

Role of cofilin-1 in arsenic trioxide-induced apoptosis of NB4-R1 cells

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Received November 16, 2019; Accepted September 15, 2020

DOI: 10.3892/mmr.2020.11570

Abstract. All-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) are currently first-line treatments for acute promyelocytic leukemia (APL). However, a number of patients with APL are resistant to ATRA but still sensitive to As_2O_3 , and the underlying mechanisms of this remain unclear. In the present study, two-dimensional gel electrophoresis, mass spectrometry and other proteomic methods were applied to screen and identify the differentially expressed proteins between the retinoic acid-sensitive cell lines and drug-resistant cell lines. The results demonstrated that in retinoic acid-resistant NB4-R1 cells, the protein expression of cofilin-1 was markedly increased compared with that in the drug-sensitive NB4 cells. Subsequently, the effects of cofilin-1 on As_2O_3 -induced apoptosis in NB4-R1 cells were further investigated. The results revealed that cell viability was markedly suppressed and apoptosis was increased in the As_2O_3 -treated NB4-R1 cells, with increased expression levels of cleaved-poly (ADP-ribose) polymerase and cleaved-caspase 12. Cofilin-1 expression was significantly decreased at both the mRNA and protein levels in the As_2O_3 -treated group compared with the control. Western blotting further revealed that As_2O_3 treatment decreased the cytoplasmic cofilin-1 level but increased its expression in the mitochondrion. However, the opposite effects of As_2O_3 on the cytochrome C distribution were found in NB4-R1 cells. This suggested that As_2O_3 can induce the transfer of cofilin-1 from the cytoplasm to mitochondria and trigger the release of mitochondrial cytochrome C in NB4-R1 cells. Moreover, cofilin-1 knockdown by its specific short hairpin RNA significantly suppressed As_2O_3 -induced NB4-R1 cell apoptosis and inhibited the release of mitochondrial cytochrome C. Whereas, overexpression of cofilin-1 using a plasmid vector carrying cofilin-1 increased the release of cytochrome C into the

cytoplasm from the mitochondria in As_2O_3 -treated NB4-R1 cells. In conclusion, cofilin-1 played a role in As_2O_3 -induced NB4-R1 cell apoptosis and it might be a novel target for APL treatment.

Introduction

Acute promyelocytic leukemia (APL) accounts for 10-12% of all cases of acute myeloid leukemia worldwide, with a characteristic chromosomal abnormality t(15;17)(q22;q23) and a specific promyelocytic leukemia protein (PML)/retinoic acid receptor α (RAR α) fusion protein (1,2). APL results from a blockade of granulocyte differentiation at the promyelocytic stage and is associated with a high incidence of coagulopathy, including disseminated intravascular coagulation, fibrinolysis and proteolysis (3). In APL, there is a tendency to bleed, disproportionate to thrombocytopenia (4). APL blasts in the bone marrow can lead to megakaryocyte inhibition. Besides, the promyelocytes of APL are able to activate the coagulation cascade and increase procoagulant activity in endothelial cells (3). The coagulopathy would also induce thrombocytopenia. Currently, combined all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) treatment is recommended as the first-line treatment for low-risk APL worldwide (5), with a complete remission rate of 94-100%, a 2-year overall survival rate of 97% and a 4-year survival rate of 93% (6,7). It has been reported that arsenic agent can bind to the PML portion of PML/RAR α protein and activate the ubiquitin-proteasome system, leading to the degradation of PML/RAR α protein (8) and the apoptosis of APL cells. When ATRA combines with RARs or retinoid X receptors of PML/RAR α protein, RAR α can further induce APL cell differentiation, and promote myeloid and granulocyte maturation (9). However, the key pathways and proteins involved in the degradation of PML/RAR α protein and apoptosis remain unclear; and certain patients relapse with resistance to ATRA or As_2O_3 . Therefore, there have been numerous studies on the APL apoptosis pathway and the mechanism of resistance to ATRA and As_2O_3 . Several studies have found mutations in the PML gene that contribute to resistance to arsenic. Goto *et al* (10) reported A216V and L218P mutations of the PML protein in two arsenic-resistant cases. In 2014, a previous study found a PML gene mutation through gene sequencing and proposed a 'mutation hotspot' (c202-s220) of PML in the case of arsenic resistance (11).

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Key words: cofilin-1, apoptosis, arsenic trioxide, acute promyelocytic leukemia

Patients with PML mutations have a high fatality rate when arsenic resistance occurs. A French study successfully established a mouse model of the A216V mutation, which presented resistance to arsenic treatment (12). ATRA resistance has a more complex mechanism. Prolonged oral administration of ATRA can lead to increased cytochrome P450 oxidase activity, which results in decreased blood ATRA concentration (13). Elevation of cellular retinoic acid-binding protein contributes to a decrease in free ATRA, and a decreased ATRA concentration in the nucleus also results in reduced efficacy (14,15). In the present study, using proteomics research methods, a total of 21 differentially expressed proteins between retinoic-resistant cell lines and non-resistant cell lines were screened and identified, among which the expression of cofilin-1 was upregulated.

Cofilin is a 21-kD actin-binding protein that is universally present in eukaryotes and is crucially involved in regulating the reorganization of the cytoskeleton and muscle development (16). In humans, there are two cofilin gene subtypes, cofilin-1 and cofilin-2, which encode different proteins. The former is expressed in a variety of tissues except for muscle, whereas the latter is mainly expressed in muscle. Cofilin can bind to F-actin, accelerate the dissociation of actin monomers from the filament, and lead to the depolymerization of F-actin (17). Besides the basic function of regulating the actin cytoskeleton, cofilin plays a role in the metastasis, infiltration and apoptosis of tumor cells (18,19). It was previously reported that at the early stage of apoptosis, cofilin transferred from the cytoplasm to the mitochondria and altered the permeability of mitochondrial membranes, leading to the release of cytochrome C and triggering cell apoptosis via the mitochondrial apoptosis pathway (20).

In the present study, a significant increase of cofilin-1 expression was found in retinoic acid-resistant NB4-R1 cells. Following which, the effects of cofilin-1 in As₂O₃-induced apoptosis in NB4-R1 cells were evaluated, and the possible underlying mechanisms were explored. The present study could provide novel strategies for APL treatment.

Materials and methods

Reagents and antibodies. As₂O₃ was purchased from Sigma-Aldrich (Merck KGaA) and dissolved at a concentration of 5 μ M in DMSO. Primary antibodies against poly (ADP-ribose) polymerase (PARP; cat. no. 9532), cleaved PARP (cat. no. 5625), caspase 12/cleaved caspase 12 (cat. no. 2202), cofilin (cat. no. 5175), cytochrome C (cat. no. 11940), cytochrome c oxidase subunit 4 isoform 1 mitochondrial (COX IV; cat. no. 4850) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. The annexin V-FITC Apoptosis kit was purchased from BD Biosciences.

