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DADS on the Biological Behavior of Human Leukemia HL−60 Cells with Nuclear Localization and Overexpression of DJ−1*

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Abstract Recent studies have revealed that DJ-1 is overexpressed in multiple forms of human tumors, and nuclear overexpression of DJ-1 is correlated with the biological behavior of tumor. The purpose of the present study was to test for possible DADS effects on Human Leukemia HL-60 cells which DJ-1 is overexpressed, and to clarify the function of DJ-1 in the nucleus. Gene transfection technology was used to establish stable HL-60 cell lines with nuclear overexpression of DJ-1 (DJ-1/HL-60). Soft agar colony formation, MTT, NBT and indirect immunofluorescence were used to evaluate the proliferation, differentiation, migration and invasion of HL-60 cells. The nuclear overexpression of DJ-1 protein may promote the proliferation and weaken the differentiation of HL-60 cells. Transwell migration and invasion assays showed that the nuclear localization and overexpression of DJ-1 can promote the migration and invasion capability of HL-60. Western blot analysis revealed that the expression of DJ-1 was inhibited in HL-60 cells with nuclear overexpression of DJ-1 after treatment with DADS. These results defined the impacts of DJ-1 on biological behavior of the Human Leukemia HL-60 cells by DADS, the proliferation, migration and invasion were inhibited and the differentiation was induced in HL-60 cells with nuclear overexpression of DJ-1, after treatment with DADS.

Key words HL-60, diallyl disulfide, DJ-1, nuclear localization, differentiation
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Leukemia is a malignant clonal disease derived from hematopoietic stem cells, and it causes a serious threat to human health, with over 300 000 new cases and 70% deaths in the world each year[1]. Diallyl disulfide (DADS) is a major organosulfur compound extracted from garlic and some other plants from the genus, Allium[2]. Our previous studies have confirmed that DADS can induce differentiation and apoptosis and also suppress the growth in human acute promyelocytic leukemia HL-60 cells[3-4].

DJ-1 is a member of DJ-1/ThiJ/Pfpl superfamily, which is involved in multiple cellular biological functions[5]. Accumulating evidences suggest that DJ-1 plays a vital role in tumorigenesis, and in the progression, prognosis, and drug resistance of tumors[6-10]. Many studies have confirmed that DJ-1 is overexpressed in multiple human tumors, including cholangiocarcinoma[11], breast cancer[12], thyroid cancer[13], non-small cell lung cancer[14]and leukemia cell lines[15].

In earlier reports, DJ-1 has been shown to express in both nuclear and cytoplasmic regions[16-17], and it carries out different functions in different cellular compartments. For example, nuclear localization of DJ-1 mediates the mitochondrial antioxidant function[18-19]. Additionally, nuclear localization of DJ-1 is important to carry out certain functions, such as sequestering of Daxx in the nucleus

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to prevent Daxx-mediated apoptosis[20]. Nuclear localized DJ-1 may also interact with other transcription cofactors to modify their transcription activity[21]. In esophageal squamous cell carcinoma, the overexpression of DJ-1 in the cytoplasm is correlated with tumorigenesis, and a high level of nuclear expression of DJ-1 is correlated with poor prognosis[22]. Therefore, the present study aimed at demonstrating the role of DADS in regulating nuclear localization of DJ-1 for the suppression of proliferation and invasion of HL-60 cell lines, induction of differentiation.

1 Materials and methods

1.1 Cell culture

The HL-60 human Leukemia cell line was obtained from the Cancer Institute of Xiangya Medical College, Changsha, China. The cells were passaged routinely in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Shanghai, China) and incubated in a 5% CO_2 air humidified atmosphere. Cells were tested during the logarithmic growing phase. DADS (Fluka Chemika, Ronkonkoma, NY, USA) was diluted using the culture medium to the required concentration.

1.2 Plasmid construction and transfection

The cDNA of DJ-1 isoforms was cloned into pCMV/myc/nuc-EGFP vector (Invitrogen, Shanghai, China). The expression vectors were verified by DNA sequencing. The recombinant plasmid pCMV/myc/nuc-EGFP-DJ-1 was stably transfected into HL-60 cell nucleus by Lipofectamine2000 (Invitrogen, Shanghai, China), and G418 was used to screen out the positive clone. The nuclear overexpression of DJ-1 in HL-60 cells was verified fluorescence microscope and Western blot assay.

