

Triptolide promotes ferroptosis by suppressing Nrf2 to overcome leukemia cell resistance to doxorubicin

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Received July 1, 2022; Accepted October 19, 2022

DOI: 10.3892/mmr.2022.12904

Abstract. Doxorubicin (DOX) is an extensively used chemotherapeutic drug to treat leukemia. However, there remains a pivotal clinical problem of resistance to DOX in patients with leukemia. Erythroid 2-related factor 2 (Nrf2) is a master regulator of antioxidation response which serves a critical role in maintaining cellular oxidative homeostasis. However, whether Nrf2 is involved in DOX resistance is not totally clear. It is well-documented that triptolide, a widely used drug to treat autoimmune disorders, possesses anti-cancer activities, yet whether triptolide affects leukemia cell sensitivity to DOX remains to be elucidated. The present study aimed to determine the role of triptolide-mediated downregulation of Nrf2 in regulating leukemia cell ferroptosis and resistance to DOX. For this purpose, low-dose DOX was used to establish DOX-resistant K562 cells and HL-60 cells. Nrf2 mRNA and protein expression were examined by quantitative PCR and western blotting assays. The effects of triptolide on leukemia cell viability, reactive oxygen species (ROS) levels, or lipid oxidation were determined by CCK8 assay, DCFH-DA assay, or BODIPY 581/591 C11 assay, respectively. The results show that Nrf2 expression was significantly upregulated in DOX-resistant leukemia cells and clinical leukemia samples. Silencing of Nrf2 significantly sensitized leukemia cells to DOX. Furthermore, it was demonstrated that triptolide inhibited Nrf2 expression and induced leukemia cell ferroptosis, as evidenced by increased ROS levels and lipid oxidation as well as decreased glutathione peroxidase 4 expression. Ectopic expression of Nrf2 significantly rescued triptolide-induced leukemia cell ferroptosis. Notably, the present study showed that triptolide re-sensitized DOX-resistant leukemia cells to

DOX. In conclusion, the present study indicated that Nrf2 served a critical role in leukemia cell resistance to DOX and triptolide-induced ferroptosis and suggested a potential strategy of combination therapy using triptolide and DOX in leukemia treatment.

Introduction

Leukemia is the sixth most lethal cancer accounting for 4% of all cancer cases (1) and arises from the bone marrow and the lymphatic system (2). Based on the rapidity of proliferation (acute or chronic) and originator cell (myeloid cell or lymphoid cell), leukemia can be classified into four types: Acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML) and chronic myelomonocytic leukemia (CMML) (3). In 2020, the American Cancer Society reported that leukemia developed 474,519 new cases globally and caused 311,594 deaths (4). Clinical treatment for leukemia includes chemotherapy, radiotherapy, immunotherapy, bone marrow transplantation and even traditional Chinese medicine (5,6). Among them, chemotherapy is the first choice for leukemia therapy (2,7). Doxorubicin (DOX), an anthracycline antibiotic that can inhibit topoisomerase and induce oxidative stress to kill cells, is a well-established chemotherapeutic drug for leukemia treatment (8-10). DOX resistance is a major clinical problem in the leukemia treatment and can lead to rapid deterioration in leukemia (10). It has been demonstrated that the amplification of the multi-drug resistance gene *mdr1* or increased expression of glyoxalase I contributes to DOX resistance of leukemia cells (11-13). In addition, epigenetic mechanisms, such as DNA modification and histone modification, have been involved in DOX resistance of leukemia cells (14-16).

Ferroptosis is a novel form of cell death, first defined in 2012 (17). Intracellular iron ion accumulation and reactive oxygen species (ROS)-mediated lipid peroxidation are two hallmarks of ferroptosis (18). It has been documented that a series of extrinsic or intrinsic pathways can trigger ferroptosis, such as inhibition of cystine/glutamate transporter, activation of the iron transporter transferrin, or blockade of glutathione peroxidase GPX4 (19). Ferroptosis has been shown to link

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Key words: leukemia, doxorubicin resistance, Nrf2, triptolide, ferroptosis

a number of human diseases including neurodegenerative diseases, organ injury and cardiovascular diseases (20). In addition, recent evidence indicates that induction of ferroptosis is a potential strategy to eliminate cancer cells (21). Currently, a series of anti-tumor drugs associated with ferroptosis have been developed, such as nuclear factor erythroid 2-related factor 2 (Nrf2) inhibitors, GSH inhibitors and iron activators (21).

Triptolide is a natural diterpenoid epoxide, extracted from the Chinese traditional herb thunder god vine (*Tripterygium wilfordii*) (22). Triptolide has been used to treat autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus for a number of years (23). Accumulating evidence indicates that triptolide also exhibits anti-tumor activities in multiple types of human cancer, including leukemia, lung cancer, breast cancer, colon cancer and prostate cancer (24-28). Currently, several triptolide derivatives are in clinical phase I/II trials for cancer therapy (29). However, the molecular mechanisms underlying the anti-cancer activity of triptolide remain to be elucidated.

The present study showed that Nrf2 served a critical role in leukemia cell resistance to DOX. Triptolide can induce leukemia cell ferroptosis via downregulation of Nrf2 to overcome leukemia cell resistance to DOX. Thus, the present study suggested that a combination of triptolide and DOX is a potential strategy for leukemia treatment.

Materials and methods

Collection of patient samples. A total of 30 patients with leukemia (15 men and 15 women; age range, 15-35 years) admitted to Jin'an District Hospital (Fuzhou, China) between May 1, 2020, and December 1, 2020, were enrolled in the current study. Leukemia patient blood samples (n=10; 4 men and 6 women) and DOX-resistant Leukemia patient blood samples (n=20; 11 men and 14 women) were collected according to institutional regulation of the Hospital Clinic Ethical Committee and according to the declaration of Helsinki. Informed written consent was given by all patients. The blood samples were separated to obtain peripheral blood mononuclear cells (PBMCs) using a cell isolator (LTS1077-1; Tianjin Haoyang Biological Manufacture Co., Ltd.) (30). Cells were subjected to western blot analyses or reverse transcription-quantitative (RT-q) PCR analyses.