Cell culture. Human APL cell lines, NB4-R1 and NB4 cells were obtained from the Institute of Hematology, Shanghai Ruijin Hospital (Shanghai, China). The NB4 cell line was derived from a patient with APL who underwent relapse and was established by Dr Lanotte (Saint Louis Hospital, France) in 1991, with characteristic chromosome translocation t(15;17) and positive PML-RAR α (L type) gene. The NB4-R1 cell line is an APL subclone resistant to ATRA and is derived from NB4 cells. The NB4-R1 and NB4 cells were maintained in

RPMI-1640 medium (Cytiva) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The culture medium was replaced every 3 days.

Two-dimensional gel electrophoresis (2-DE) and image analysis. Cells were collected and solubilized in lysis buffer containing 7 mol/l urea, 2 mol/l thiourea, 4% (w/v) 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 1% protease inhibitor cocktail (v/v), and 2% (v/v) IPG buffer at 11,000 IU/min on ice. The agents used were purchased from Promega Corporation. Suspensions were then placed at 4°C for 1 h followed by centrifugation at 10,000 \times g for 30 min at 4°C. Supernatants were obtained and protein concentrations were measured using the Bradford method. Each protein sample (90 μ g) was loaded on 24-cm immobilized pH gradient (IPG) strips (pH 3-10; non-linear; Cytiva). Following the rehydration of the IPG-strips and isoelectric focusing, the strips were equilibrated. Subsequently, 2-DE was performed in an Ettan-Dalt twelve electrophoresis system (Cytiva). Following silver-staining, as previously described (21), all the gels were scanned using ImageScanner™ (Cytiva) and the resulting images were used for detection, quantification, matching and analysis using ImageMaster™ 2D Platinum software (version 5.0; Cytiva). Each sample was run three times to minimize the variation and an average gel was made to represent the medium protein expression level of each group. The protein spots that changed in all three gels were compared between each conditioned group. The differentially expressed proteins were further identified by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).

Protein identification by MS. The selected protein spots were excised from silver-stained gels, cut into small pieces, and dehydrated in 50 μ l acetonitrile (ACN) for 5 min at room temperature. The gel pieces were dried following removal of the acetonitrile and incubated in 50 μ l 10 mM DTT at 56°C for 1 h, followed by an alkylating incubation in 50 μ l 55 mM iodoacetamide in the dark. Subsequently, the spots were dehydrated with 50 μ l ACN, rehydrated in 5 μ l trypsin for 30 min, and then 10 μ l 25 mM ammonium bicarbonate was added. Proteolysis continued overnight at 37°C and was then stopped by adding 10 μ l 2% formic acid and desalted using C18 ZipTips (EMD Millipore). The resulting peptides were concentrated, mixed with α -cyano-4-hydroxycinnamic acid (α -HCCA), deposited on a 384-well MALDI target and air-dried. All samples were analyzed in the positive-ion, reflectron mode on a TOF Ultraflex II mass spectrometer (Bruker Corporation). The accelerating potential was 20 kV with eight shots per second. Trypsin autodigestion peaks were used for internal calibration.

Database searching using MS/MS data. All peptide mass fingerprinting (PMF) and MS/MS data were used for protein identification using the MASCOT search program (<http://www.matrixscience.com>) based on the Uniprot protein database (<http://www.uniprot.org>). Up to one missed trypsin cleavage per peptide was allowed, although most matches did not contain any missed cleavages. A mass tolerance of 100 ppm was the window of error allowed for matching the peptide

mass values. Proteins with a score >56 identified by MASCOT were considered significant and were verified manually for spectral quality.

Lentiviral vector infection. Three short hairpin RNAs (shRNAs) targeting the human cofilin-1 gene (GenBank no. NM_005507) for RNA interference were designed using siRNA Wizard™ software version 3.1 (<https://www.invivogen.com/sirnazard/design.php>). The efficacy of the sequence for cofilin-1 knockdown was evaluated using western blotting (data not shown) and the most effective one was screened as follows: 5'-GACAGGGATCAAGCATGAA-3'. The scrambled sequence (5'-AAUCGCAUAGCGUAUGCCGUU-3') was used as a negative control. Following heating and annealing, the sequence was ligated into the *Age*I and *Eco*RI sites of pGCSIL-GFP (containing human U6 promoter; Shanghai GeneChem Co., Ltd.) to generate a pGCSIL-GFP-cofilin vector, which was then transformed into *E. coli*. Positive recombinant clones were selected by DNA sequencing. The new recombinant viral vector was generated by co-transfecting 293T cells (American Type Culture Collection) with the lentivirus expression plasmid and packaging plasmids (pHelper 1.0 and pHelper 2.0) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 48 h after transfection, the recombinant lentiviral vector was harvested for the subsequent viral infection.

NB4-R1 cells were divided into two groups: i) Sc, infected with negative control lentiviral vector; and ii) shCofilin, infected with the pGCSIL-GFP-cofilin lentiviral vector. Cells were cultured at a density of 6×10^5 /well in 6-well plates with addition of 8 μ g/ml polybrene (Sigma-Aldrich; Merck KGaA) and infected with specific or negative control lentiviral vectors (viral titer range, 2×10^8 - 2×10^9 TU/ml). Following incubation for 48 h, cells were observed under a fluorescence microscope (magnification, x200 or x400). The knockdown efficiency of transfection was analyzed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. For the subsequent experiments, 48 h after infection, cells were divided to four groups, sc, sc+As₂O₃, shCofilin and shCofilin+As₂O₃. Cells in the sc+As₂O₃ or shCofilin+As₂O₃ groups were then treated with 2.5 μ M of As₂O₃, while the other two groups were treated with an equal volume of culture medium. One day later, cells were collected for the subsequent experiments.

Overexpression plasmid. Cofilin-1 overexpression plasmid (pcDNA3.1-cofilin) and a control vector were designed and constructed by Shanghai GeneChem Co., Ltd. For transfection, NB4-R1 cells at a density of 5×10^5 /ml in 6-well plates were transfected with 3 μ g expression plasmid in each group using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h of transfection, cells were divided into three groups, control, As₂O₃ and As₂O₃ + oeCofilin groups. The cells were then treated with 2.5 μ M of As₂O₃ (or an equal volume of culture medium for the control). One day later, cells were collected for subsequent experiments. The present study aimed to investigate the effects of As₂O₃ on drug-resistant cell NB4-R1, so NB4 cells were not used for the overexpression experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was determined using the MTT method. The NB4-R1 cells were inoculated in 96-well plates at 5×10^3 cells/well and then treated with As₂O₃ of different concentrations (0, 0.625, 1.25, 2.50, 5.0, 7.5 and 10 mmol/l). After 24 h, 20 μ l MTT reagent (cat. no. ab211091; Abcam) was added into each well and incubated for 4 h, then 150 μ l DMSO was added and the plates were oscillated until the crystalline matter was fully dissolved. The absorbance at 490 nm was measured using a spectrophotometric plate reader.

