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Silencing of EDAG Induces Degradation of NPM1 and Sensitivity to Chemotherapeutic Drug in Acute Myeloid Leukemia Patients*

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Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; EDAG, erythroid differentiation-associated gene; GFP, green fluorescence protein; IL-3, interleukin-3; LMB, leptomycin B; MT, mutated; NPM1, nucleophosmin; NPMc$^+$, cytoplasmic NPM1; NK, normal karyotype; WT, wild type.

Abstract Nucleophosmin (NPM1 or B23.1) is a ubiquitously expressed nuclear phosphoprotein that plays key role in several cellular processes, including ribosome biogenesis, centrosome duplication, cell cycle progression, cell growth, and transformation. NPM1 is one of the most frequently mutated genes in AML. EDAG is a hematopoietic tissue-specific transcription regulator that plays a key role in maintaining the homeostasis of hematopoietic lineage commitment. In AML patients, the high expression level of EDAG is associated with poor prognosis. Our previous study suggest that EDAG is a physiological binding partner of NPM1 and regulates NPM1 protein stability, however, whether EDAG regulates NPM1 in AML patients and whether EDAG regulates NPM1 mutations remain unknown. In the present study, we found that in bone marrow CD34$^+$ cells from AML patients, silencing of EDAG led to decreased protein stability of NPM1 protein and increased cell sensitivity to daunorubicin. Although EDAG failed to interact with NPMc$^+$ and regulate its protein stability in normal culture condition, with leptomycin B treatment, EDAG overexpression enhanced the protein stability of NPMc$^+$. In AML patients with NPMc$^+$, the CD34$^+$ cells were more responsive to daunorubicin treatment than the cells from AML with wild type NPM1, and silencing of EDAG weakly increased the sensitivity to daunorubicin. These results suggested a potential role of EDAG in chemotherapy of AML and the "escape" of NPMc$^+$ protein from EDAG stabilization might contribute to the favorable prognosis of AML with NPMc$^+$.

Key words EDAG, NPM1, NPMc$^+$, daunorubicin, AML
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Nucleophosmin(NPM1 or B23.1) is a ubiquitously expressed nuclear phosphoprotein that functions as a molecular chaperone, shuttling between the nucleolus and the cytoplasm [1–2]. NPM1 plays multiple roles in cell growth and proliferation by participating in diverse biological processes, including ribosome biogenesis and transport [3], centrosome duplication [4], DNA repair [5], transcriptional regulation and histone chaperoning [6]. NPM1 is essential for development, since ablation of the *NPM1* gene in mice results in early embryonic lethality [7], and there is growing evidence that protein dosage is fundamental for many of its function[8].

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NPM1 is directly invovled in the pathogenesis of diverse human malignancies. An increase in its protein levels has been reported in solid tumors of different cellular origin, including gastric[9], colon[10], prostate[11], bladder[12] and ovarian carcinomas[13], as well as during melanoma progression[14]. Also, the overexpression of NPM1 has been detected in leukemia blast and cell lines[15-16]. Overexpression of NPM1 in HL-60 cells or K562 cells can lead to resistance to RA- or TPA-induced differentiation[17-18]. Blockage of NPM1 expression with its antisense oligonucleotides or specific siRNA oligos significantly potentiates the induction of differentiation, inhibits cell proliferation, blocks cell cycle progression and induces apoptosis in leukemic cells, implicating that NPM1 might be a potential target for leukemia gene therapy[18-19]. In addition, genetic abnormalities involving the *NPM1* gene have been described in different types of hematologic malignancies[20]. NPM1 is frequently involved in chromosomal translocation in hematological malignancies, forming fusion proteins (NPM1-ALK[21], NPM1-RARa[22] and NPM1-MLF1[23]). More importantly, the relevance of NPM1 in acute myeloid leukemia (AML) was demonstrated recently by the discovery of mutations in the NPM1 gene in a large proportion of AML patients with a normal karyotype (NK), which identified NPM1 as one of the most frequently mutated genes in AML[24-25]. NPM1 mutations are heterozygous and, in the majority, localized to exon 12 of the gene, creating the cytoplasm-dislocated mutant (Mt) NPM1 ($NPMc^+$) protein[25-26]. AML-NK carrying mutated/cytoplasmic NPM shows a higher complete remission rate than AML-NK without NPM1 mutations, though the mechanism remains unknown[27-29].