1.3 Cell viability and cell proliferation assay

Cell viability was determined by the MTT (Fluka Chemika, Ronkonkoma, NY, USA) assay. HL-60 cells, vector and DJ-1 / HL-60 were incubated in culture media alone, or in culture media containing 1.25 mg/L DADS for 24, 48, 72, and 96 h. Cells were seeded at an initial density of 3×10^4/ml in 96 well culture plates. After centrifugation, supernatant was discharged, and remaining crystals were dissolved in 100 μl DMSO. The absorbance of the sample was read at 570 nm (BIO-RAD MR17260, USA). Cell Inhibition rate (IR%) = [A_{570} (control) − A_{570} (treatment)]/(A_{570} (control)×100%. A_{570} (control) is the absorbance of cells without DADS treatment, A_{570} (treatment) is the absorbance of cells exposed to the DADS.

Cell proliferation inhibition was assessed by soft agar colony-forming experiments. Cells were incubated as above, and the cell concentration was adjusted to 3×10^4/ml. 1.5 ml of 5% agar and complete medium were dissolved at a ratio of 1∶9, and added to the lower 6-well plates. 1 ml of cell suspension and 0.5% agar were dissolved at a ratio of 1∶1, and added to the upper 6-well plates. The cells were cultured for 10-15 days and the number of cells was counted using an inverted microscope at x100 magnification and a digital camera.

1.4 NBT assay

NBT (Nitroblue Tetrazolium) assay was used to determine the proliferation and differentiation of HL-60 cells. Cells were incubated in culture media alone, or in culture media containing 1.25 mg / L DADS, at 37℃ for 72 h. Briefly, a suspension containing approximately 2×10^3 cells and 200 μl NBT-TPA (Sigma, Shanghai, China) media were added to each well of the 96-well culture plate and incubated for 1 h at 37℃ in a humidified atmosphere with 95% air and 5% CO_2. Subsequently, the cultures were incubated in 200 μl of DMSO solution along with shaking for 30 min. A microplate reader was used to measure absorbance at 570 nm for each well.

1.5 Indirect immunofluorescence assay

Immunofluorescence was accomplished to confirm expression of a general myeloid differentiation marker CD11b (existed in mature blood cells) and CD33 (existed in leukemia cells). Cells were permeabilized with acetone, washed with PBS, and incubated with primary CD11b or CD33 antibody (Epitomics, Burlingame, CA, USA) for 2 h. Subsequent to washing and application of the secondary cy3 goat anti-rabbit IgG for 30 min, the cells were visualized using a fluorescence microscope.

1.6 Transwell migration assay

The transwell system was used to explore migration of cells. For the cell invasion assay *in vitro*, we used 24-well Transwell chambers with a pore size of 8.0 μm. The experimental procedure was as follows: cells density was adjusted to 2×10^5/ml, cells suspension (200 μl) was added to the upper chamber, and 600 μl of complete medium were added to the

lower chamber, the medium was disposed 24 h after incubation at 37℃. Next, the cells were counted by MTT method and a microplate reader was used to measure absorbance at 570 nm for each well.

1.7 Transwell invasion assay

The invasion assay protocol was the same as of the migration assay except that the upper chambers were first coated with 1 g/L Matrigel. The cells were stained in Giemsa solution for 30 min and washed with double distilled water three times. The wells were gently wiped with a swab. Number of cells was counted using an inverted microscope (× 100 magnification) with 5 elds of view, and the mean values were taken as the invasive cell number.

1.8 Subcellular fractionation analysis

Cells were harvested and rinsed twice with cold PBS. The cell cytoplasm protein and nucleoprotein were prepared using the Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Pittsburgh, PA, USA), according to the manufacturer's instructions. The amount of protein was determined using BCA protein assay reagent.