Isolation of mitochondria. The cytoplasm and mitochondria extraction was conducted using a Mitochondria Isolation kit (cat. no. MITOISO2; Sigma-Aldrich; Merck KGaA) in accordance with the manufacturer's protocols. Briefly, cells from each group were collected, washed with ice-cold PBS, and subsequently were subjected to a 5 min 600 x g centrifugation step at 4°C. The supernatant was discarded and cells were incubated and homogenized with Extraction Buffer A on ice for 10 min. Then, the homogenate was pelleted for 10 min at 700 x g at 4°C. The post-nuclear supernatant was subjected to a 25 min, 10,000 x g centrifugation step at 4°C to pellet the mitochondrial fraction. The post-mitochondrial supernatant was collected and regarded as the remaining cellular cytoplasm for further analysis. The pellet was suspended with Fractionation Buffer Mix as the mitochondrial fraction. To confirm the protein origin, GAPDH was applied as the internal reference for cytoplasm, while COX IV, a membrane protein in the inner mitochondrial membrane, was the internal reference for mitochondria.

Western blot analysis. NB4-R1 cells were cultured in 6-well plates and following the different treatments, proteins were collected and lysed with RIPA buffer (Thermo Fisher Scientific, Inc.) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) for western blotting. Protein concentration was determined using the BCA method (Pierce; Thermo Fisher Scientific, Inc.) and lysates (20-40 μ g/sample) were subjected to 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked at room temperature for 1 h in 5% non-fat dry milk in Tris-HCl buffer followed by incubation with primary antibodies overnight at 4°C. Primary antibodies (1:1,000 dilution) against COX IV and GAPDH served as loading controls, GAPDH for cytoplasm (22) and COX IV for mitochondria (23). Membranes were then incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. Following a thorough rinse, immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.) and were visualized by the charge-coupled device imaging system (Bio-Rad Laboratories, Inc.). The protein expression was quantified using ImageJ 3.5 software (National Institutes of Health). The values were normalized to the GAPDH band.

RT-qPCR. NB4-R1 cells were cultured in 6-well plates and treated with different concentrations (0.625, 1.25, 2.5, 5, 7.5 and 10 μ mol/l) of As₂O₃ for 24 h. Total RNA was extracted from cultured cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RT was performed using the RevertAid First

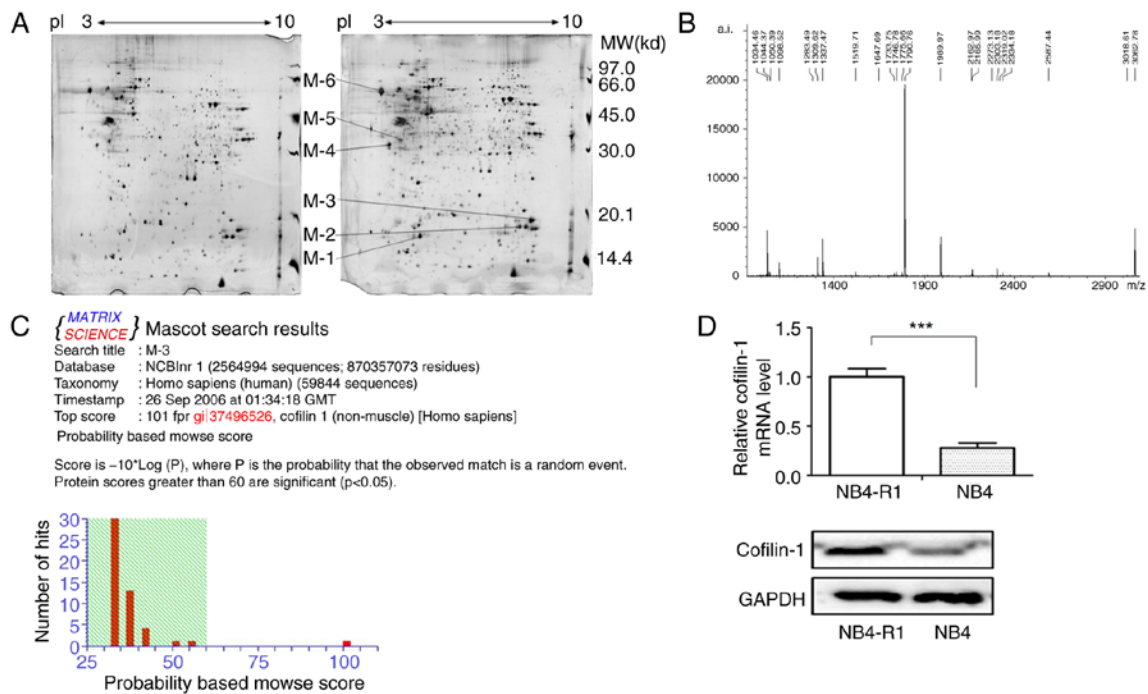


Figure 1. Analysis of differentially expressed proteins between NB4-R1 and NB4 cells. (A) Detection of protein spots by 2-dimensional electrophoresis in NB4-R1 and NB4 cells. (B) Matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry analysis of the M3 spot. (C) Identification of the M3 spot by the MASCOT search program. (D) Cofilin-1 expression in NB4-R1 and NB4 cells, as indicated by reverse transcription-quantitative PCR and western blotting. *** $P < 0.001$.

Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) and the reaction was carried out in a thermocycler at 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The relative abundance of mRNA in each sample was determined by qPCR using the SYBR Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.) on an iQ™ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Inc.). The corresponding primers were designed and synthesized by Takara Biotechnology Co., Ltd., as follows: Cofilin-1 forward, 5'-CACCTTTGT CAAGATGCT-3' and reverse, 5'-GGAGCTGGCATAAAT CAT-3'; GAPDH forward, 5'-GTCATCCCAGAGCTGAAC-3' and reverse, 5'-TCAGTGTAGCCCAAGATG-3'. The PCR cycles were performed under the following conditions: 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Data were analyzed using the $2^{-\Delta\Delta C_q}$ method (24) and GAPDH served as the internal control. The results are presented as the mean \pm standard deviation (SD) of triplicate reactions from three separate experiments.

Flow cytometry analysis. NB4-R1 cells with or without cofilin-1 shRNA treatment were routinely cultured in the 6-well plates, and cell density was adjusted to 4.0×10^5 /well. Subsequently, cells were treated with As_2O_3 or an equal amount of solvent for 24 h according to the grouping. The cells were then collected, washed with cold PBS, stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using an Annexin V-FITC Apoptosis Detection kit (Merck KGaA) according to the manufacturer's instructions, and then analyzed by a FACSCalibur (BD Biosciences) accompanied with CellQuest™ software (version 5.1; BD Biosciences). Each experiment was performed in triplicate wells and repeated three times.