Human erythroid differentiation-associated gene (EDAG) (also known as hemgn in mouse, and RP59 in rat) is a hematopoietic tissue-specific gene which is primarily expressed in the $lin^{lo}c\text{-}kit^+Sca\text{-}1^+$ hematopoietic stem cell population and $CD34^+$ progenitor cells, and is down-regulated in mature blood cells[30]. EDAG is overexpressed in PBMCs of patients with leukemia, and the association of EDAG overexpression and poor prognosis in *de novo* AML has also been identified, suggesting that EDAG may play a modulator role in AML[31]. EDAG is supposed to be involved in the regulation of proliferation, differentiation and apoptosis of hematopoietic cells[32]. Down-regulation of EDAG expression using antisense in K562 cells results in inhibition of cell growth and colony formation, and enhancement of sensitivity to erythroid differentiation induced by hemin. Overexpression of EDAG in HL-60 cells significantly blocked the expression of the monocyte/macrophage differentiation marker CD11b after pentahydroxytiglia myristate acetate induction[32]. Moreover, enforced expression of EDAG in pro-B Ba/F3 cells prolonged survival and increased the expression of c-Myc, Bcl-2 and Bcl-xL in the absence of interleukin-3 (IL-3)[32].

In our previous study, we found that EDAG interacts with the N-terminal (118～187aa) of NPM1 through N-terminal (1～124aa)[33]. EDAG enhances the protein stability of NPM1 by reducing the degradation rate of NPM1. Overexpression of EDAG inhibited the degradation of NPM1 in K562 cells, whereas knockdown of EDAG resulted in an increased susceptibility of cells to a chemotherapeutic drug[33]. These results suggest a possible involvement of EDAG in drug-resistance of leukemia cells. However, whether EDAG regulates the protein stability of NPM1 and cell sensitivity to chemotherapeutic drug in AML patients remains to be determined.

In the present study, we found that in bone marrow $CD34^+$ cells from AML patients, silencing of EDAG by RNA interference led to decreased protein stability of NPM1 protein and increased cell sensitivity to daunorubicin. More interestingly, we found that with the Crm1 inhibitor Leptomycin B treatment, the cytoplasmic mutated NPM1 ($NPMc^+$) shuttled from cytoplasm into nucleus and EDAG overexpression enhanced the protein stability of $NPMc^+$. In AML patients with $NPMc^+$, the $CD34^+$ cells were more responsive to daunorubicin treatment than the cells from AML with wild type NPM1, and silencing of EDAG only weakly increased the sensitivity to daunorubicin. These results suggested a potential role of EDAG in chemotherapy of AML with wild type NPM1 and the "escape" of $NPMc^+$ protein from EDAG binding and stabilization effect might contribute to the favorable prognosis of AML with $NPMc^+$.

1 Materials and methods

1.1 Cell lines and reagents

HEK293T cells were maintained in DMEM (Gibco, CA) with 10% fetal calf serum (FCS, Hyclone). HT1080 cells were cultured in MEM/EBSS medium (Hyclone) supplemented with 10% FCS and Non-Essential Amino Acid (NEAA, Gibco). Cells

were cultured in a 37℃ incubator with 5% CO_2 in the presence of 2 mmol/L glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 10 mmol/L HEPES. CHX was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Daunorubicin was purchased from Pfizer (Pfizer Inc, USA).

1.2 Plasmid constructs

The human full-length NPM1 and $NPMc^+$ were amplified by PCR and cloned into the pcDNA3.1/myc-His B vector. The human full-length of EDAG was cloned into pFLAG-CMV2 vector. For construction of lentivirus-mediated RNA interference, three siRNA oligos against EDAG (siEDAG) were synthesized in GenePharma Biotechnology, the sequences are as follows: siEDAG-1, 5′ GCA CAC CAG GAA GAT GCT A 3′, siEDAG-2, 5′ GCC AGG AAT ACC AGC AAT T 3′; siEDAG-3, 5′ GGA TGC TCT CTT CAA GCA T 3′. The siRNA sequences were cloned into a psicoR-IRES-GFP vector to generate siEDAG lentivirus. The siEDAG lentivirus expresses CMV promoter-driven GFP protein and U6 promoter-driven siRNA targeting EDAG. A negative control siRNA was cloned into psicoR-GFP as a control.

1.3 Co-immunoprecipitation

Cells were washed once in PBS, and lysed in 1 ml of lysis buffer(50 mmol/L Tris HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100), and centrifuged for 15 min at 12 000 r/min at 4℃. The supernatant was transferred to a fresh tube, and immunoprecipitations were performed with anti-Flag affinity gel (A2220, Sigma) followed by adsorption to protein A/G plus-agarose beads (sc-2003, Santa Cruz). After SDS-PAGE, the samples were transferred onto polyvinylidene difluoride membranes (Amersham Life Science) and probed with a variety of antibodies.

1.4 Western blotting analysis

For Western blotting, cells were lysed with M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Then, Western blot analysis was performed according to standard procedures. Antibodies were used at the following concentrations: EDAG (sc-68361, Santa Cruz), 1∶1 000; NPM1 (sc-47725, Santa Cruz), 1∶1 000; c-Myc (sc-40, Santa Cruz), 1∶1 000; Flag (F 3165, Sigma), 1∶5 000; GAPDH (10494-1-AP, Proteintech Group), 1∶2 000. Chemiluminescent detection was conducted using supersignal substrate (Pierce) according to the manufacturer's specifications.