1.9 Western blot assay

Equal amount of protein samples were completely vortexed with 1 × SDS-gel buffer, and boiled for 5 min at 100℃ to dissolve the bound proteins. The samples were segregated on 100 g / L SDS acrylamide gel, transferred onto a nitrocellulose membrane, blocked with 50 g / L defatted milk, and subsequently probed with primary DJ-1 antibodies (Millipore, Billerica, MA, USA). Anti-mouse IgG conjugated peroxidase was used as a secondary antibody. The filters were then incubated in Super Signal ECL-HRP detection reagent (ComWin Biotech, Beijing, China) for 1 min followed by exposure to film in a visualizer.

1.10 Statistical analysis

For statistical analyses, the difference between two groups was analyzed by 2-tailed Student *t* test using GraphPad Prism software. In all the cases, a *P* value of less than 0.05 was considered significant.

2 Results

2.1 Establishment of pCMV / myc / nuc−EGFP−DJ−1−HL−60 （DJ−1/HL−60） cell lines

After transfecting pCMV / myc / nuc-EGFP-DJ-1 into HL-60 cells by Lipofectamine 2000, the nucleus was found to overexpress DJ-1 protein in DJ-1/HL-60 cells. We verified by fluorescence microscope and Western blot (Figure 1a, 1b), Leukemia HL-60 cell

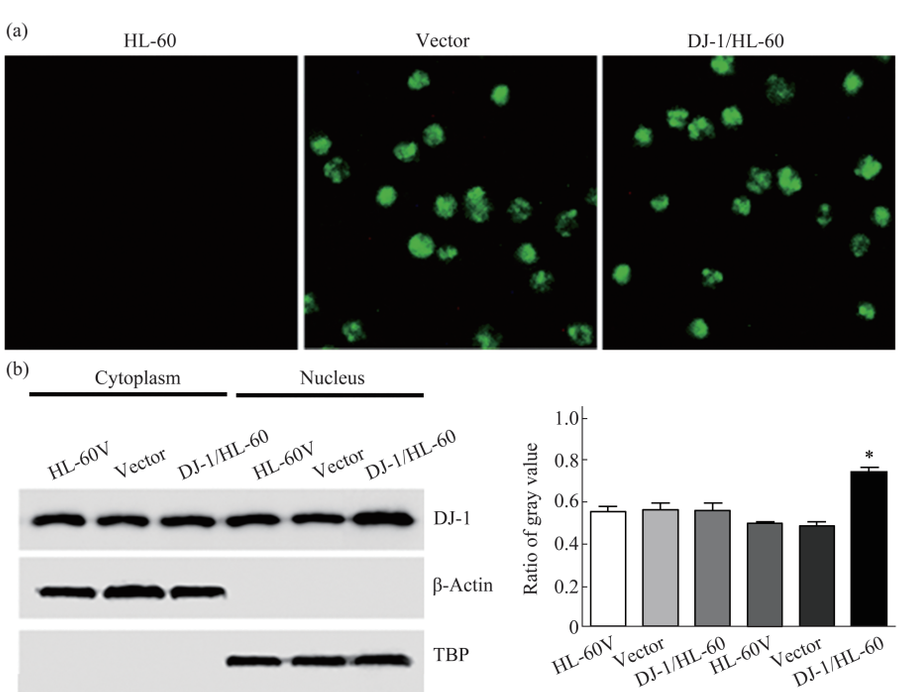


Fig. 1 HL−60 cell lines with the nuclear overexpression of DJ−1 were established

（a） HL-60 cells were transfected with EGFP-tagged DJ-1 or an empty vector expressing EGFP alone. In each experiment， a group of cells untransfected as control（Vector）. EGFP fluorescence served to confirm transfection efficiencies. （b）DJ-1 expression in cytoplasm and nucleus of HL-60 cells were measured by Western blot. * P<0.05（Student t test） with respect to control for necleus of HL-60 cells.

lines with a stable high-level nuclear expression of DJ-1, were successfully constructed.