Fluorescence. NB4-R1 cells were cultured at a density of 1×10^5 /well in 6-well plates, following the different treatments. After fixation in 4% formaldehyde in PBS (pH 7.2) at room temperature for 15 min, cells were stained with 20 μ M DAPI in antifade solution (Qbiogene, Inc.) at room temperature for 5 min. The stained cells were visualized using a BX51 fluorescent microscope (magnification, x200 or x400) equipped with a DP70 digital camera (Olympus Corporation).

Statistical analysis. All the data are reported as the mean \pm SD from at least three independent *in vitro* experiments. Shapiro-Wilk test was used to test normality of data. Differences among groups were analyzed using one-way analysis of variance, followed by Tukey's post hoc test. All statistical analyses were performed using the SPSS statistical software (version 19.0; IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Protein expression profiles of NB4-R1 and NB4 cells. In the present study, 2-DE and silver staining were used to analyze the proteomic profile of NB4-R1 and NB4 cells from three independent biological replicates. The representative gel images are shown in Fig. 1, which indicates the total differential spots. In total, $>1,000$ protein spots per gel ($1,068 \pm 33$ for NB4-R1 cells and $1,160 \pm 51$ for NB4 cells) were detected using ImageMaster™ 2D Platinum software. One 2-DE gel of the NB4 cells was randomly selected as the reference gel, and other gels were matched with it for analysis. The results showed that the matching rate of protein spots was 81% in NB4 cells and 78% in NB4-R1 cells. The matching rate between NB4-R1

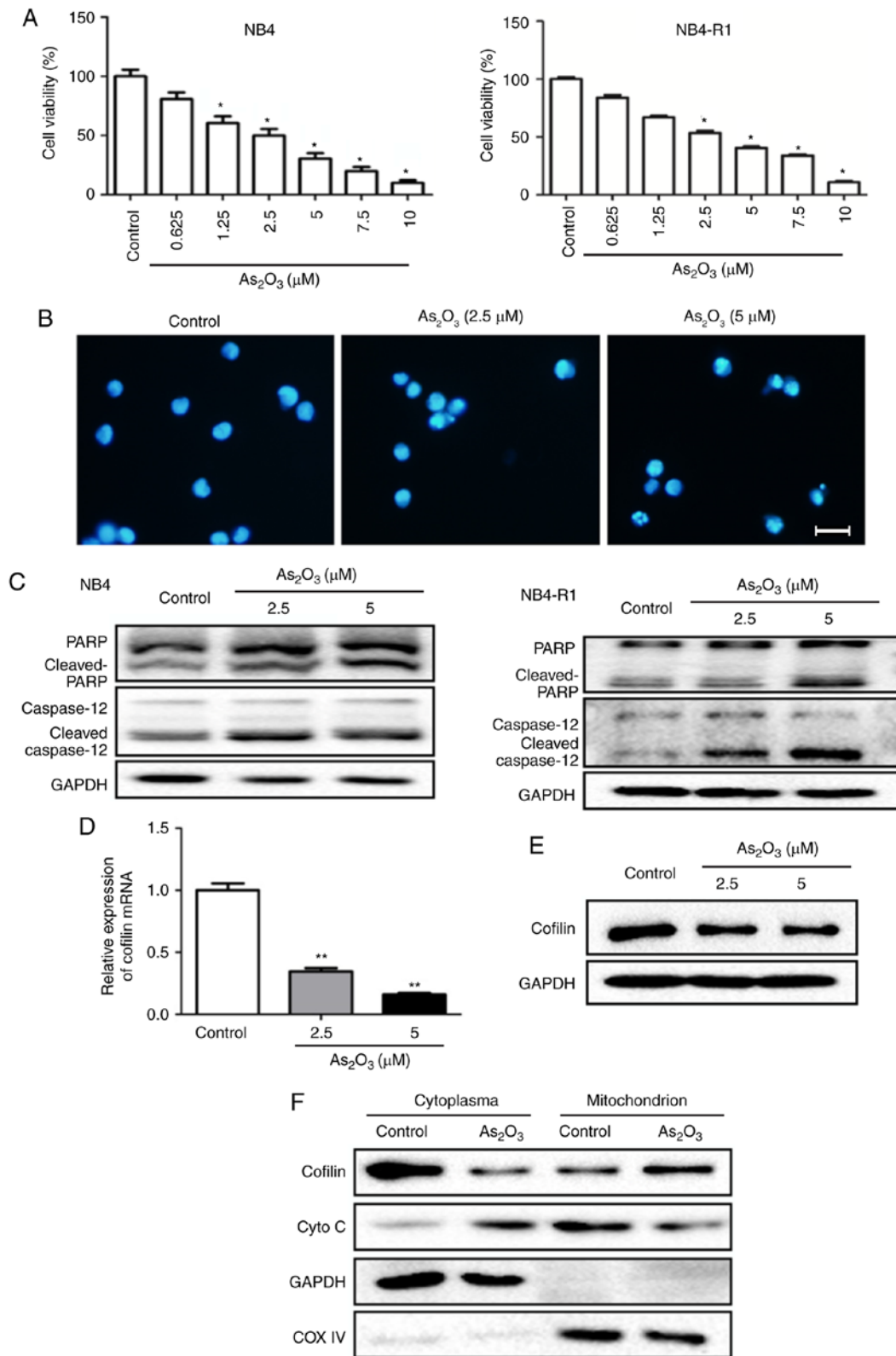


Figure 2. Effects of As₂O₃ in NB4-R1 or NB4 cells. (A) As₂O₃ treatment with a concentration ≥ 2.5 $\mu\text{mol/l}$ significantly inhibited cell viability of NB4 and NB4-R1 cells. (B) As₂O₃ inhibited NB4-R1 cell growth, as indicated by immunofluorescence (scale bar, 50 μm). (C) As₂O₃ treatment at concentrations of 2.5 and 5 $\mu\text{mol/l}$ notably increased cleaved-PARP and cleaved-caspase 12 expression in NB4 and NB4-R1 cells, as indicated by western blotting. As₂O₃ decreased cofilin expression at the (D) mRNA and (E) protein levels in NB4-R1 cells. (F) As₂O₃ notably reduced cytoplasmic cofilin expression, whereas it markedly increased the mitochondrial content, as indicated by western blotting. The opposite result was found for the cytoplasmic and mitochondrial levels of cytochrome C. * $P < 0.05$, ** $P < 0.01$ vs. control group. As₂O₃, arsenic trioxide; PARP, poly (ADP-ribose) polymerase; COX IV, cytochrome c oxidase subunit 4 isoform 1 mitochondrial.

and NB4 cells was 67%. Moreover, there were 21 protein spots found to be significantly up- or downregulated, ≥ 2 or ≤ 0.5 -fold,

respectively, in NB4-R1 cells (data not shown). Among these, six proteins were upregulated, M1-M6 shown in Fig. 1A.