1.5 Immunofluorescence assays

To analyze the localization of NPM1 or $NPMc^+$, cells were fixed for 30 min at room temperature with 4% paraformaldehyde in PBS, perforated with 1% Triton X-100 in PBS for 15 min, blocked with 3% BSA in PBS for 2 h, incubated with primary antibody for 1 h and probed with secondary antibody for 45 min, and nuclei were counterstained with Hoechst 33258 for 10 min. Cells were washed with PBS three times between each step. Primary mouse monoclonal antibodies (NPM1 (sc-47725, Santa Cruz); c-Myc (sc-40, Santa Cruz)) were used at 1∶100. Secondary antibodies were used with TRITC-labeled anti-mouse antibody at 1∶500. Confocal imaging was performed using Zeiss 510 META system. The green fluorescence was excited at 488nm with 505～530 nm barrier filter and red fluorescence was simultaneously excited at 543 nm with 560 nm barrier filter.

1.6 Lentiviral production

For production of lentivirus, HEK293T cells were cotransfected with transfer vectors pSicoR-GFP-siEDAG and the packaging vectors including pMD2G and pSPAX2. Lentiviruses were harvested 24 h after transfection, passed through a 0.45 μm filter, and concentrated 500 fold by PEG precipitation. Titers of viral stock were determined on HT1080 cells.

1.7 Primary AML blasts

Primary AML samples (AML with $NPMc^+$ and FLT3-ITD negative, and AML with wild type AML) were obtained with informed consent in accordance with the declaration of Helsinki. Bone marrow (BM) aspirate samples were collected and mononuclear cells were separated using Ficoll. $CD34^+$ cells were isolated from mononuclear cells by positive selection using anti-CD34-tagged magnetic beads and a mini-magnet (StemCell Technologies, Vancouver, BC, Canada). The purity of the recovered cells was typically more than 95%. The $CD34^+$ cells were cultured in serum-free liquid medium using SFEM (StemCell technology), supplemented with 100 U/ml penicillin/streptomycin and a cocktail of cytokines (R&D Systems) as follows: 25 μg/L interleukin-3 (IL-3), 25 μg/L stem cell factor (SCF) 25 μg/L TPO and 25 μg/L Flt3 ligand.

For lentivirus infection, cells were prestimulated with cytokines described above for 24 h and then infected with lentivirus at a MOI of 10 in the presence of 8 mg/L polybrene(Sigma), followed by spinoculation (600 *g* for 30 min, 30℃) and after 12 h cells were subjected to additional infection. Cells were washed

24 h after infection, sorted for GFP$^+$ cells by FACSAria (BD Biosciences) and cultured for further experiment.

1.8 Apoptosis analysis

Cell apoptosis was performed using Annexin V Apoptosis Detection Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were harvested, washed and resuspended in 1× binding buffer. The cells were incubated with APC-conjugated Annexin V for 15 min, and then washed in 1 × binding buffer for three times, and finally resuspended in 200 μl 1× binding buffer. Then the Annexin V positive cells were detected using FACSCalibur (BD Biosciences, San Jose, CA, USA).

1.9 Statistical analysis

Error bars represent the $\bar{x} \pm s$ determined from three separate experiments. All statistical analysis was performed by Student's *t*-test.

2 Results

2.1 Knockdown of EDAG in CD34$^+$ cells of AML patients enhanced degradation of NPM1 protein

We first detected the expression level in bone marrow CD34$^+$ cells in AML patients. As Figure 1a shows, in 6 AML samples, the mRNA level of EDAG