2.2 Effect of DADS on cell proliferation of DJ−1/HL−60 cells

MTT and soft agar assay revealed that the proliferation rate of DJ-1 / HL-60 cells was significantly enhanced as compared to the control and vector groups ($P < 0.05$), however the cell proliferation rate was found to be significantly reduced ($P < 0.05$) following the DADS (1.25 mg/L) treatment after 72 h (Figure 2a, 2c). Furthermore, the DJ-1 / HL-60 cell inhibition rate was significantly lower ($P < 0.05$) when compared with the control and vector groups, whereas DADS reduced the suppressive effect ($P < 0.05$) (Figure 2b). The results, therefore, showed that DJ-1 gene overexpression positively correlated with the proliferation ability of HL-60 cells, while DADS suppressed the HL-60 cell proliferation. The nuclear localization of overexpressed DJ-1 may weaken the ability of proliferation of DJ-1/HL-60 cells by DADS.

2.3 Effect of DADS on differentiation of DJ−1/HL−60 cells

CD33 expression was significantly enhanced in the DJ-1/HL-60 cells as compared to the control and vector cells, as shown by the indirect immunofluorescence assay. Furthermore, in comparison to the non-treated groups, the CD11b expression was significantly enhanced ($P < 0.05$) and the CD33 expression was significantly decreased ($P < 0.05$) in the DADS (1.25 mg/L) treatment group after 72 h. The results, thus, revealed that DADS induce differentiation in HL-60 and DJ-1/HL-60 cells (Figure 3a, 3b).

The observations made through the NBT assay revealed that the cell percent reduction was significantly decreased ($P < 0.05$) in the DJ-1/HL-60 cells as compared to the control and vector cells. In addition, the results showed that DJ-1 gene expression positively correlated with the differentiation ability of HL-60 cells. The cell percent reduction was significantly enhanced ($P < 0.05$) after 72 h of DADS (1.25 mg / L) treatment (Table 1). Therefore, the nuclear overexpression of DJ-1 protein may weaken HL-60 cell differentiation, and DADS has the ability to induce DJ/HL-60 cells differentiation.

2.4 Effect of DADS on HL−60 cell migration

To determine whether DADS suppresses HL-60 cell migration, the transwell migration assay was performed. Compared to the control and vector cells, the cell migration rate was significantly increased ($P < 0.05$) in the DJ-1/HL-60 cells. In contrast, the cell migration rate was significantly reduced ($P < 0.05$) in the group treated with DADS (1.25 mg/L) after 24 h (Table 2). The results showed that DADS suppressed the migration of DJ-1/HL-60 cells.

2.5 Effect of DADS on HL−60 cell invasion

Invasion assays aided in determining whether DADS suppresses HL-60 cell invasion and showed a significant increase in the number of penetrating cells along with a significant improvement in the cell penetrating ability (Figure 3c) ($P < 0.05$) of the DJ-1/HL-60 cells in comparison to the control and vector cells. In contrast, the cell penetrating ability and the number of penetrating cells significantly decreased ($P < 0.05$) in the experimental cells treated with DADS (1.25 mg/L) for 24 h, compared with the non-treated cells (Figure 3c). Therefore, these findings showed that the DJ-1 gene overexpression positively correlated with HL-60 cells, and that DADS suppressed the invasion of DJ-1/HL-60 cells.

2.6 Effect of DADS on DJ−1 expression in HL−60 cells

Previous construction of the cell line shown that DJ-1 is highly overexpressed cell in the nucleus, whereas the highly expressed DJ-1 can better preserved cell survival, which can be inhibited by DADS, so we suspect that DADS can inhibit the expression of DJ-1 in the nucleus. To confirm the effect of DADS on the DJ-1 expression, 1.25mg/L DADS treatment cells after extraction of nuclear protein, and the expression of DJ-1 in the cells was detected by Western blot. In the Figure 4, the results show DADS downregulated the expression of DJ-1 protein in the nuclear of DJ-1/HL-60 cells, compared to the control and vector cells ($P < 0.05$) .