Cell culture and drug treatment. Human leukemia chronic myelogenous leukemia K562 and acute promyelocytic leukemia HL-60 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; Cytiva). HEK293T (Human embryonic kidney) cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Hyclone; Cytiva). All cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were grown in a medium supplemented with 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified 37°C incubator under a 5% CO₂ atmosphere. Cells at 80% confluence were treated with 1 µg/ml DOX (cat. no. HY-15142A;

MedChemExpress) for 48 h, 40 nM berberine (cat. no. HY-N0716; MedChemExpress) for 24 h, 40 nM metformin (cat. no. HY-15763; MedChemExpress), 40 nM artemisinin (cat. no. HY-B0094; MedChemExpress) for 24 h, 40 nM curcumin (cat. no. HY-N0005; MedChemExpress) for 24 h, 2 µM erastin (cat. no. HY-B0627; MedChemExpress) for 48 h and 40 nM triptolide (cat. no. HY-32735; MedChemExpress) for 24 or 48 h at 37°C.

Plasmid transfection and lentiviral infection. Recombinant lentiviruses were amplified by transfecting HEK 293T cells at 75% confluence with 6 µg pMD2.G and 6 µg psPAX2 packaging plasmids (the 2nd generation lentiviral packaging plasmid; Addgene, Inc.) and 6 µg lentivirus-based Nrf2 expression plasmid (pLVX-puro; Addgene, Inc.) or 6 µg lentiviral-based short hairpin (sh)RNAs (pLKO.1-puro; Addgene, Inc.) specific for green fluorescent protein (GFP, CAAATCACAGAATCG TCGTAT; negative control) or Nrf2 (#1, GCTCCTACTGTG ATGTGAAAT; 2#, GGAGTGTCAGTATGTTGAA) using Lipofectamine® 2000 (cat. no. 11668; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. Viruses were collected at 72 h after transfection. K562 or HL-60 cells at 40% confluence were infected with a recombinant lentivirus in the presence of 10 µg/ml polybrene, followed by 12 h incubation at 37°C with 5% CO₂. After 36 h of infection, cells were treated with 2 µg/ml puromycin for 24 h. The viable cells were used to further experiments. The infection efficiency was ~85%.

Western blot analyses. Cells were collected, washed with cold PBS and resuspended in EBC250 lysis buffer (250 mM NaCl, 50 mM Tris pH 8.0, 0.5% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin and 2 µg/ml leupeptin) (31). The protein concentration was assessed using the BCA method. Equal amounts of total protein (20 µg) were loaded, separated by SDS-PAGE (4% stacking gel, 10% running gel), transferred to PVDF membranes (MilliporeSigma). The membrane was then blocked with 5% skimmed milk powder for 1 h at room temperature and hybridized to an appropriate primary antibody (4°C, overnight) and HRP-conjugated secondary antibody (anti-rabbit: 1:3,000; cat. no. AB0101; anti-mouse: 1:3,000, cat. no. AB0102; Shanghai Abways Biotechnology Co., Ltd.; room temperature, 1 h) for subsequent detection by enhanced chemiluminescence using an ECL kit (cat. no. P0018S; Beyotime Institute of Biotechnology). The images were analyzed using ImageJ software 1.8.0 (National Institutes of Health). Primary antibodies for GAPDH (cat. no. AF7021; 1:1,000), Nrf2 (cat. no. AF0639; 1:1,000), catalase (cat. no. DF7545; 1:1,000), superoxide dismutase (SOD)2 (cat. no. AF5144; 1:1,000), glutathione peroxidase (GPX)4 (cat. no. DF6701; 1:1,000) and were purchased from Affinity Biosciences. Antibody for Lamin B (cat. no. ab32535; 1:1,000) was purchased from Abcam.

RT-qPCR. Total RNA was extracted from 1x10⁶ cells using RNA easy Plus Mini kit (Qiagen) according to the manufacturer's protocol. RNA was reverse-transcribed into cDNAs using M-MLV First Strand kit (Invitrogen) according to the manufacturer's protocol. qPCR analyses of Nrf2 (forward: GGTTCCTTCGGCTACGTTT; reverse: ACTTCTTTTTC ATTGAGGGTATA), catalase (forward: CTCCGGAACAAC AGCCTTCT; reverse: ATAGAATGCCCCGCACCTGAG),