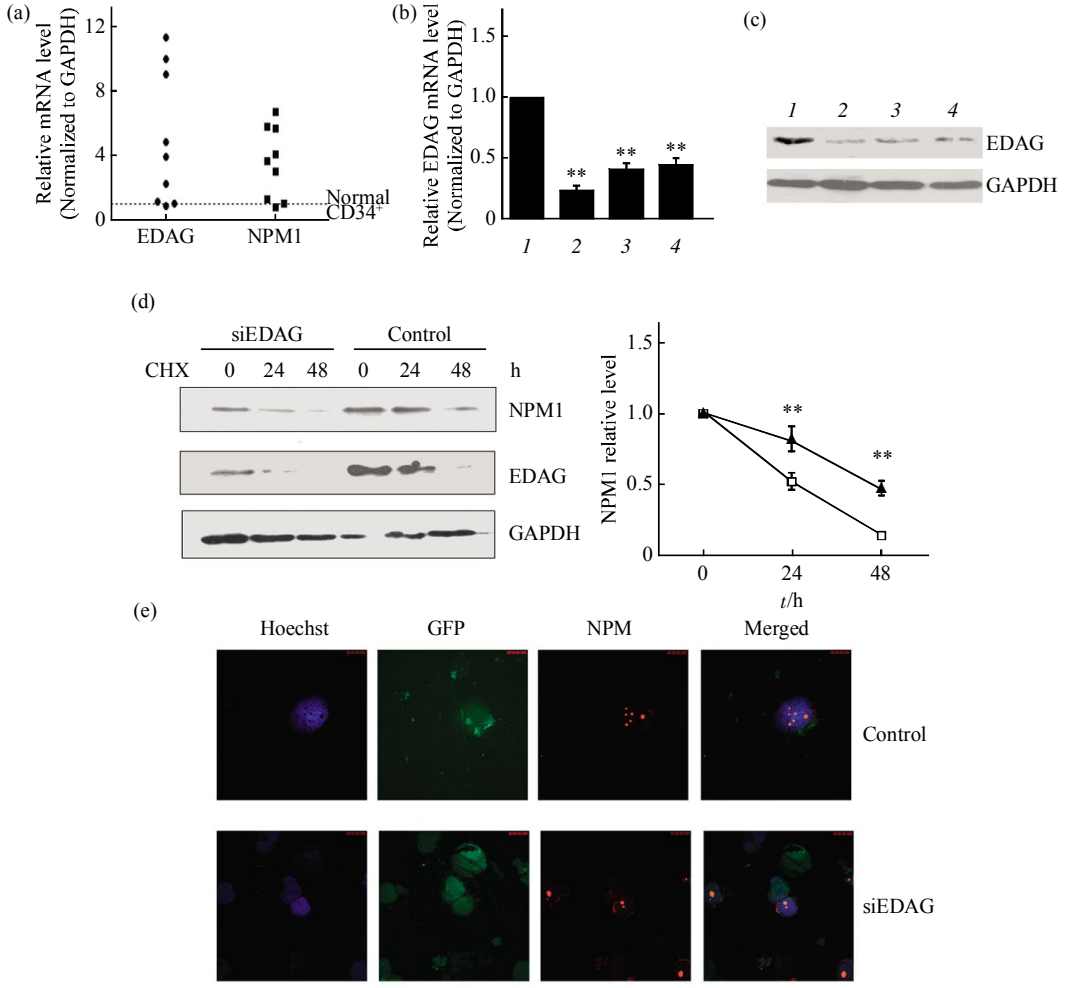

Fig. 1 Knockdown of EDAG decreased the stability of NPM1 in AML CD34$^+$ cells

(a) Bone marrow CD34$^+$ cells from 6 AML patients and 3 healthy people were isolated, RNA was extracted and the mRNA level of EDAG and NPM1 were examined by Real-time PCR. The results were expressed as fold induction compared to one normal CD34$^+$ sample and normalize to GAPDH. (b, c) Bone marrow CD34$^+$ cells from AML patients expressing wild type NPM1 were infected with lentivirus-based EDAG siRNA(siEDAG-1, siEDAG-2, and siEDAG-3) or universal scramble siRNA (Control) and then GFP positive cells were purified by FACS sorter. Total RNA was isolated for Real-time PCR analysis (b) and total cell lysates were prepared for Western blot (c). GAPDH was used as internal control. Results represented $\bar{x} \pm s$ of 3 independent experiments. The statistical difference between the samples was demonstrated as $^{**}P < 0.001$. *1*: Control; *2*: siEDAG-1; *3*: siEDAG-2; *4*: siEDAG-3. (d) BM CD34$^+$ cells from AML patients infected with EDAG siRNA lentivirus (siEDAG-1) or control lentivirus were treated with 100 mg/L CHX for indicated time, and total cell lysates were prepared for Western blot analysis for the antibody indicated. GAPDH were used as internal control. The immunoblot bands were scanned for densitometry analysis with the value obtained from control cells set as 1 (right panel). The values were normalized to GAPDH. Statistical analysis was performed and the results represented $\bar{x} \pm s$ of 6 AML pateints. The statistical difference between the samples was demonstrated as $^{**}P < 0.001$. □—□:siEDAG ; ▲—▲: Control. (e) BM CD34$^+$ cells from an AML patient were immunostained with NPM1 antibody after infected with EDAG siRNA or control lentivirus. 400×.

increased from 2.22 to 11.29 fold compared to normal control and NPM1 increased from 2.98 to 6.69 fold to control. Both of the two proteins expressed at a higher level in bone marrow CD34$^+$ cells from AML patients, which is consistent with our previous findings. Next, we determined whether EDAG affected NPM1 stability in AML patients. Bone marrow CD34$^+$ cells were isolated from AML patients with wild type NPM1 and infected with EDAG siRNA lentivirus or control lentivirus. Then the GFP positive cells were purified using a FACS sorter followed by treatment with CHX for the indicated time. As Figure 1b shows, EDAG siRNA lentivirus infection led to significant reduction of EDAG mRNA level and protein level (Figure 1c) in AML CD34$^+$ cells. Under CHX treatment, NPM1 protein was degraded to 50% at 48 h in control siRNA lentivirus-infected cells, while in EDAG siRNA-1 lentivirus-infected cells, NPM1 protein was degraded at 24 h and its level decreased to about 14% at 48 h (Figure 1d). This result suggested that knockdown of EDAG led to accelerated degradation of NPM1 protein in AML patients. The similar result was obtained with immunofluorescence assay using NPM1 antibody (Figure 1e) and in EDAG siRNA-2 and siRNA-3 infected cells (data not shown).