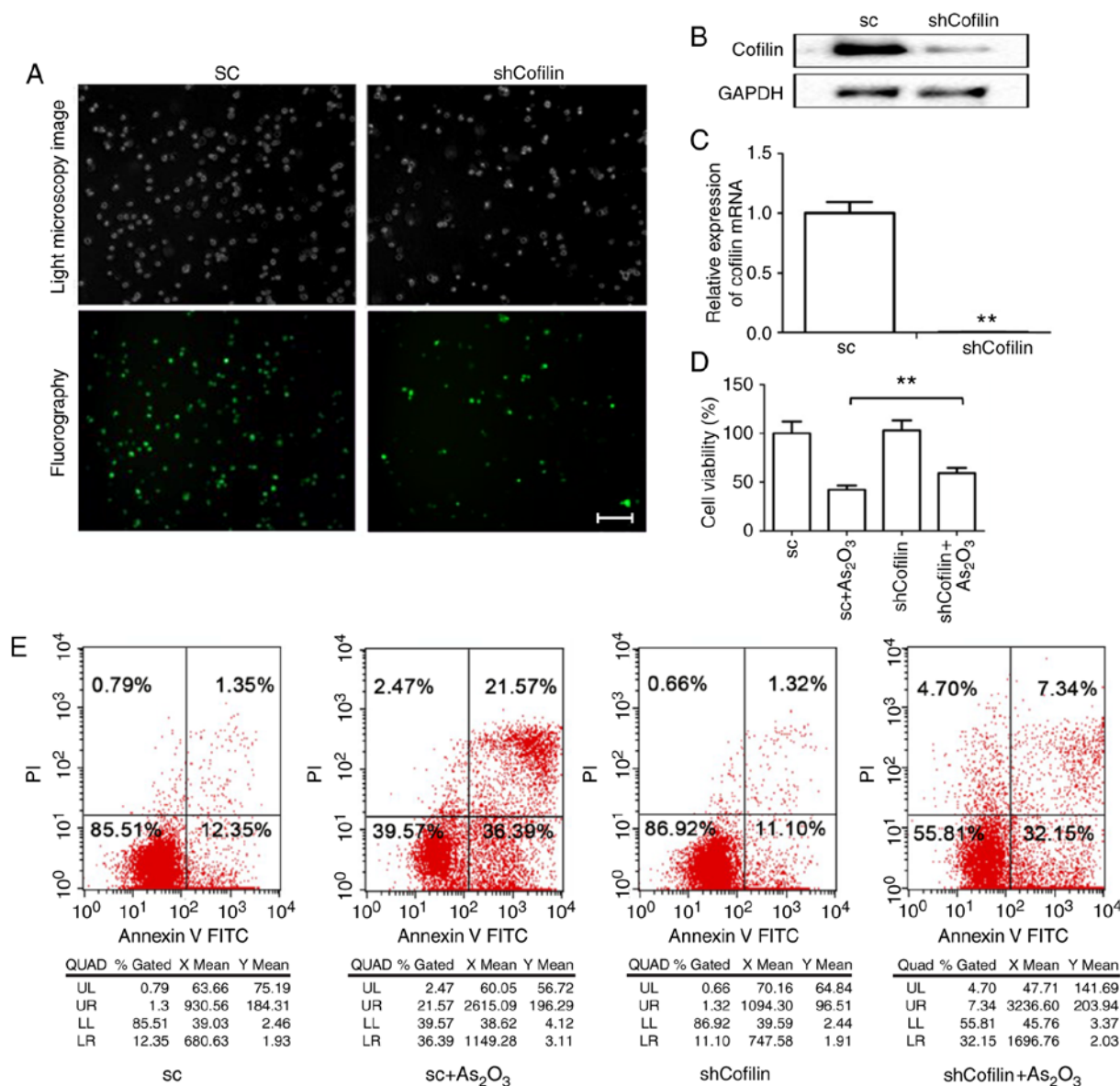


Figure 3. Cofilin knockdown suppresses the effects of As₂O₃ on cell viability and apoptosis in NB4-R1 cells. (A) Immunofluorescence staining showed that the recombinant viral vectors containing shRNA were successfully transfected into the NB4-R1 cells (scale bar, 100 μ m). The expression of cofilin was markedly decreased by shRNA at the (B) mRNA and (C) protein levels. (D) Cofilin knockdown increased cell viability in As₂O₃-treated NB4-R1 cells. (E) Cofilin knockdown inhibited apoptosis in As₂O₃-treated NB4-R1 cells. **P<0.01 vs. sc or sc + As₂O₃ group. As₂O₃, arsenic trioxide; shRNA, short hairpin RNA; sc, negative control.

The M3 spot was further studied using PMF and MALDI-TOF-MS analysis (Fig. 1B) and the data were analyzed using the MASCOT search program (<http://www.matrixscience.com>) (25,26). The results revealed that M3 corresponded to cofilin-1 (Fig. 1C). The RT-qPCR and western blotting results further confirmed the significantly increased expression of cofilin-1 at the mRNA and protein levels in NB4-R1 cells, compared with that in the NB4 cells (Fig. 1D).

As₂O₃ suppresses cell viability and promotes apoptosis in NB4-R1 or NB4 cells. NB4 and NB4-R1 cells were treated with different concentrations (0.625, 1.25, 2.5, 5, 7.5 and 10 μ mol/l) of As₂O₃ for 24 h, and the results showed that cell viability was significantly inhibited by As₂O₃ at a concentration ≥ 2.5 μ mol/l in both cell types (Fig. 2A). Immunofluorescence staining further indicated the inhibitory effect of As₂O₃ on NB4-R1 cell growth (Fig. 2B). A similar result that As₂O₃ suppressed

the proliferation was also seen in NB4 cells (data not shown). Besides, western blotting demonstrated that both cleaved-PARP and cleaved-caspase 12 were notably increased following As₂O₃ treatment at a concentration of 2.5 and 5 μ mol/l in NB4-R1 cells. Similar results were found in NB4 cells (Fig. 2C).

Effects of As₂O₃ on cofilin-1 expression and transfer in NB4-R1 cells. Cytoplasmic and mitochondrial cofilin levels in NB4-R1 cells were detected using RT-qPCR and western blotting, with or without As₂O₃ treatment. The results showed that following As₂O₃ treatment at 5 μ mol/l for 24 h, both the mRNA level and cytoplasmic protein content of cofilin-1 were significantly decreased, whereas the protein level of cofilin-1 in mitochondria was markedly increased (Fig. 2D-F). This indicated that As₂O₃ could decrease the production of cofilin-1, as well as induce cofilin-1 transfer to the mitochondria from the cytoplasm in NB4-R1 cells. Besides, experiments were