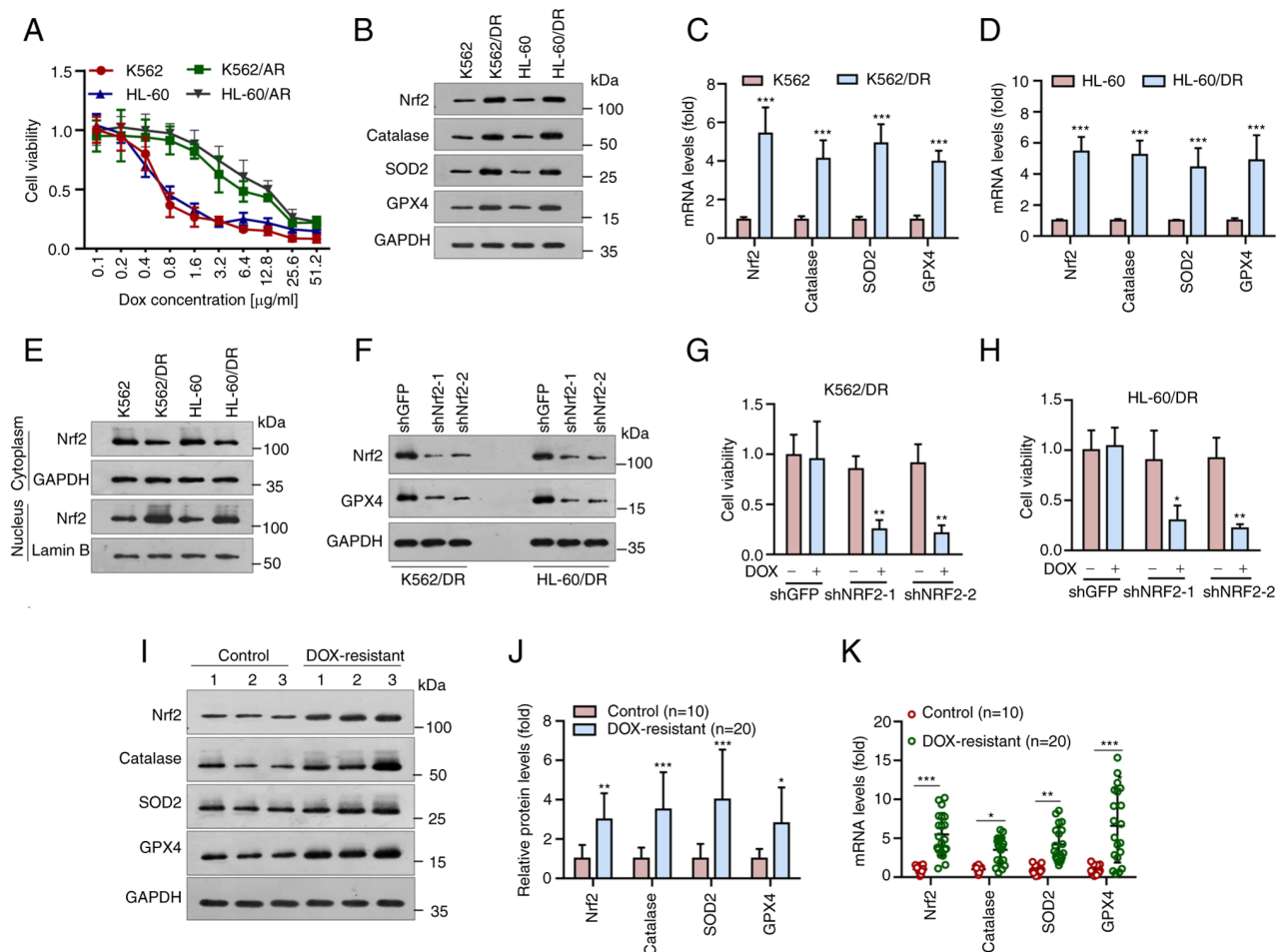


Figure 1. Nrf2 serves a critical role in leukemia cell resistance to DOX. K562 or HL-60 cells were grown in a regular RPMI-1640 medium in the presence or absence of 0.8 $\mu\text{g/ml}$ DOX for 24 h alternatively. On day 40, viable DOX-resistant K562 or HL-60 cells (K562-DR or HL-60-DR) were collected for further experiments. (A) K562 cells, K562/DR, HL-60 cells, or HL-60/DR were treated with an indicated concentration of DOX (DOX) for 48 h. Cell viability was determined using CCK8 assays. K562, K562/DR, HL-60, or HL-60/DR cells were subjected to (B) western blot or (C and D) qPCR analyses. (E) K562, K562/DR, HL-60, or HL-60/DR cells were subjected to fractionation of cytoplasm and nucleus, followed by western blot analyses. (F) K562/DR or HL-60/DR cells stably expressing shGFP, shNrf2-1, or shNrf2-2 were subjected to western blot analyses. (G and H) Cells were treated with 1 $\mu\text{g/ml}$ DOX for 48 h. Cell viability was determined using CCK8 assays. Peripheral blood mononuclear cells (PBMC) from leukemia patients (n=10) or DOX-resistance leukemia patients (n=20) were examined by (I and J) western blot analyses or (K) qPCR analyses. Data were derived from at least three independent experiments and were presented as means \pm standard deviation. *** P <0.001; ** P <0.01; * P <0.05. Nrf2, nuclear factor erythroid 2-related factor 2; DOX, doxorubicin; qPCR, quantitative PCR.

SOD2 (forward: TAGCTCTTCAGCCTGCACTG; reverse: GCTTCCAGCAACTCCCCTTT), GPX4 (forward: TGGACG AGGGGAGGAGC; reverse: GGGACGCGCACATGGT) and GAPDH (forward: TCAAGAAGGTGGTGAAGCAGG; reverse: TCAAAGGTGGAGGAGTGGT) were performed in CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.) using SoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. The reactions were carried out in a 96-well plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 58°C for 30 sec. GAPDH expression was used as an inner control to normalize gene expression by the $2^{-\Delta\Delta C_q}$ method (31). All experiments were performed three times in triplicate.

Cell viability analyses. Cell viability assay (CCK-8) was performed using a CCK-8 assay Kit (cat. no. CK04; Dojindo Molecular Technologies, Inc.) as described in the manufacturer's instruction (32). Briefly, 10 μl CCK-8 reagent was incubated with the cells at 37°C for 4 h, and absorbance was

quantified at 450 nm using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc.).

Measurement of ROS levels and lipid oxidation by flow cytometry. K562 or HL-60 cells (2×10^5) were seeded in 24-well plates in the absence or presence of triptolide. For the measurement of ROS levels, cells were washed and subjected to the procedures as described in the Reactive Oxygen Species Assay kit (cat. no. D6470, Beijing Solarbio Science & Technology Co., Ltd.) (33). Measurement of lipid oxidation was performed using BODIPY 581/591 C11 assay kit (cat. no. D3861; Invitrogen; Thermo Fisher Scientific, Inc.) as described in the manufacturer's instruction (33). Then the 2×10^4 cells were analyzed using a flow cytometer (Beckman Coulter, Inc.) and the data were analyzed using FlowJo software V10 (FlowJo, LLC).

Statistical analyses. GraphPad Prism 6.0 (GraphPad Software Inc.) was used for data recording and calculation. All