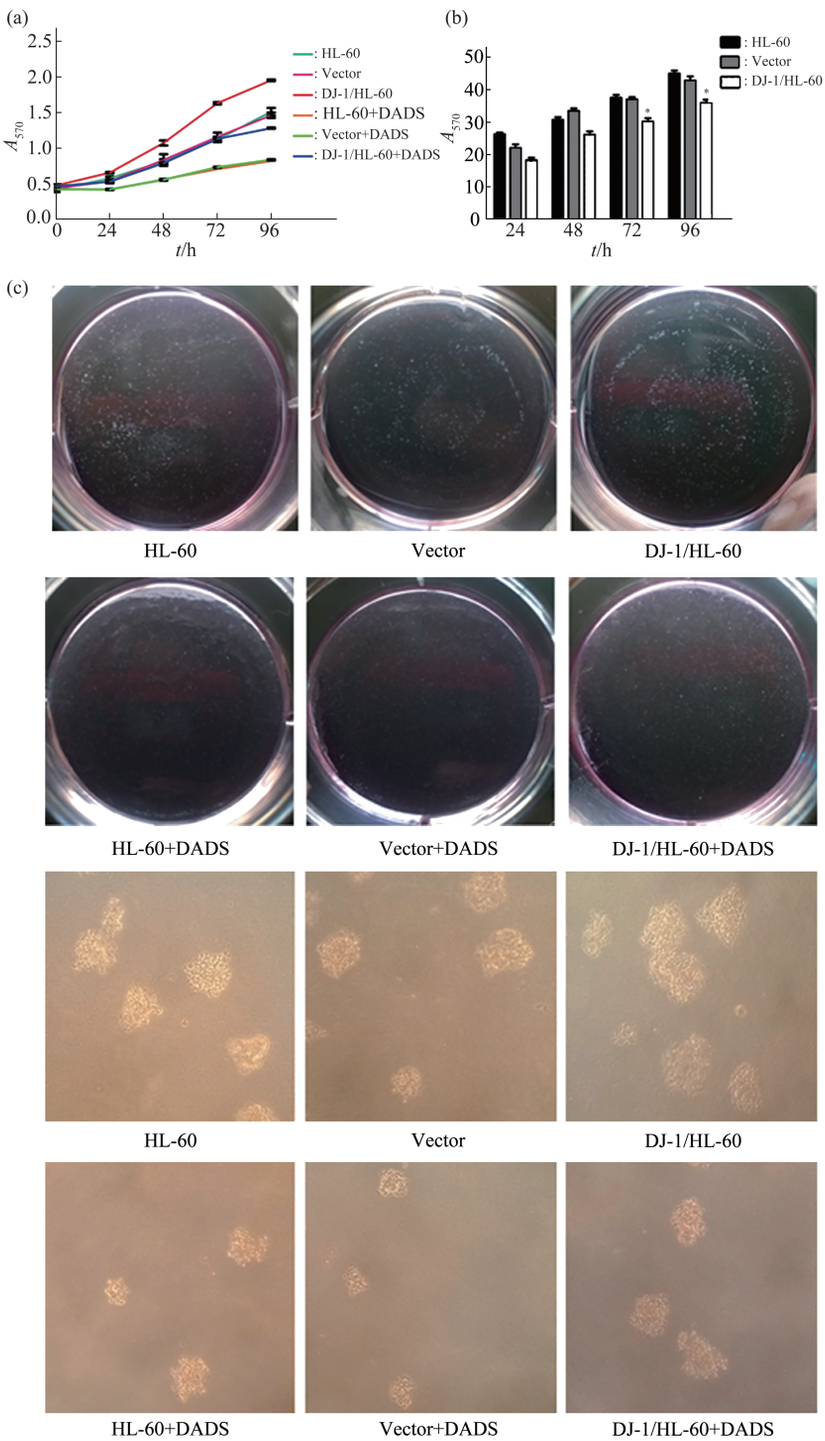


Fig. 2　The nuclear overexpression of DJ−1 protein may promote the proliferation and weaken the ability of proliferation of DJ−1/HL−60 cells by DADS

（a）HL-60，Vector，HL-60 / DJ-1 cells were seeded at an initial concentration of 3×10^4/ml and incubated for 24，48，72 and 96 h with DADS（1.25mg/L） or not. After incubation，the absorbances of cells at 570 nm were measured in a microplate reader.（b）The cell viability was determined using MTT assay. Cell Inhibition rate（IR%） = ［A_{570}（control）−A_{570}（treatment）］/（A_{570}（control）×100%. A_{570}（control）is the absorbance of cells without DADS treatment，A_{570}（treatment） is the absorbance of cells exposed to the DADS.（c）Cell inhibition rates were evaluated by Soft Agar Colony Formation，cells were incubated as above，and the cell concentration was adjusted to 3×10^4/ml. The cells were cultured for 10 days，photographed using a digital camera（upper panels）and a microscope（lower panels）at 100 magnification.