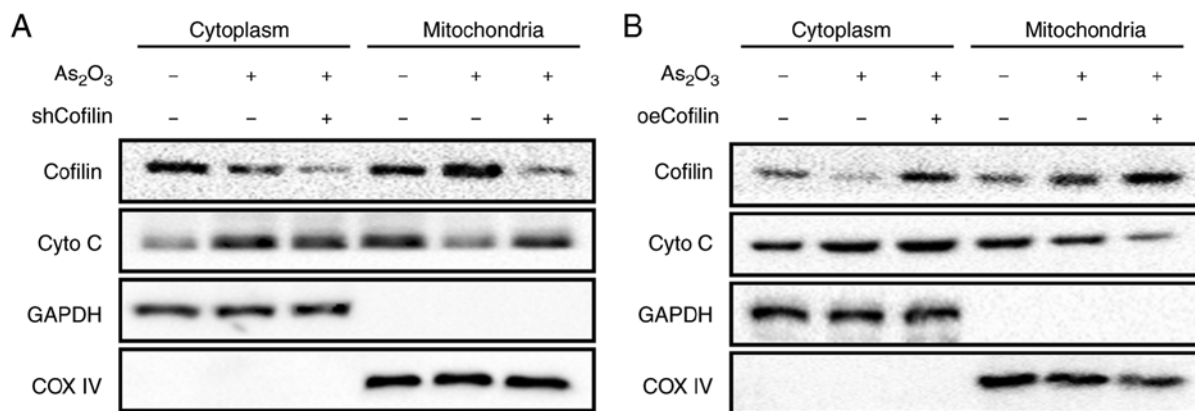


Figure 4. Role of cofilin-1 in mitochondrial cytochrome C release. (A) Knockdown of cofilin-1 increased the mitochondrial expression of cytochrome C, whereas it decreased the cytoplasmic content of cytochrome C in As₂O₃-treated NB4-R1 cells. (B) Overexpression of cofilin-1 markedly decreased the mitochondrial expression of cytochrome C, whereas it increased its cytoplasmic content in As₂O₃-treated NB4-R1 cells. As₂O₃, arsenic trioxide; shRNA, short hairpin RNA; oe, overexpression vector; COX IV, cytochrome c oxidase subunit 4 isoform 1 mitochondrial.

conducted to study the effects of As₂O₃ on cofilin-1 translocation in NB4 cells. The results showed that As₂O₃ treatment could also induce cofilin-1 transfer to the mitochondria from the cytoplasm in NB4 cells (data not shown).

Effects of As₂O₃ on the production and transfer of cytochrome C in NB4-R1 cells. In NB4-R1 cells, mitochondrial expression of cytochrome C was notably decreased, whereas the cytoplasmic level was markedly increased after As₂O₃ treatment, compared with the control group (Fig. 2F). Combined with the result of increased cleaved-caspase 12 following As₂O₃ treatment (Fig. 2C), the data suggested that cytochrome C was released to the cytoplasm, which activated the mitochondrial apoptosis pathway at the early stage of cell apoptosis.

Role of cofilin-1 in As₂O₃-induced apoptosis of NB4-R1 cells. A stable shCofilin cell line was obtained with a down-regulated expression of the cofilin gene (target gene sequence: GACAGGGATCAAGCATGAA) by transfection of NB4-R1 cells with shRNA. Immunofluorescence staining demonstrated that the recombinant viral vectors containing shRNA were successfully transfected into the NB4-R1 cells, and RT-qPCR and western blotting indicated that cofilin expression was significantly decreased by shRNA (Fig. 3A-C). No significant alterations in viability and apoptosis were found between the shRNA control and shCofilin groups. Compared with the sc + As₂O₃ group, cell viability was increased (Fig. 3D) and apoptosis was significantly decreased in the shCofilin + As₂O₃ group (Fig. 3E).

Association between cofilin-1 expression and mitochondrial cytochrome C release. The cytoplasmic and mitochondrial levels of cytochrome C in the shCofilin and oeCofilin groups with or without As₂O₃ treatment were detected using western blotting. The results showed that in the absence of As₂O₃ treatment, the cytoplasmic and mitochondrial levels of cytochrome C were hardly affected by either shCofilin or oeCofilin (data not shown). As₂O₃ treatment markedly increased cofilin expression and decreased cytochrome C expression in the mitochondria, whereas cytoplasmic cytochrome C expression was remarkably increased (Fig. 4A and B). This result was consistent with the

finding in Fig. 2F. However, in the shCofilin + As₂O₃ group, mitochondrial cytochrome C levels were notably increased while its cytoplasmic level was markedly reduced, compared with the As₂O₃ group (Fig. 4A). Opposite results were found in the oeCofilin + As₂O₃ group; the mitochondrial level of cytochrome C was notably decreased and the cytoplasmic level of cytochrome C was marginally increased, compared with the As₂O₃ group (Fig. 4B). It revealed that alterations in cofilin-1 levels had little influence on the release of cytochrome C from the mitochondria, whereas As₂O₃-induced cofilin-1 translocation was the primary factor triggering cytochrome C release.

Discussion

Differential proteomics has been widely used in hematological tumors. In the study, comparative proteomics were applied to screen and identify the differentially expressed proteins between retinoic acid-sensitive cell lines and drug-resistant cell lines. Key proteins that may be related to apoptosis and drug resistance were identified and the effects of cofilin-1 on As₂O₃-induced apoptosis in NB4-R1 cells were further investigated. The results of the primary study indicated that cofilin-1 serves a role in the mitochondrial apoptosis pathway in As₂O₃-treated NB4-R1 cells.

The combined treatment of ATRA and As₂O₃ has achieved favorable clinical efficacy in APL, which can be mainly attributed to the targeted mechanism of inducing cell differentiation and apoptosis (27). However, these results are not seen in patients with other types of acute leukemia as there are no specific targeted drugs. Therefore, further studies are required to illustrate the molecular mechanisms in APL and identify novel factors that regulate differentiation and apoptosis. NB4-R1 cells are naturally resistant to ATRA but sensitive to As₂O₃, thus this cell line is a useful model for the study of drug resistance and apoptosis (28,29). Through protein spectrum analysis, the present study identified that the expression of a number of proteins was significantly increased in NB4-R1 cells, a retinoid-resistant cell line, compared with that in NB4 cells, a retinoid-sensitive cell line. Particularly, the expression of M3 protein was increased by 2.3-fold. Following the PMF and database retrieval, cofilin-1 was identified for

M3. Increased expression of cofilin-1 in non-small cell lung cancer has been reported, suggesting cisplatin resistance and poor prognosis (30). Liao *et al* (31) found that cofilin-1 expression was markedly increased in drug-resistant liver cancer cell lines and an inhibitor of cofilin-1 could abolish drug resistance and induce tumor cell apoptosis. Nevertheless, little is known about the role of cofilin-1 in APL. Cofilin-1 may be involved in the resistance of NB4-R1 cells to retinoic acid, but hardly affect the pro-apoptotic potency of As₂O₃ in NB4-R1. Thus, the present study further investigated the role of cofilin-1 in the As₂O₃-induced apoptosis of NB4-R1 cells.