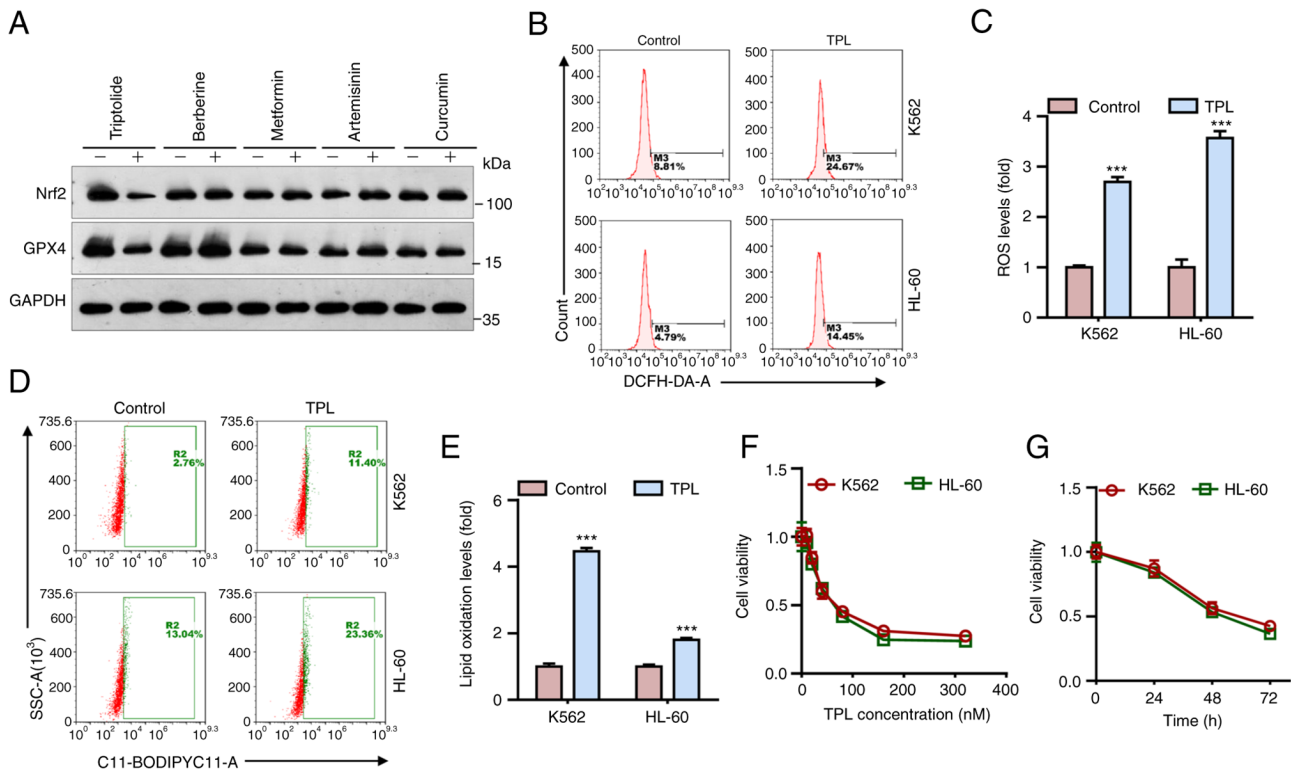


Figure 2. Triptolide inhibits Nrf2 expression and induces leukemia cells ferroptosis. (A) K562 cells were treated with an indicated chemical compound (40 nM) for 24 h prior to western blot analyses. K562 or HL-60 cells were treated with or without triptolide (40 nM) for 24 h. Cells were subjected to the measurement of ROS levels (B and C) or lipid oxidation (D and E), as described in Materials and methods. (F) K562 or HL-60 cells were treated with an indicated concentration of triptolide for 48 h or (G) were treated with 40 nM triptolide for the indicated time course. Cell viability was determined using CCK8 assays. Data were derived from at least three independent experiments and were presented as means \pm standard deviation. *** $P < 0.001$. Nrf2, nuclear factor erythroid 2-related factor 2; TPL, triptolide.

experiments were performed at least three times. Data were presented as means \pm standard deviation. Quantitative data were analyzed statistically using unpaired Student's t-test for two groups (Figs. 1 and 2) and one-way ANOVA followed by Tukey's post-hoc test for >2 groups (Figs. 3 and 4) to assess the significance.

Results

Nrf2 is essential for leukemia cell resistance to DOX. Chemotherapy is the first choice for leukemia treatment. DOX is a well-established chemotherapeutic drug for leukemia treatment. However, DOX resistance is a major clinical problem in leukemia treatment. Currently, the molecular mechanism(s) by which leukemia cells resistance to DOX remains to be elucidated. To explore this issue, DOX-resistant K562 cells (K562/DR) and DOX-resistant HL-60 cells (HL-60/DR) were first established upon a low dose of DOX treatment. As shown in Fig. 1A, compared with normal K562 or HL-60 cells, K562/DR or HL-60/DR cells exhibited strong resistance to DOX. Oxidative stress is an important hallmark of cancer cells (34). It is reported that DOX can induce the production of ROS to kill cancer cells (9). Next, the present study examined whether oxidative stress also served a role in leukemia cell resistance to DOX. As shown in Fig. 1B, K562/DR or HL-60/DR cells had higher Nrf2, a master regulator of the antioxidant response, protein expression than normal K562 or HL-60 cells, as evidenced by western blot analyses. It has been documented that Nrf2 is a

transcription factor that regulates a series of genes expression involved in oxidative stress response, including catalase, SOD2 and GPX4. Consistent with Nrf2, K562/DR or HL-60/DR cells also exhibited higher catalase, SOD2 and GPX4 protein expression than normal K562 or HL-60 cells (Fig. 1B). In addition, qPCR analyses also showed that compared with normal K562 or HL-60 cells, K562/DR or HL-60/DR cells had higher Nrf2, catalase SOD2 and GPX4 mRNA levels (Fig. 1C and D; $P < 0.001$). It is reported that Nrf2 can both localize in the cytoplasm and nucleus (35). However, only nuclear localization of Nrf2 can regulate antioxidant genes transcription. Therefore, the localization of Nrf2 was also examined in our system. As shown in Fig. 1E, compared with normal leukemia cells, nuclear localization of Nrf2 protein expression significantly increased in K562/DR or HL-60/DR cells. Notably, silencing of Nrf2 significantly sensitized K562/DR or HL-60/DR cells to DOX (Fig. 1F-H; $P < 0.05$).

To further investigate whether the expression of Nrf2 and its downstream target genes are also upregulated in clinical DOX-resistant leukemia samples, leukemia patient blood samples ($n=10$) and DOX-resistant leukemia patient blood samples ($n=20$) were harvested. Levels of Nrf2, catalase, SOD2 and GPX4 in leukemia PBMC ($n=10$) and DOX-resistant leukemia PBMC ($n=20$) were then examined by western blot and quantitative PCR analyses. As shown in Fig. 1I-K, DOX-resistant leukemia PBMC exhibited significantly upregulated Nrf2, catalase, SOD2 and GPX4 protein and mRNA expression than normal leukemia PBMC ($P < 0.05$).