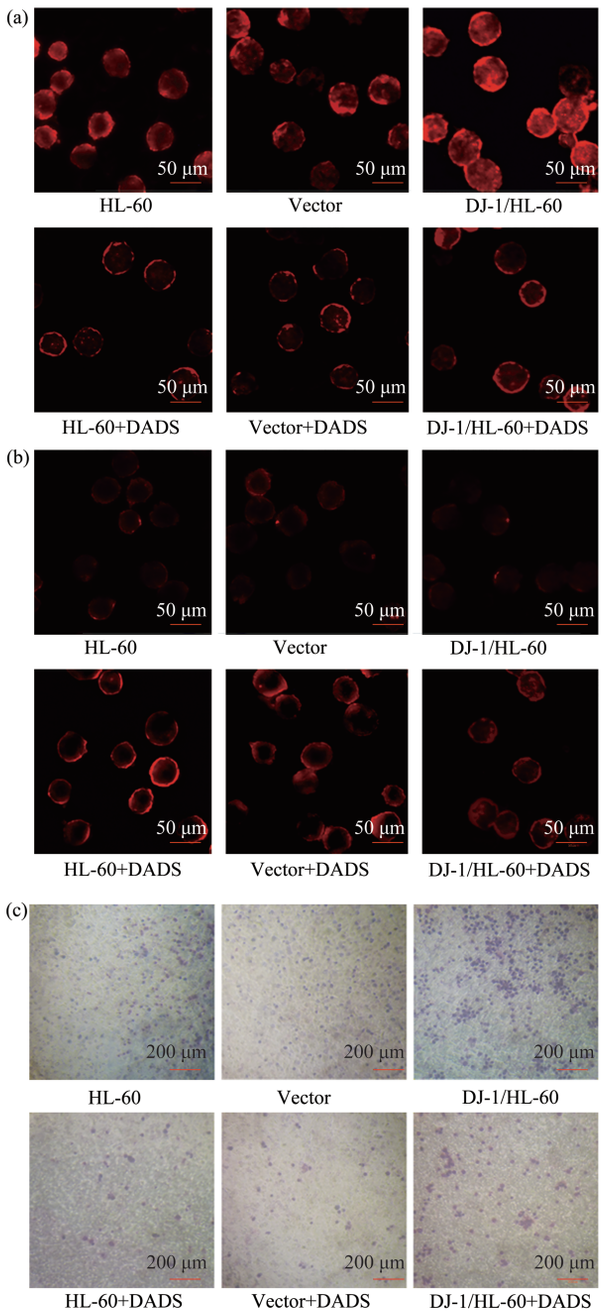


Fig. 3　DADS induced the differentiation of HL−60 and DJ−1 / HL−60 cells, suppressed the invasion of DJ−1/ HL−60 cells

HL-60, Vector, HL-60 / DJ-1 cells were incubated in the presence or absence of DADS. (a) Cell surface differentiation marker CD33 was detected by immunofluorescence after 72 h treatment of 1.25 mg/L DADS. (b) Cell surface differentiation marker CD11b was detected by immunofluorescence after 72 h treatment of 1.25 mg / L DADS. (c) Cell invasion were examined by invasion assays after 24 h treatment of 1.25 mg/L DADS .

Table 1　Distribution of the cell percent reduction in DJ−1/ HL−60 cells

Variant	HL-60	Vector	DJ-1/HL-60
DADS（-）	0.141±0.010	0.140±0.008	0.102±0.011*
DADS（+）24h	0.164±0.012	0.163±0.010	0.130±0.008#
DADS（+）48h	0.182±0.011	0.179±0.009	0.143±0.010#
DADS（+）72h	0.189±0.007	0.193±0.006	0.153±0.008#
DADS（+）96h	0.214±0.010	0.215±0.011	0.165±0.013#

*P<0.05 *vs* HL-60 and Vector, #P<0.05 *vs* DADS（-）.