2.2 Knockdown of EDAG sensitized AML cells expressing WT NPM1 to daunorubicin-induced cell apoptosis

Since EDAG knockdown led to decreased protein stability of NPM1, we hypothesized that EDAG might regulate cell sensitivity to chemotherapy drug. Daunorubicin is an anthracycline antibiotic that induces cell toxity and apoptosis in AML and has been developed to a first line chemotherapy drug for AML[34-35]. We investigated the role of EDAG in cell apoptosis of AML CD34$^+$ cells induced by daunorubicin.

EDAG siRNA lentivirus-infected cells or control cells were incubated with 5 μmol/L daunorubicin for 3 h and thereafter cultured for 8 h in drug-free medium. The cell apoptosis was measured by Annexin V staining. As shown in Figure 2, only a small portion of cells underwent apoptosis in normal untreated conditions (9.9% in control cells and 11.1% in EDAG knockdown cells), however, after daunorubicin treatment, the apoptosis of control cells increased to 37.1% and in EDAG siRNA lentivirus-infected cells, the apoptosis was increased to 69.8%, suggesting that knockdown of EDAG increased the sensitivity to daunorubicin-induced apoptosis.

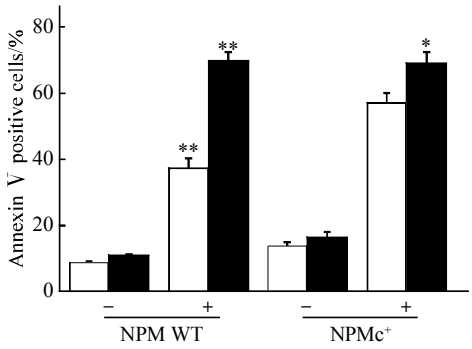

Fig. 2　The effect of EDAG silencing on the daunorubicin-induced cell apoptosis in AML patients

BM CD34$^+$ cells from AML patients with wild type NPM1 (NPM1 WT) or NPMc$^+$ were infected with EDAG siRNA or control lentivirus, sorted for GFP$^+$ cells, then treated with 5 μmol/L daunorubicin for 3 h and thereafter cultured for 8 h in drug-free medium. The apoptosis of cells were measured with Annexin V staining by FACS analysis. □: Control; ■: siEDAG. *P < 0.05; **P < 0.001.

2.3 EDAG interacts with NPMc$^+$ and enhances its protein stability in the presence of leptomycin B (LMB)

Our previous study suggested that EDAG interacts with the acidic domain/nuclear localization domain (118 ～187aa) of NPM1. Since the NPMc$^+$ mutant also contains this domain, we investigated whether EDAG interacts with NPMc$^+$ and regulates the protein stability of NPMc$^+$.

To evaluate the interaction between EDAG and NPMc$^+$ in mammalian cells, cell lysates from 293T cells co-transfected with EDAG-FLAG and Myc epitope-tagged NPMc$^+$ or WT NPM1 expression vectors were subjected to immunoprecipitation with anti-FLAG antibody. The efficiency of each transfection was monitored by GFP cotransfection and all of the transfections displayed similar efficiency (data not shown). As shown in Figure 3a, wild type NPM1 was specifically immunoprecipitated with EDAG by anti-FLAG antibody which was consistent with previous study, however, no interaction was observed between EDAG and NPMc$^+$.

To determine whether EDAG affects NPMc$^+$ stability, EDAG expression vector or control vector was cotransfected with NPMc$^+$ expression vector into 293T cells and then the cells were treated with the protein synthesis inhibitor CHX for the indicated time. Just as Figure 3b shows, in control cells, the NPMc$^+$

protein level decreased to about 50% within 24 h after CHX treatment, suggesting a lower stability of NPMc$^+$ protein compared with wild type NPM1 protein. Contrast to the effect of EDAG on wild type NPM1, EDAG did not affect the protein stability of NPMc$^+$.