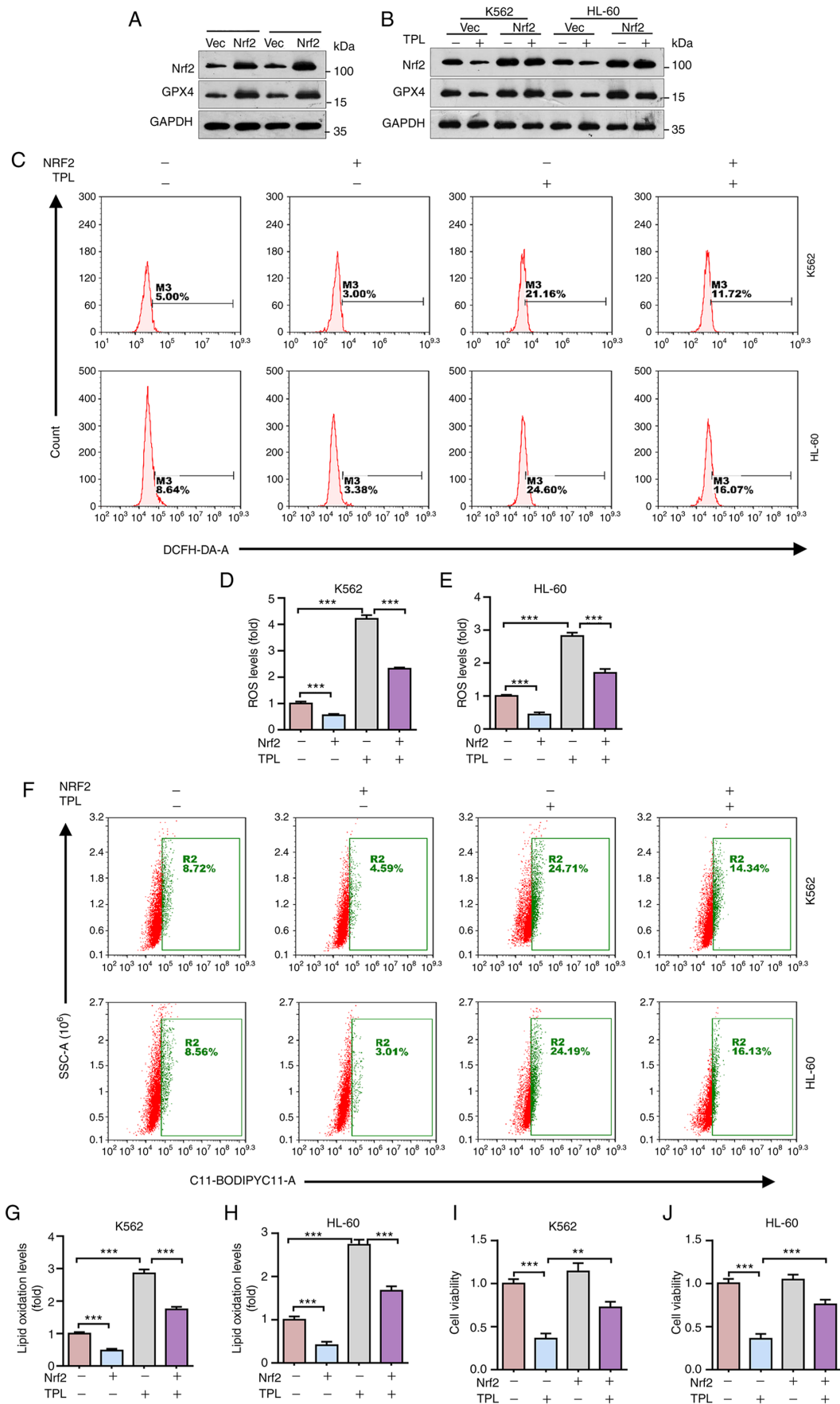


Figure 3. Ectopic expression of Nrf2 inhibits triptolide-mediated ferroptosis. (A) K562 or HL-60 cells stably expressing Nrf2 or vector control were subjected to western blot analyses. K562-Vec, K562-Nrf2, HL-60-Vec, or HL-60-Nrf2 cells were treated with or without 40 nM triptolide for 48 h. Cells were subjected to (B) western blot analyses, (C-E) the measurement of ROS levels or (F-H) lipid oxidation, or were subjected to (I and J) CCK8 analyses for cell viability. Data were derived from at least three independent experiments and were presented as means \pm standard deviation. *** P <0.001; ** P <0.05. Nrf2, nuclear factor erythroid 2-related factor 2; Vec, vector control.