The main pathways of apoptosis include extracellular signal-triggered caspase activation and intracellular apoptotic enzyme release from mitochondria, which activates caspase (32). At the early stage of apoptosis, cytochrome C is released by mitochondria and binds to apoptotic protease-activating factor 1, ATP/dATP and pro-caspase-9 in the cytoplasm, forming an apoptotic complex. Then, the complex activates caspase-9 and leads to apoptosis (20,33,34). Increasing evidence has suggested that mitochondrial translocation of cofilin seems to be necessary in regulating apoptosis. It was reported that in the pathogenesis of Alzheimer's disease activated cofilin could form a complex with p53 and promote its mitochondrial localization, resulting in promotion of apoptosis (35,36). Moreover, Li *et al* (37) demonstrated that allyl isothiocyanate (AITC) could induce dephosphorylation of cofilin, which then translocated to mitochondria, leading to the release of cytochrome C and apoptosis. Furthermore, it was revealed that the underlying mechanisms of cofilin activation by AITC might involve the ROCK1/PTEN/PI3K signaling pathway (37). In the present study, the results indicated that As₂O₃ treatment significantly decreased the expression of cytoplasmic cofilin-1 and increased the mitochondrial expression of cofilin-1 in NB4-R1 cells. Downregulation of cofilin-1 expression using specific shRNAs did not have much of an effect on the proliferation and apoptosis of NB4-R1 cells. This suggested that alterations in the location of cofilin-1 rather than its expression level were the primary cause of As₂O₃-induced apoptosis in NB4-R1 cells, which was consistent with the aforementioned studies (35-37). Besides, Xing *et al* (38) demonstrated that isoalantolactone inhibits IKK β kinase activity and promotes apoptosis of glioblastoma cells by inducing translocation of cofilin-1 to the mitochondria and the release of mitochondrial cytochrome C to the cytoplasm, which activates caspase-3/9. In human leukemia cells, cofilin-1 translocation to the mitochondria could induce mitochondrial injury and cell apoptosis (39). Xiao *et al* (40) revealed that docetaxel induced apoptosis of prostate cancer cells by inhibiting cofilin-1 and paxillin signaling pathways. These results were consistent with the data of the present study. However, others found that genetic ablation of cofilin does not affect apoptosis in mouse embryonic fibroblasts (MEF), suggesting that cofilin activity is not generally required for inducing apoptosis (17). The observation that cytochrome C release from mitochondria proceeds normally in the absence of cofilin in MEF suggests that cell-type-specific functions for cofilin in apoptosis might exist. Further studies are needed to explore the underlying mechanisms.

The current results confirmed for the first time that the downregulation of cofilin-1 expression suppressed As₂O₃-induced apoptosis in NB4-R1 cells. The underlying mechanism may involve the reduction of cytochrome C release from mitochondria following cofilin-1 translocation to the mitochondria. These results suggested that the change of cofilin-1 location occurs earlier than the release of cytochrome C in the mitochondrial apoptosis pathway. Cofilin-1 serves as a key regulatory point in apoptosis, and it may be a novel potential drug target for APL treatment. Both NB4 and NB4-R1 cells are sensitive to As₂O₃ (41,42). Dual induction therapy is effective in certain patients with APL and arsenic-resistant APL. Besides, the effectiveness of combination therapy is higher compared with that of single drug use (43), which is consistent with the real-world conclusions. Further studies are needed to investigate mechanisms by which cofilin-1 regulates apoptosis in APL cells.