Previous studies suggest that in cells carrying NPMc$^+$ mutant treatment with specific exportin-1/Crm-1 inhibitor leptomycin B (LMB) led to the mutant re-location into the nucleus [36-37]. Using red immunofluoresence antibody stained with NPM, we confirmed that in the presence of LMB, the NPMc$^+$ protein translocated from cytoplasm into nucleus, while the nucleolus location of the wild type NPM1 protein did not change (Figure 4). Since EDAG protein mainly localizes in the nucleus, we investigated whether EDAG interacts with NPMc$^+$ in the presence of LMB. As shown in Figure 3a, LMB treatment led to a high affinity interaction between EDAG and NPMc$^+$. Correspondingly, overexpression of EDAG increased the protein stability of NPMc$^+$ in the presence of LMB (Figure 3c).

These results suggest that although NPMc$^+$ contains the binding domain with EDAG, the cytoplasm location result in an escape of NPMc$^+$ protein from EDAG binding and stabilization.

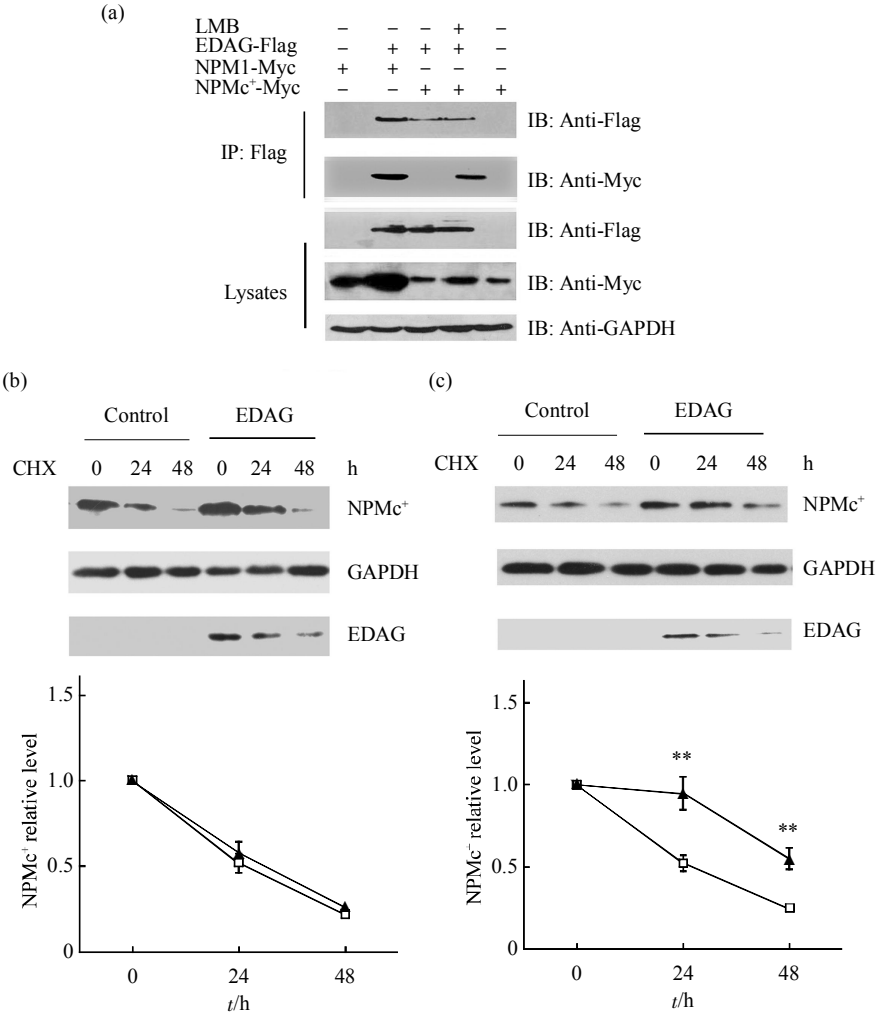

Fig. 3 EDAG regulates NPMc$^+$ protein stability in the presence of LMB

(a) 293T cells were co-transfected with the expression vectors as indicated with or without 20 μg/L LMB, then cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were analyzed with the indicated antibodies. (b, c) 293T cells were co-transfected with the vectors indicated and pretreated with (c) or without (b) 20 μg/L LMB for 2 h and then treated with 100 mg/L CHX for indicated time. Then total cell lysates were prepared for Westeren blot analysis for the antibody indicated. GAPDH were used as internal control. The immunoblot bands were scanned for densitometry analysis with the value obtained from control cells set as 1(lower panel). The values were normalized to GAPDH. Statistical analysis was performed and the results represented $\bar{x} \pm s$ of 3 independent experiments. The statistical difference between the samples was demonstrated as $^{**}P < 0.001$.□—□: Control; ▲—▲: EDAG.