Table 2　Detection of migration in DJ−1/HL−60 cells

DADS	Group	Absorbance
DADS（-）	HL-60	0.556±0.040
	Vector	0.560±0.039
	DJ-1/HL-60	0.833±0.038*
DADS（+）	HL-60	0.252±0.037
	Vector	0.244±0.035
	DJ-1/HL-60	0.558±0.033#

*P < 0.05 *vs* HL-60 and Vector, #P < 0.05 *vs* DJ-1/HL-60.

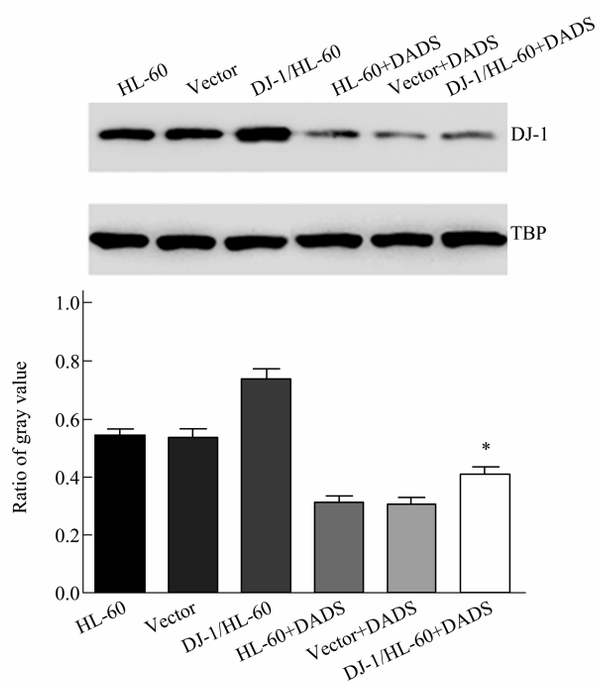


Fig. 4　DADS decreases DJ−1 protein expression

HL-60, Vector, HL-60 / DJ-1 cells were incubated in the presence or absence of DADS for 72 h. DJ-1 in the nucleus of HL-60 cell was detected by Western blot. *P＜0.05.

3 Discussion

The well-known characteristics of unlimited cell proliferation, invasion and migration are the leading causes of leukemia-related deaths. Diallyl disulfide (DADS), an anti-neoplastic compound found naturally in garlic, is an effective inhibitor of growth in many tumor cells. DADS can induce the apoptosis of HL-60 cells, by blocking the G2 / M phase[23], and by regulating the correlated signal pathway *via* inhibition of ERK and upregulation of Rac2 at moderate doses (> 1.25 mg/L)[24] . The small level of dose (< 1.25 mg/L) can block the HL-60 cells at the G0 / G1 phase and thereby, prevent DNA duplication by inhibiting phosphorylation of JAK1 and STAT3 tyrosine kinase, this in turn can inhibit the proliferation of HL-60 cells and induce differentiation[4].

DJ-1 protein, a member of the ThiJ / PfpI / DJ-1 superfamily, appears to be involved in diverse biological processes at disparate subcellular levels. Although DJ-1 is mainly localized in the cytosol, it is also present in the mitochondria and nucleus[25]. It is transferred from cytoplasm to the nucleus and combines with specific transcription factors or cofactors in S phase of cells[26-27]. DJ-1 protein can regulate activities of transcription genes through acetylation or deactivation, cause alteration of the structure of chromatin, or regulate the activity of transcription factors[28]. The present study indicated that DJ-1 shows abnormal expression and occurs in a wide variety of tumors. In bladder cancer, a negative correlation between the high expression of DJ-1 or loss of PTEN, and tumor invasiveness and pathological grade, have been clearly identified[29]. Higher DJ-1 and lower PTEN expression in the ovarian cancer exudate, after chemotherapy, has been correlated with its prognosis[30]. In addition, DJ-1 involved in leukemia through regulation of PTEN, promotes cell proliferation and malignant transformation[15]. And may become the new anti-leukemia treatment target.