In conclusion, the present study demonstrated that during the process of As₂O₃-induced apoptosis in NB4-R1 cells, cofilin-1 is transferred to mitochondria from the cytoplasm, which promotes the release of cytochrome C from mitochondria and further activates the mitochondrial apoptosis pathway. Cofilin-1 may play a key role in the mitochondrial apoptosis pathway and has the potential to be used as a drug target in APL treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Key Research and Development Project of Shaanxi Province (grant no. 2017sf-185).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ and PH were involved in developing the concept and design of the study, and are guarantors of the integrity of the study. HZ and XZ performed the experiments, and prepared and revised the manuscript. HF and WW performed the experiments. ML and JZ were responsible for the literature review and assisted with data analysis. XW and HW performed the data analysis and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Park JH, Qiao B, Panageas KS, Schymura MJ, Jurcic JG, Rosenblat TL, Altman JK, Douer D, Rowe JM and Tallman MS: Early death rate in acute promyelocytic leukemia remains high despite all-trans retinoic acid. *Blood* 118: 1248-1254, 2011.
- Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Grignani F, Peschle C, Nicoletti I, *et al*: The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* 74: 423-431, 1993.
- Yang L, Chai W, Wang Y, Cao L, Xie M, Yang M, Kang R and Yu Y: Reactive oxygen species regulate the differentiation of acute promyelocytic leukemia cells through HMGB1-mediated autophagy. *Am J Cancer Res* 5: 714-725, 2015.
- Bassi SC and Rego EM: Molecular basis for the diagnosis and treatment of acute promyelocytic leukemia. *Rev Bras Hematol Hemoter* 34: 134-139, 2012.
- Sanz MA, Fenaux P, Tallman MS, Estey EH, Löwenberg B, Naoe T, Lengfelder E, Döhner H, Burnett AK, Chen SJ, *et al*: Management of acute promyelocytic leukemia: Updated recommendations from an expert panel of the European LeukemiaNet. *Blood* 133: 1630-1643, 2019.
- Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM, Iacobelli S, Ferrara F, Fazi P, Cicconi L, Di Bona E, *et al*: Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* 369: 111-121, 2013.
- Burnett AK, Russell NH, Hills RK, Bowen D, Kell J, Knapper S, Morgan YG, Lok J, Grech A, Jones G, *et al*: Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): Results of a randomised, controlled, phase 3 trial. *Lancet Oncol* 16: 1295-1305, 2015.
- Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, *et al*: Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. *Science* 328: 240-243, 2010.
- Esnault C, Rahmé R, Rice KL, Berthier C, Gaillard C, Quentin S, Maubert AL, Kogan S and de Thé H: FLT3-ITD impedes retinoic acid, but not arsenic, responses in murine acute promyelocytic leukemias. *Blood* 133: 1495-1506, 2019.
- Goto E, Tomita A, Hayakawa F, Atsumi A, Kiyoi H and Naoe T: Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment. *Blood* 118: 1600-1609, 2011.
- Zhu HH, Qin YZ and Huang XJ: Resistance to arsenic therapy in acute promyelocytic leukemia. *N Engl J Med* 370: 1864-1866, 2014.
- Lehmann-Che J, Bally C and de Thé H: Resistance to therapy in acute promyelocytic leukemia. *N Engl J Med* 371: 1170-1172, 2014.
- Ozpolat B, Mehta K and Lopez-Berestein G: Regulation of a highly specific retinoic acid-4-hydroxylase (CYP26A1) enzyme and all-trans-retinoic acid metabolism in human intestinal, liver, endothelial, and acute promyelocytic leukemia cells. *Leuk Lymphoma* 46: 1497-1506, 2005.
- de Thé H and Chen Z: Acute promyelocytic leukaemia: Novel insights into the mechanisms of cure. *Nat Rev Cancer* 10: 775-783, 2010.
- Liang C, Ding M, Weng XQ, Sheng Y, Wu J, Li ZY and Cai X: Combination of enzastaurin and ATRA exerts dose-dependent dual effects on ATRA-resistant acute promyelocytic leukemia cells. *Am J Cancer Res* 9: 906-926, 2019.
- Chua BT, Volbracht C, Tan KO, Li R, Yu VC and Li P: Mitochondrial translocation of cofilin is an early step in apoptosis induction. *Nat Cell Biol* 5: 1083-1089, 2003.
- Rehklau K, Gurniak CB, Conrad M, Friauf E, Ott M and Rust MB: ADF/cofilin proteins translocate to mitochondria during apoptosis but are not generally required for cell death signaling. *Cell Death Differ* 19: 958-967, 2012.
- Satoh M, Takano S, Sogawa K, Noda K, Yoshitomi H, Ishibashi M, Mogushi K, Takizawa H, Otsuka M, Shimizu H, *et al*: Immune-complex level of cofilin-1 in sera is associated with cancer progression and poor prognosis in pancreatic cancer. *Cancer Sci* 108: 795-803, 2017.
- Tahtamouni LH, Shaw AE, Hasan MH, Yasin SR and Bamberg JR: Non-overlapping activities of ADF and cofilin-1 during the migration of metastatic breast tumor cells. *BMC Cell Biol* 14: 45, 2013.
- Wang C, Zhou GL, Vedantam S, Li P and Field J: Mitochondrial shuttling of CAPI promotes actin- and cofilin-dependent apoptosis. *J Cell Sci* 121: 2913-2920, 2008.
- Blough ER, Rennie ER, Zhang F and Reiser PJ: Enhanced electrophoretic separation and resolution of myosin heavy chains in mammalian and avian skeletal muscles. *Anal Biochem* 233: 31-35, 1996.
- Barber RD, Harmer DW, Coleman RA and Clark BJ: GAPDH as a housekeeping gene: Analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 21: 389-395, 2005.
- Barrientos A, Barros MH, Valnot I, Rötig A, Rustin P and Tzagoloff A: Cytochrome oxidase in health and disease. *Gene* 286: 53-63, 2002.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Villanueva J, Lawlor K, Toledo-Crow R and Tempst P: Automated serum peptide profiling. *Nat Protoc* 1: 880-891, 2006.
- Strupat K: Molecular weight determination of peptides and proteins by ESI and MALDI. *Methods Enzymol* 405: 1-36, 2005.
- McCulloch D, Brown C and Iland H: Retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia: Current perspectives. *Onco Targets Ther* 10: 1585-1601, 2017.
- Wang L, Xiao H, Zhang X, Liao W, Fu S and Huang H: Restoration of CCAAT enhancer binding protein α P42 induces myeloid differentiation and overcomes all-trans retinoic acid resistance in human acute promyelocytic leukemia NB4-R1 cells. *Int J Oncol* 47: 1685-1695, 2015.
- Bruel A, Benoit G, De Nay D, Brown S and Lanotte M: Distinct apoptotic responses in maturation sensitive and resistant t(15;17) acute promyelocytic leukemia NB4 cells. 9-cis retinoic acid induces apoptosis independent of maturation and Bcl-2 expression. *Leukemia* 9: 1173-1184, 1995.
- Becker M, De Bastiani MA, Müller CB, Markoski MM, Castro MA and Klamt F: High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas. *Tumour Biol* 35: 1233-1238, 2014.
- Liao PH, Hsu HH, Chen TS, Chen MC, Day CH, Tu CC, Lin YM, Tsai FJ, Kuo WW and Huang CY: Phosphorylation of cofilin-1 by ERK confers HDAC inhibitor resistance in hepatocellular carcinoma cells via decreased ROS-mediated mitochondria injury. *Oncogene* 36: 1978-1990, 2017.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA and Yuan J: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403: 98-103, 2000.
- Elena-Real CA, Díaz-Quintana A, González-Arzola K, Velázquez-Campoy A, Orzáez M, López-Rivas A, Gil-Caballero S, De la Rosa MA and Díaz-Moreno I: Cytochrome c speeds up caspase cascade activation by blocking 14-3-3epsilon-dependent Apaf-1 inhibition. *Cell Death Dis* 9: 365, 2018.
- Babbitt SE, Sutherland MC, San Francisco B, Mendez DL and Kranz RG: Mitochondrial cytochrome c biogenesis: No longer an enigma. *Trends Biochem Sci* 40: 446-455, 2015.
- Liu T, Wang F, LePochat P, Woo JA, Bukhari MZ, Hong KW, Trotter C and Kang DE: Cofilin-mediated neuronal apoptosis via p53 translocation and PLD1 regulation. *Sci Rep* 7: 11532, 2017.
- Kang DE and Woo JA: Cofilin, a master node regulating cytoskeletal pathogenesis in Alzheimer's disease. *J Alzheimers Dis* 72 (Suppl 1): S131-S144, 2019.
- Li GB, Cheng Q, Liu L, Zhou T, Shan CY, Hu XY, Zhou J, Liu EH, Li P and Gao N: Mitochondrial translocation of cofilin is required for allyl isothiocyanate-mediated cell death via ROCK1/PTEN/PI3K signaling pathway. *Cell Commun Signal* 11: 50, 2013.
- Xing JS, Wang X, Lan YL, Lou JC, Ma B, Zhu T, Zhang H, Wang D, Yu Z, Yuan Z, *et al*: Isoalantolactone inhibits IKK β kinase activity to interrupt the NF- κ B/COX-2-mediated signaling cascade and induces apoptosis regulated by the mitochondrial translocation of cofilin in glioblastoma. *Cancer Med* 8: 1655-1670, 2019.
- Lu Z, Jia X, Chen Y, Han X, Chen F, Tian S, Su X, Li Z, Zhao J, Zhang X, *et al*: Identification and characterization of key charged residues in the cofilin protein involved in azole susceptibility, apoptosis, and virulence of *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 62: e01659-17, 2018.

40. Xiao P, Ma T, Zhou C, Xu Y, Liu Y and Zhang H: Anticancer effect of docetaxel induces apoptosis of prostate cancer via the cofilin-1 and paxillin signaling pathway. *Mol Med Rep* 13: 4079-4084, 2016.
41. Dong X, Ma N, Liu M and Liu Z: Effects of As₂O₃ nanoparticles on cell growth and apoptosis of NB4 cells. *Exp Ther Med* 10: 1271-1276, 2015.
42. Chelbi-alix MK, Bobé P, Benoit G, Canova A and Pine R: Arsenic enhances the activation of Stat1 by interferon gamma leading to synergistic expression of IRF-1. *Oncogene* 22: 9121-9130, 2003.
43. de Thé H, Pandolfi PP and Chen Z: Acute promyelocytic leukemia: A paradigm for oncoprotein-targeted cure. *Cancer Cell* 32: 552-560, 2017.



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