJ-1 was not only associated with leukemia, but also with drug resistance, in

---

**Figure 4.** Interference of DJ-1 significantly attenuated HL-60/ADR cell proliferation and drug resistance. A. Western blot detection of protein expression; B. Caspase-3 activity detection; C. Flow cytometry detection of cell apoptosis; D. Flow cytometry detection of cell proliferation. *P < 0.05.
DJ-1 affects leukemia

accord with previous studies. In one study of the relationship between PTEN and leukemia, Yao et al. demonstrated that expression of PTEN in leukemia NB-4 cells was significantly decreased [26]. Wang et al. reported that expression of the tumor suppressor gene PTEN in leukemia HL-60 cells was obviously reduced [27]. In this study, expression of PTEN in leukemia cells was declined, in accord with Yao [26] and Wang [27].

Compared with the siRNA-NC group, expression of DJ-1 mRNA and protein in HL-60/ADR cells was significantly decreased after siRNA-DJ-1 transfection, indicating that transfection efficiency was high. Further detection showed that transfection of siRNA-DJ-1 significantly upregulated expression of PTEN proteins, downregulated expression of p-AKT proteins in drug-resistant HL-60/ADR cells, enhanced caspase-3 activity, elevated cell apoptosis, and attenuated cell proliferation induced by ADR. It was found that increased expression of DJ-1 could restrain tumor suppressor gene PTEN expression, enhance the activity of PI3K/AKT pathways, and participate in the regulation of drug resistance in HL-60 cells. Moreover, siRNA interference can reduce expression of DJ-1, upregulate PTEN levels, inhibit the activity of PI3K/AKT pathways, and attenuate the drug resistance of leukemia HL-60 cells. In a study of the relationship between DJ-1 and tumor cell resistance, Gao et al. showed that expression of DJ-1 was related to the chemosensitivity of lung cancer H69AR cells [12]. Also, siRNAs affecting DJ-1 expression can significantly promote lung cancer H69AR cell apoptosis, induce cell cycle arrest, and reduce drug resistance. Zhu et al. revealed that interference of DJ-1 expression in the dihydroartemisinin (DHA) resistant cell line Hela/DHA can reduce drug resistance against DHA, whereas overexpression of DJ-1 in drug-sensitive parental Hela cells may develop resistance to DHA [14]. Zhang et al. demonstrated that an increase of DJ-1 was related to drug resistance of breast cancer MCF-7 cells. Interference of siRNAs to DJ-1 significantly reduced the drug resistance of MCF-7 cells [28]. This study revealed that elevated expression of DJ-1 plays a role in regulating PTEN-Pi3K/AKT pathway activity and mediating ADR resistance in leukemia HL-60 cells, while interference of DJ-1 expression can reduce ADR resistance. However, whether DJ-1 regulating PTEN-Pi3K/AKT is related to drug resistance in leukemia patients remains unclear.

Conclusion

Increased expression of DJ-1 plays a role in regulating PTEN-Pi3K/AKT pathway activities and mediating ADR resistance in leukemia HL-60 cells, while interference with DJ-1 expression reduces ADR resistance in leukemia HL-60 cells.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Donghu Wen, Department of Hematology, The First Affiliated Hospital of Qiqihar Medical School, No. 26 Xiangyang Street, Fularki District, Qiqihar City 161041, Heilongjiang, China. Tel: +86-0452-6867646; Fax: +86-0452-6867646; E-mail: goubaitao8887@163.com

References

DJ-1 affects leukemia