In the present study, we constructed DJ-1 nuclear localized expression vector and set up steady models of HL-60 cell line with over expression of DJ-1 in the nucleus. These experiments were undertaken to further test the biological features of subcellular localization of DJ-1 in leukemia HL-60 cells induced by DADS, and detect the role of DJ-1 during the inhibition of proliferation, invasion, and induction of differentiation of HL-60 cells by DADS. Our results showed that overexpression of DJ-1 protein in the nucleus positively correlates with HL-60 cell proliferation ability, DADS suppresses HL-60 cell proliferation, and the ability of proliferation of DJ-1 / HL-60 cells may weaken by DADS.

CD11b is a member of the integrin family, and is regarded as an antigen marker of mature granulocytes with an expression on neutrophils, however it is weakly expressed (or not expressed) on myeloblasts and promyelocytic cells[31-32]. CD33, a myeloid differentiating antigen, particularly overexpressed during the early stages of differentiation, are found on more than 90% APL patients[33]. In contrast, it shows almost no expression on hematopoietic stem cells, mature granulocytes, and other tissues, providing further evidence that CD33 as a good identifying sign of myeloid leukemia. NBT assay and indirect immunofluorescence assay showed that DADS induce the differentiation in DJ-1/HL-60 cells.

DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC / ERK / Upa[34], Transwell Chambers is a commonly used test to detect *vitro* tumor cell migration and invasion. The results showed that overexpression of DJ-1 protein in the nucleus positively correlates with the migration and invasion of HL-60 cells, DADS suppresses invasion of DJ-1/HL-60 cells. Western blotting results detected overexpression of DJ-1 protein in the nucleus of DJ-1/HL-60 cells, which significantly reduced after treatment with DADS. Nuclear localized DJ-1 might interact with other transcription cofactors to modify their transcription activity[21]. In prostate cancer, the subcellular localization of DJ-1 is regulated by androgens and antiandrogens, which in turn leads to differential binding to androgen receptor[35]. Due to the central role of DJ-1 in transcriptional regulation, identification of DJ-1 inhibitors, such as DADS, may lead to novel treatment regimens that modulate cancer hallmarks including cancer cell growth, survival, angiogenesis, progression, invasion, and metastasis as well as boosting the efficacy of current Chemotherapeutics.

In summary, overexpression of DJ-1 protein in the nucleus involved in promoting cell proliferation and malignant transformation in leukemia. DADS can induce differentiation of human leukemia HL-60 cells, inhibit their proliferation and invasion by

downregulating the nuclear expression of DJ-1 protein. Further investigations are required to reveal how subcellular localization of DJ-1 is controlled and how this affects its activity in promoting leukemia tumorigenesis and progression. Our findings on the functions of DADS and nucleus-localized expression of DJ-1 may help in the development of potential biomarkers and drugs for the prognosis and therapy of cancer.

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DADS对DJ-1核定位高表达人白血病HL-60细胞的影响*

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摘要　最近研究表明，DJ-1在许多肿瘤中过表达，而且DJ-1的核表达与肿瘤的生物学行为有关. 本文主要研究二烯丙基二硫（DADS）对DJ-1核定位高表达人白血病HL-60细胞生物行为学的影响，阐明DJ-1在细胞核内的功能，为临床诊断及治疗过程提供一个潜在的治疗靶点. 通过基因转染技术建立核内高表达HL-60细胞株（DJ-1/HL-60），利用软琼脂集落形成实验、MTT法、间接免疫荧光细胞化学实验、硝基蓝四氮唑（NBT）还原比色实验评估HL-60细胞的增殖与分化，DJ-1核定位过表达促进HL-60细胞的增殖并抑制其分化. Transwell迁移侵袭小室实验表明DJ-1核定位过表达可以促进HL-60的迁移和侵袭能力. Western blot结果表明DADS具有抑制HL-60细胞中核内DJ-1蛋白表达的能力. 说明DJ-1核定位高表达具有促进HL-60细胞增殖和迁移侵袭及抑制HL-60细胞分化的作用，DADS可以诱导DJ-1核定位高表达HL-60细胞分化及抑制迁移侵袭，DJ-1核定位高表达可减弱DADS抑制HL-60细胞增殖的作用.

关键词　HL-60细胞，二烯丙基二硫，DJ-1，细胞核定位，分化

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