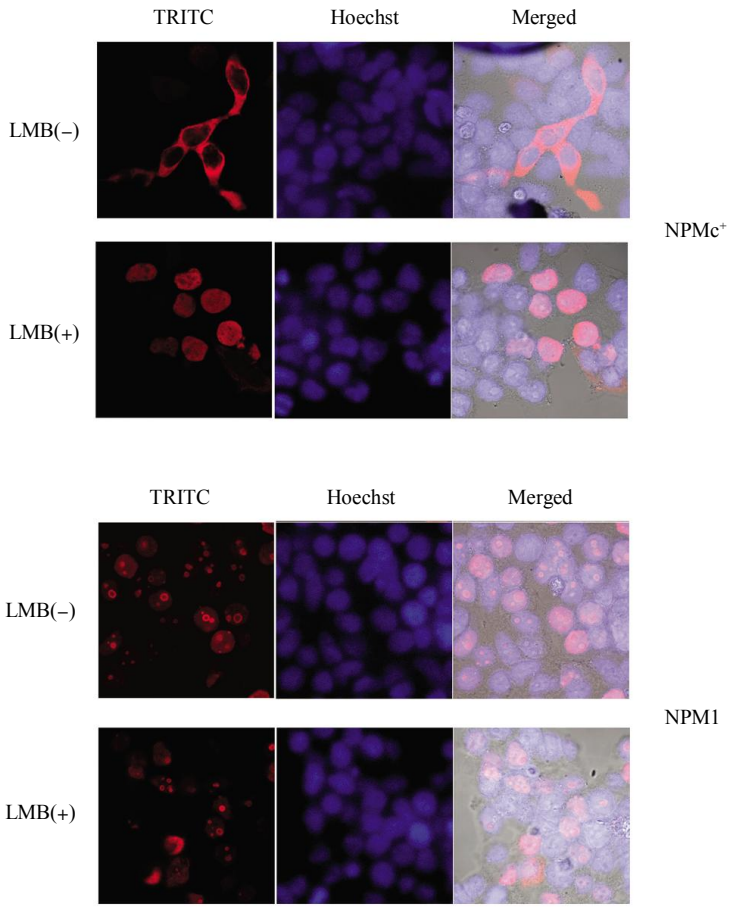

Fig. 4　LMB treatment led to the NPMc$^+$ re-location into the nucleus

293T cells were co-transfected with NPM1 or NPMc$^+$ expression vectors, and 24 h later the cells were treated with or without 20 μg/L LMB for 8 h before harvested. Cells were fixed and immunostained with anti-Myc antibody for NPM1 or NPMc$^+$ location. 400×.

2.4　The effect of EDAG silencing on the daunorubicin-induced cell apoptosis in AML patients with NPMc$^+$

We further examine whether EDAG silencing affect the sensitivity of CD34$^+$ cells from AML with NPMc$^+$ to daunorubicin. EDAG siRNA lentivirus-infected cells or control cells were incubated with 5 μmol/L daunorubicin for 3 h and thereafter cultured for 8 h in drug-free medium. The cell apoptosis was measured by Annexin V staining. As Figure 2 shows, daunorubicin treatment induced a significant apoptosis in control cells (57.1%) which was much higher than that in AML cells expressing wild type NPM1. EDAG silencing weakly increased the apoptosis to 71.6%. This result suggest that in contrast to the AML expressing wild type NPM1, EDAG knockdown only weakly increased the sensitivity to daunorubicin in AML expressing NPMc$^+$.

3　Discussion

Although our previous study suggest that EDAG interacts with NPM1 and enhances the protein stability of NPM1, whether EDAG regulates NPM1 in AML patients and the effect of EDAG on AML expressing NPMc$^+$ remains unknown. In the present study, we found that in AML patients expressing wild type NPM1, silencing of EDAG led to decreased protein stability of NPM1 protein and increased cell sensitivity to daunorubicin. More interestingly, we found that with Leptomycin B treatment, NPMc$^+$ shuttled from cytoplasm into nucleus and EDAG overexpression enhanced the protein stability of NPMc$^+$. In AML patients with NPMc$^+$, the CD34$^+$ cells were more responsive to daunorubicin treatment than the cells

from AML with wild type NPM1, and silencing of EDAG only weakly increased the sensitivity to daunorubicin. These results suggested a potential role of EDAG in chemotherapy of AML with wild type NPM1 and the "escape" of $NPMc^+$ protein from EDAG stabilization might contribute to the favorable prognosis of AML with $NPMc^+$.