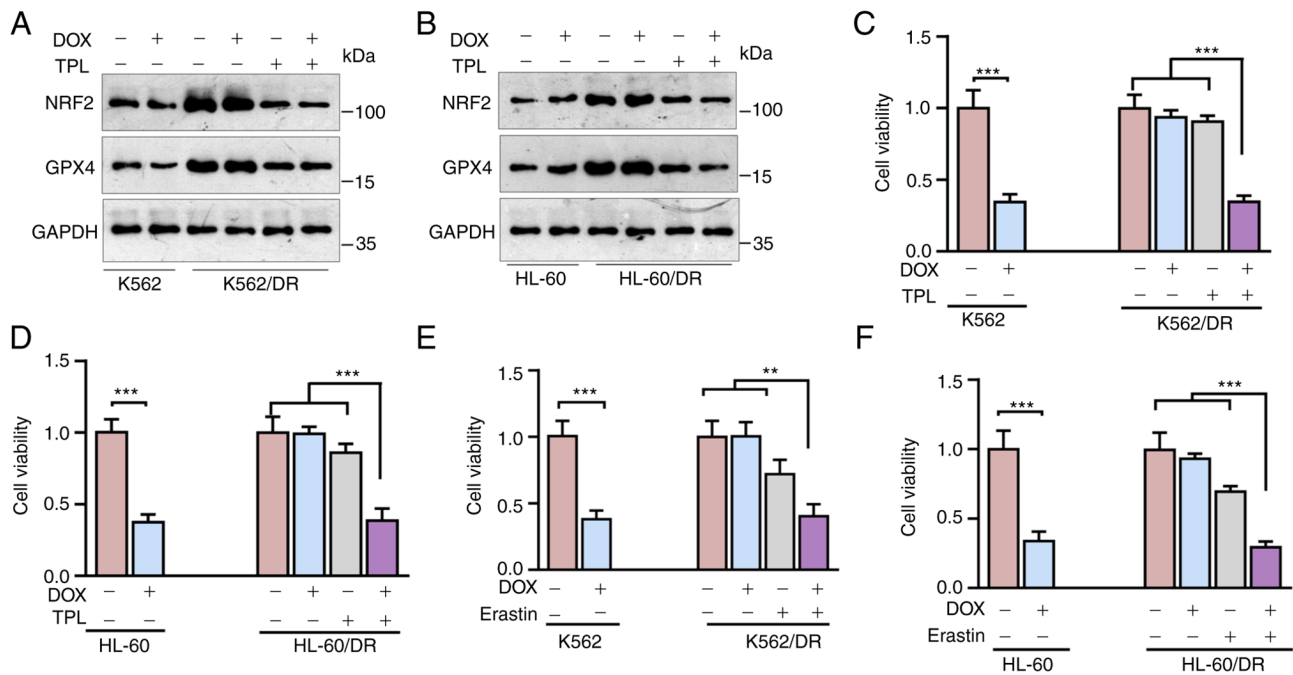


Figure 4. Triptolide sensitizes leukemia cells to DOX. K562, K562/DR, HL-60, or HL-60/DR cells were treated with or without 1 μ g/ml DOX in the presence or absence of 10 nM triptolide for 48 h. Cells were subjected to (A and B) western blot analyses or (C and D) CCK8 analyses for cell viability. (E and F) K562, K562/DR, HL-60, or HL-60/DR cells were treated with or without 1 μ g/ml DOX in the presence or absence of 2 μ M erastin for 48 h. Cells were subjected to CCK8 analyses for cell viability. Data were derived from at least three independent experiments and were presented as means \pm standard deviation. ***P<0.001. DOX, doxorubicin.

Together, these results suggested that Nrf2 may play a role in leukemia cell resistance to DOX.

Triptolide inhibits Nrf2 expression and induces leukemia cells ferroptosis. The aforementioned data indicated that Nrf2 served a critical role in leukemia cell resistance to DOX. It was thus hypothesized that inhibition of Nrf2 may be a potential strategy for overcoming DOX resistance in leukemia cells. To search for potential small chemical Nrf2 inhibitors, the effects of several known chemical compounds on Nrf2 expression were examined. As shown in Fig. 2A, triptolide, a clinical-approved chemical drug used to treat autoimmune disorders, remarkably inhibited Nrf2 protein expression, concomitant with reduced GPX4 expression. The effects of triptolide on cellular ROS levels were also examined. As shown in Fig. 2B and C, triptolide markedly increased ROS levels in K562 and HL-60 cells, as evidenced by DCFH-DA analyses (P<0.001). Triptolide also significantly promoted lipid oxidation in K562 and HL-60 cells, as evidenced by BODIPY 581/591 C11 analyses (Fig. 2D and E; P<0.001). Since increased ROS and lipid oxidation and decreased GPX4 expression are hallmarks of ferroptosis, the effects of triptolide on leukemia cell viability were therefore examined. As shown in Fig. 2F and G, triptolide markedly inhibited K562 or HL-60 cell viability, indicating that triptolide induced leukemia cell ferroptosis. Together, these results demonstrated that triptolide can inhibit Nrf2 expression and induce leukemia cell ferroptosis.

Ectopic expression of Nrf2 inhibits triptolide-induced ferroptosis. Next, the present study investigated the role of Nrf2 in triptolide-induced leukemia cell ferroptosis. To examine this

issue, K562 or HL-60 cells which stably express Nrf2 were first established. As shown in Fig. 3A, ectopic expression Nrf2 significantly increased GPX4 protein expression, consistent with the previous report (36). Furthermore, ectopic expression of Nrf2 restored GPX4 protein expression inhibited by triptolide (Fig. 3B). In addition, ectopic expression of Nrf2 also markedly reduced endogenous ROS levels and triptolide-induced ROS levels in K562 and HL-60 cells (Fig. 3C-E; P<0.001). Next, the role of Nrf2 on triptolide-induced leukemia cell lipid oxidation was examined. As shown in Fig. 3F-H, triptolide significantly induced lipid oxidation in K562 and HL-60 cells, which can be significantly rescued by ectopic expression of Nrf2 (P<0.001), suggesting that Nrf2 is critical in triptolide-induced ferroptosis. Indeed, it was found that ectopic expression of Nrf2 can inhibit triptolide-induced downregulation of K562 and HL-60 cell viability (Fig. 3I-J; P<0.001). Together, these results indicated that triptolide promotes leukemia cell ferroptosis via downregulation of Nrf2 expression.

Triptolide sensitizes leukemia cells to DOX. The aforementioned data indicated that Nrf2 served a critical role in leukemia cell resistance to DOX (Fig. 1) and triptolide inhibits Nrf2 expression to induce leukemia cell ferroptosis (Figs. 2 and 3). Therefore, it was hypothesized that treatment with triptolide may overcome leukemia cell resistance to DOX. To examine this hypothesis, triptolide was used to treat K562/DR or HL-60 cells. As shown in Fig. 4A and B, triptolide significantly suppressed Nrf2 and GPX4 expression. Furthermore, DOX significantly inhibited normal K562 or HL-60 cell viability (P<0.001), but it had little effect on K562/DR or HL-60 cell viability (Fig. 4C and D). Notably, treatment with low-dose triptolide did not affect K562/DR or HL-60 cell viability, but it could re-sensitize K562/DR or HL-60 cells

to DOX (Fig. 4C and D). In addition, consistent with triptolide, erastin, a ferroptosis inducer, could also re-sensitize K562/DR or HL-60 cells to DOX (Fig. 4E and F). Together, these results suggested that triptolide promotes ferroptosis via suppressing Nrf2 to sensitize leukemia cells to DOX.

Discussion

The present study showed that the expression of Nrf2, the master regulator of cellular antioxidation response, is significantly increased in the clinical DOX-resistant leukemia sample. Notably, the silencing of Nrf2 markedly sensitized DOX-resistant leukemia cells to DOX. In addition, the present study showed that triptolide, a natural diterpenoid epoxide used to treat autoimmune disorders, can inhibit Nrf2 expression to induce leukemia cells ferroptosis and sensitize DOX-resistant leukemia cells to DOX.

DOX is widely used to treat leukemia. However, DOX resistance is a major clinical problem for leukemia therapy. Therefore, it is important to investigate the molecular mechanism by which leukemia cells resistance to DOX and explore new strategies to overcome DOX resistance. It is reported that piperlongumine, a ROS inducer, can reverse leukemia cell resistance to DOX via the PI3K/Akt pathway (37). In addition, indomethacin, a cyclooxygenase inhibitor, can also overcome DOX resistance by decreasing glutathione (38). The present study indicated that Nrf2 served an important role in leukemia cell resistance to DOX and highlighted a potential strategy of combination therapy using triptolide and DOX in leukemia treatment.

Nrf2 mRNA and protein levels were significantly upregulated in DOX-resistant leukemia cells and clinical DOX-resistant leukemia samples. An important question is: How Nrf2 is upregulated in DOX-resistant leukemia? It is reported that Nrf2 protein stability is tightly regulated by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm (34). Upon oxidative stress, Keap1 separates from Nrf2, which leads to the stabilization of Nrf2 protein and results in Nrf2 translocation to the nucleus (34). In addition, activation of ERK signaling also can promote Nrf2 translocation to the nucleus and stabilize Nrf2 protein (35,39). It has been documented that oncogenic K-Ras^{G12D} can increase the transcription of Nrf2 via activating ERK signaling (40). Notably, DOX can induce ROS production and activate ERK signaling (41,42). Therefore, it is plausible that DOX promotes Nrf2 expression via induction of ROS or activation of ERK, which needs to be further investigated.

The present study clearly demonstrated that triptolide can significantly inhibit Nrf2 expression in leukemia cells. A recent report shows that triptolide can suppress Nrf2 target genes expression via decreasing nuclear localization of Nrf2 in lung cancer cells (43). In addition, triptolide can also serve as an inhibitor of Nrf2, which suppresses Nrf2 transcriptional activity in glioma cells (44). Together, these observations demonstrate that triptolide can inhibit Nrf2 in multiple levels, including protein expression, nuclear localization and transcriptional activity.

Notably, the present study indicated that triptolide-mediated downregulation of Nrf2 led to leukemia cells ferroptosis. It also showed that Nrf2 serves a critical role in the DOX resistance of leukemia cells. Therefore, it is plausible that triptolide can promote leukemia cell sensitivity to DOX via downregulation

of Nrf2 expression. Indeed, the present study indicated that treatment with triptolide sensitized DOX-resistant leukemia cells to DOX. Together, the present study suggested that the combined use of triptolide and DOX may be a promising therapeutic strategy for leukemia therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Jin'an District Hospital Research Foundation (grant no. JA2021KJ005) to GL.

Availability of data and materials

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

Authors' contributions

XW and GL conceived and designed the experiments. XW, SC and KH performed the experiments. XW, SC and KH analyzed the data. XW and GL wrote the manuscript. XW and GL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Fuzhou Jin'an District Hospital (Fuzhou, China; approval no. JA-KJ2021-011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Du Y and Chen B: Detection approaches for multidrug resistance genes of leukemia. *Drug Des Devel Ther* 11: 1255-1261, 2017.
2. Yi Y, Gao L, Wu M, Ao J, Zhang C, Wang X, Lin M, Bergholz J, Zhang Y and Xiao ZJ: Metformin sensitizes leukemia cells to vincristine via activation of AMP-activated protein kinase. *J Cancer* 8: 2636-2642, 2017.
3. Brunning RD: Classification of acute leukemias. *Semin Diagn Pathol* 20: 142-153, 2003.
4. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71: 209-249, 2021.
5. Burnett AK, Kell J and Rowntree C: Acute myeloid leukemia: Therapeutic indications. *Curr Opin Hematol* 7: 333-338, 2000.
6. Burnett A, Wetzler M and Löwenberg B: Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* 29: 487-494, 2011.
7. Wang HW, Ma KL, Liu H and Zhou JY: Reversal of multidrug resistance in leukemia cells using a transferrin-modified nanomicelle encapsulating both doxorubicin and psoralen. *Aging (Albany NY)* 12: 6018-6029, 2020.