NPM1 gene is mutated in one third of adult AML, especially those with normal karyotype [38]. Approximately 50 molecular variants of NPM1 mutations have been identified to date in AML. In spite of this molecular heterogeneity, all mutations create the cytoplasm-dislocated mutant (Mt) NPM1 ($NPMc^+$) protein[25-26]. The most common is the type-A mutation, accounting for 75% of cases, which consists of TCTG tetra-nucleotide tandem duplication at position 956～959 of the NPM1 coding sequence[7, 26, 39]. This mutation causes the loss of tryptophans 288 and 290 (or 290 alone) from the carboxy-terminus and the creation of an additional leucine-rich nuclear export motif in the NPM1 protein, which causes the aberrant cytoplasmic dislocation of $NPMc^+$[7, 26, 39]. Although *in vivo* studies suggested that $NPMc^+$ mutants conferred a proliferative advantage in the myeloid lineage[40-41] and resulted in the expansion of hematopoietic cells, AML-NK carrying $NPMc^+$ is highly responsive to induction chemotherapy [20]. Approximately 80% of patients achieve complete remission with clearance of leukemic cells as early as 16 days after starting treatment. The exquisite chemosensitivity of NPM1-mutated AML is probably related to the aberrant dislocation of nucleophosmin from nucleolus to cytoplasm, but the underlying mechanism through which this occurs remains unknown. Our present study suggests that in normal culture condition, $NPMc^+$ localizes in the cytoplasm and EDAG fails to interact with $NPMc^+$ and then increase its protein stability, suggesting that the $NPMc^+$ might escape the stabilization effect by EDAG dependent on its cytoplasm dislocation. In fact, we also found that the protein stability of $NPMc^+$ is much lower than wild type NPM1 with a 24 h-halflife time. So the decreased protein stability of $NPMc^+$ might contribute to the favorable prognosis of AML-NK with $NPMc^+$. Further detailed studies are needed to illustrate this possibility.

Since the NPM1 mutations are always heterozygous and the primary blasts from NPM1-mutated AML patients retain in the nucleolus a certain amount of wild-type NPM1 protein, the residual dose of nuclear normal NPM1 may be necessary for the survival of the NPM1-mutated leukemia cells [42]. The use of drugs able to interfere with the localization/function of the wild type NPM1 encoded by the normal allele is a potential therapeutic intervention for NPM1-mutated AML. Small molecules could be developed to selectively bind to the residual nuclear NPM1WT protein[43] with the aim of interfering with its nuclear building capacity [44]. They would hopefully enhance the propensity of NPM1-mutated AML cells to die or be sensitized to the action of chemotherapeutic agents. The anti-cancer peptide CIGB-300 binds to nucleophosmin and leads to apoptosis through its nuclear disassembly activity[43]. In the present study we found that EDAG silencing in AML without $NPMc^+$ result in decreased protein stability of NPM1 and significant increase of the sensitivity to daunorubicin treatment. More interestingly, although EDAG does not alter the protein stability of $NPMc^+$, silencing of EDAG in AML with $NPMc^+$ still leads to a weak increase of sensitivity to daunorubicin. The increased sensitivity might be due to a down-regulation of residual wild type NPM1 protein. These results raise the possibility that targeting EDAG might be a potential therapeutic strategy for AML with NPM1 mutations.

In conclusion, EDAG knockdown in AML cells reduces the NPM1 protein stability and increases the susceptibility to chemotherapy drug, suggesting a potential role of EDAG in AML therapy.

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敲低 EDAG 加强 NPM1 蛋白的降解并增加 AML 病人对药物的敏感性 *

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摘要 Nucleophosmin (NPM1 或 B23.1)是在细胞核内广泛表达的蛋白磷酸酶，在多方面发挥重要作用，如核糖体合成、中心体复制、细胞周期控制、细胞增殖及转化．NPM1 是急性粒细胞白血病(acute myeloid leukemia, AML)中最常见的突变基因之一．红系分化相关基因(erythroid differentiation associated gene, EDAG)是在造血组织特异表达的基因，在造血细胞的增殖与谱系分化调节方面发挥重要作用．在 AML 病人中，高表达的 EDAG 与较差的预后相关联．我们前期研究结果显示，EDAG 与 NPM1 相结合并调节 NPM1 稳定性，但在 AML 病人体内 EDAG 与 NPM1 的关系，及 EDAG 与 NPM 突变体(NPMc+)的关系尚未明确．在本文中发现：在 AML 病人骨髓 CD34+ 细胞中，敲低 EDAG 表达导致 NPM1 蛋白稳定性降低并提高了对柔红霉素的敏感性； EDAG 虽不与突变体 NPMc+ 相互作用，但在蛋白出核抑制剂(leptomycin B, LMB)作用下，过表达 EDAG 提高 NPMc+ 蛋白稳定性；表达突变 NPMc+ 的 AML 病人与表达 NPM1 蛋白的病人相比，其骨髓 CD34+ 细胞对柔红霉素具有更高的敏感性，且敲低 EDAG 能微弱提高其敏感性．上述结果表明，EDAG 在 AML 病人药物治疗中发挥的可能作用以及 NPMc+ “逃脱”，使 EDAG 无法保护其稳定性，这提示了在 AML 病人药物治疗过程中 EDAG 的潜在作用，同时也提示，携带 NPMc+ 蛋白的 AML 患者具有较好预后，可能与 NPMc+ 蛋白“逃脱”出 EDAG 对其稳定性的保护有关．

关键词 EDAG，NPM1，NPMc+，柔红霉素，AML
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