8. Lothstein L, Israel M and Sweatman TW: Anthracycline drug targeting: Cytoplasmic versus nuclear-a fork in the road. *Drug Resist Updat* 4: 169-177, 2001.
9. Kruk I, Michalska T, Kładny J and Kubera-Nowakowska L: Luminescence investigations of redox cycling of adriamycin. *Chemosphere* 44: 83-90, 2001.
10. Davies GF, Roesler WJ, Juurlink BH and Harkness TA: Troglitazone overcomes doxorubicin-resistance in resistant K562 leukemia cells. *Leuk Lymphoma* 46: 1199-1206, 2005.
11. Sakamoto H, Mashima T, Kizaki A, Dan S, Hashimoto Y, Naito M and Tsuruo T: Glyoxalase I is involved in resistance of human leukemia cells to antitumor agent-induced apoptosis. *Blood* 95: 3214-3218, 2000.
12. Sakamoto H, Mashima T, Sato S, Hashimoto Y, Yamori T and Tsuruo T: Selective activation of apoptosis program by S-p-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells. *Clin Cancer Res* 7: 2513-2518, 2001.
13. Xia CQ and Smith PG: Drug efflux transporters and multidrug resistance in acute leukemia: Therapeutic impact and novel approaches to mediation. *Mol Pharmacol* 82: 1008-1021, 2012.
14. Altucci L, Clarke N, Nebbioso A, Scognamiglio A and Gronemeyer H: Acute myeloid leukemia: Therapeutic impact of epigenetic drugs. *Int J Biochem Cell Biol* 37: 1752-1762, 2005.
15. Waldmann T and Schneider R: Targeting histone modifications-epigenetics in cancer. *Curr Opin Cell Biol* 25: 184-189, 2013.
16. Liu T, Guo Q, Guo H, Hou S, Li J and Wang H: Quantitative analysis of histone H3 and H4 post-translational modifications in doxorubicin-resistant leukemia cells. *Biomed Chromatogr* 30: 638-644, 2016.
17. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, *et al*: Ferroptosis: An iron-dependent form of nonapoptotic cell death. *Cell* 149: 1060-1072, 2012.
18. Bebbier CM, Müller F, Prieto Clemente L, Weber J and von Karstedt S: Ferroptosis in cancer cell biology. *Cancers (Basel)* 12: 164, 2020.
19. Chen X, Kang R, Kroemer G and Tang D: Broadening horizons: The role of ferroptosis in cancer. *Nat Rev Clin Oncol* 18: 280-296, 2021.
20. Shi Z, Zhang L, Zheng J, Sun H and Shao C: Ferroptosis: Biochemistry and biology in cancers. *Front Oncol* 11: 579286, 2021.
21. Su Y, Zhao B, Zhou L, Zhang Z, Shen Y, Lv H, AlQudsy LHH and Shang P: Ferroptosis, a novel pharmacological mechanism of anti-cancer drugs. *Cancer Lett* 483: 127-136, 2020.
22. Cai J, Yi M, Tan Y, Li X, Li G, Zeng Z, Xiong W and Xiang B: Natural product triptolide induces GSDME-mediated pyroptosis in head and neck cancer through suppressing mitochondrial hexokinase-II. *J Exp Clin Cancer Res* 40: 190, 2021.
23. Liu Q: Triptolide and its expanding multiple pharmacological functions. *Int Immunopharmacol* 11: 377-383, 2011.
24. Pigneux A, Mahon FX, Uhalde M, Jeanneteau M, Lacombe F, Milpied N, Reiffers J and Belloc F: Triptolide cooperates with chemotherapy to induce apoptosis in acute myeloid leukemia cells. *Exp Hematol* 36: 1648-1659, 2008.
25. Shao H, Ma J, Guo T and Hu R: Triptolide induces apoptosis of breast cancer cells via a mechanism associated with the Wnt/ β -catenin signaling pathway. *Exp Ther Med* 8: 505-508, 2014.
26. Isharwal S, Modi S, Arora N, Uhlrich C III, Giri B, Barlass U, Soubra A, Chugh R, Dehm SM, Dudeja V, *et al*: Minnelide inhibits androgen dependent, castration resistant prostate cancer growth by decreasing expression of androgen receptor full length and splice variants. *Prostate* 77: 584-596, 2017.
27. Oliveira A, Beyer G, Chugh R, Skube SJ, Majumder K, Banerjee S, Sangwan V, Li L, Dawra R, Subramanian S, *et al*: Triptolide abrogates growth of colon cancer and induces cell cycle arrest by inhibiting transcriptional activation of E2F. *Lab Invest* 95: 648-659, 2015.
28. Phillips BJ, Kumar A, Burki S, Ryan JP, Noda K and D'Cunha J: Triptolide-induced apoptosis in non-small cell lung cancer via a novel miR204-5p/Caveolin-1/Akt-mediated pathway. *Oncotarget* 11: 2793-2806, 2020.
29. Noel P, Von Hoff DD, Saluja AK, Velagapudi M, Borazanci E and Han H: Triptolide and its derivatives as cancer therapies. *Trends Pharmacol Sci* 40: 327-341, 2019.
30. Ni WJ and Leng XM: Down-regulated miR-495 can target programmed cell death 10 in ankylosing spondylitis. *Mol Med* 26: 50, 2020.
31. 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.
32. Niu W, Xu L, Li J, Zhai Y, Sun Z, Shi W, Jiang Y, Ma C, Lin H, Guo Y and Liu Z: Polyphyllin II inhibits human bladder cancer migration and invasion by regulating EMT-associated factors and MMPs. *Oncol Lett* 20: 2928-2936, 2020.
33. Tang X, Li X, Zhang D and Han W: Astragaloside-IV alleviates high glucose-induced ferroptosis in retinal pigment epithelial cells by disrupting the expression of miR-138-5p/Sirt1/Nrf2. *Bioengineered* 13: 8240-8254, 2022.
34. Deshmukh P, Unni S, Krishnappa G and Padmanabhan B: The Keap1-Nrf2 pathway: Promising therapeutic target to counteract ROS-mediated damage in cancers and neurodegenerative diseases. *Biophys Rev* 9: 41-56, 2017.
35. Zipper LM and Mulcahy RT: Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. *Toxicol Sci* 73: 124-134, 2003.
36. Ma CS, Lv QM, Zhang KR, Tang YB, Zhang YF, Shen Y, Lei HM and Zhu L: NRF2-GPX4/SOD2 axis imparts resistance to EGFR-tyrosine kinase inhibitors in non-small-cell lung cancer cells. *Acta Pharmacol Sin* 42: 613-623, 2021.
37. Kang Q and Yan S: Piperlongumine reverses doxorubicin resistance through the PI3K/Akt signaling pathway in K562/A02 human leukemia cells. *Exp Ther Med* 9: 1345-1350, 2015.
38. Asano T, Tsutsuda-Asano A and Fukunaga Y: Indomethacin overcomes doxorubicin resistance by decreasing intracellular content of glutathione and its conjugates with decreasing expression of gamma-glutamylcysteine synthetase via promoter activation in doxorubicin-resistant leukemia cells. *Cancer Chemother Pharmacol* 64: 715-721, 2009.
39. Nguyen T, Sherratt PJ, Huang HC, Yang CS and Pickett CB: Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 278: 4536-4541, 2003.
40. DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES, *et al*: Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475: 106-109, 2011.
41. Asensio-López MC, Soler F, Pascual-Figal D, Fernández-Belda F and Lax A: Doxorubicin-induced oxidative stress: The protective effect of nicorandil on HL-1 cardiomyocytes. *PLoS One* 12: e0172803, 2017.
42. Liu J, Mao W, Ding B and Liang CS: ERKs/p53 signal transduction pathway is involved in doxorubicin-induced apoptosis in H9c2 cells and cardiomyocytes. *Am J Physiol Heart Circ Physiol* 295: H1956-H1965, 2008.
43. Nam LB, Choi WJ and Keum YS: Triptolide downregulates the expression of NRF2 target genes by increasing cytoplasmic localization of NRF2 in A549 cells. *Front Pharmacol* 12: 680167, 2021.
44. Yu D, Liu Y, Zhou Y, Ruiz-Rodado V, Larion M, Xu G and Yang C: Triptolide suppresses IDH1-mutated malignancy via Nrf2-driven glutathione metabolism. *Proc Natl Acad Sci USA* 117: 9964-9972, 2020.



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