

## Upregulation of miR-142-3p Improves Drug Sensitivity of Acute Myelogenous Leukemia through Reducing P-Glycoprotein and Repressing Autophagy by Targeting HMGB1

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### Abstract

miR-142-3p was reported to be downregulated in acute myelogenous leukemia (AML) and acted as a novel diagnostic marker. However, the regulatory effect of miR-142-3p on drug resistance of AML cells and its underlying mechanism have not been elucidated. Here, we found that miR-142-3p was significantly downregulated and high mobility group box 1 (HMGB1) was dramatically upregulated in AML samples and cells, as well as drug-resistant AML cells. P-gp level and autophagy were markedly enhanced in HL-60/ADR and HL-60/ATRA cells. miR-142-3p overexpression improved drug sensitivity of AML cells by inhibiting cell viability and promoting apoptosis, and inhibited P-gp level and autophagy in drug-resistant AML cells, whereas HMGB1 overexpression obviously reversed these effect. HMGB1 was demonstrated to be a target of miR-142-3p, and miR-142-3p negatively regulated HMGB1 expression. In conclusion, our study elucidated that upregulation of miR-142-3p improves drug sensitivity of AML through reducing P-glycoprotein and repressing autophagy by targeting HMGB1, contributing to better understanding the molecular mechanism of drug resistance in AML.

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### Introduction

Leukemia, the most common type of cancer diagnosed during infancy, accounts for 25% to 35% of cases of childhood cancer in most populations [1]. Acute myeloid leukemia (AML) is a heterogeneous malignancy that is attributable to the differentiation arrest and abnormal proliferation of hematopoietic precursors in bone marrow and blood [2]. AML comprises up to approximately 20% of all pediatric leukemia cases; however, it is responsible for more than 30% of deaths from pediatric leukemia [3,4]. The 5-year disease-free survival in pediatric AML patients is approximately 50% in the most successful studies [5]. In recent years, dose-intensive chemotherapy has been widely used as a major treatment for high-risk pediatric AML [6]. However, the relapse rates still remain a major cause of therapeutic failure, and the clinical outcome of pediatric AML still remains poor [7], mainly as a result of resistance to antileukemic drugs [8]. Since chemoresistance is currently a major clinical obstacle to the pharmacological treatment of patients with AML [9], it is crucial to identify effective therapeutic targets that reverse resistance to drugs and elucidate the mechanisms of chemoresistance in AML.

The mechanisms of resistance termed multidrug resistance (MDR) are multifarious, including the altered expression of MDR-related genes, alterations in drug target sites, more effective DNA repair mechanism, escape from cell cycle checkpoints, increased drug efflux, resistance to apoptosis, and resistant stem cell development [10]. The development of refractory AML is frequently correlated with the MDR proteins of adenosine triphosphate-binding cassette (ABC) transporter in cell membranes, especially the cellular efflux protein ABCB1 (P-glycoprotein, P-gp) [11]. P-gp, a 170- to 180-kDa membrane glycoprotein encoded by multidrug resistance 1 (MDR1) gene, functions as a transmembrane drug efflux pump that decreases intracellular drug accumulation, hence reducing cellular toxicity of

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chemotherapeutic agents [12]. Accordingly, it is well established that P-gp has an important impact on the absorption, distribution, and elimination of chemotherapeutic drugs and is closely related to the development of resistance to anticancer drugs [13]. Thus, compounds that can inhibit P-gp-mediated efflux would be an effective therapeutic approach against MDR. However, modulators of MDR are not as effective as expected because, besides ABC transporters, various other mechanisms contribute to MDR. Recent studies focus on the role of autophagy, a catabolic process by which intracellular aggregated or misfolded proteins and damaged organelles are delivered to lysosomes for bulk degradation, in drug resistance [14]. It is well documented that autophagy is considered as a target for inducing cancer cell death, suggesting the functional role of autophagy in the cell survival [15]. It is stated that autophagy exerts a cytoprotective role by degrading the chemotherapeutic agents to protect cancer cells from drugs-induced apoptosis [16]. Accordingly, accumulative studies have shown that autophagy is implicated in the resistance of cancer cells to chemotherapy and is being employed to overcome MDR during anticancer therapy [17,18]. We recently found that the high mobility group box 1 (HMGB1), a well-characterized damage-associated molecular pattern molecule, can be released from leukemia cells after chemotherapy-induced cytotoxicity and contribute to chemoresistance through upregulating autophagy in leukemia [14,19].

MicroRNAs (miRNAs) represent a new class of small, noncoding endogenous RNAs with 19 to 25 nucleotides in length [20]. miRNAs can negatively regulate target gene expression in a posttranscriptional manner through its binding to the 3'-untranslated regions (3'UTRs) of target mRNAs to block mRNA translation or degrade target mRNAs [20]. Increasing evidence has demonstrated that miRNAs participate in various fundamental biological and pathological processes, such as cell differentiation, proliferation, apoptosis, as well as autophagy, indicating their important regulatory roles during carcinogenesis and chemoresistance [21]. It is well documented that dysregulation of miRNAs contributes to a variety of human disease, including AML [22,23]. In view of the significance of autophagy in the chemoresistance of tumors and the regulatory role of miRNAs in autophagy, better understanding the role of miRNA-regulated autophagy during chemotherapy in AML might contribute to clarifying its mechanism of chemoresistance.

miR-142-3p was initially identified in hematopoietic cells and served as an oncogenic biomarker for T cell acute lymphoblastic leukemia [24]. Additionally, miR-142-3p was reported to be downregulated in AML and could be used as novel diagnostic markers [25]. However, the effect of miR-142-3p on drug resistance of AML cells and its underlying mechanism have not been elucidated. In the present study, we aimed to investigate the functional role of miR-142-3p in chemoresistance of AML cells and the relationship between miR-142-3p and HMGB1 to further study the mechanism of MDR.

## Material and Methods

### Patients and AML Samples

The study protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, with prior informed consent from their legal guardians in accordance with the guidelines of the First Affiliated Hospital of Zhengzhou University, China. AML peripheral blood mononuclear cell (PBMC) samples and normal PBMC samples were collected from 23 *de novo* patients

with newly diagnosed pediatric AML in accordance with the French-American-British criteria and 15 nonmalignancies children with healthy pediatric bone marrow between January 2014 and January 2016 in the First Affiliated Hospital of Zhengzhou University. Mononuclear cells were isolated from the bone marrow samples using Ficoll-Hypaque (Sigma-Aldrich, Poole, Dorset, UK) density gradient centrifugation and stored at  $-80^{\circ}\text{C}$  until use.

### Cell Line and Treatment

Human AML cell line HL-60, adriamycin (ADR)-resistant cells (HL-60/ADR), and all-trans retinoic acid (ATRA)-resistant cells (HL-60/ATRA) were all purchased from the cell bank of the Chinese Academy of Medical Sciences (Beijing, China), and the human bone marrow stromal cell line HS-5 was obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine Serum (FBS; Gibco-BRL, Grand Island, NY), 2 mM L-glutamine, and 1% penicillin-streptomycin solution at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . To maintain the MDR phenotype, HL-60/ADR and HL-60/ATRA cells were treated with 1  $\mu\text{M}$  ADR and ATRA for 10 to 28 days and then maintained for 10 days without any additives prior to each experiment, respectively.

### Cell Transfection

miR-142-3p mimics (miR-142-3p), anti-miR-142-3p, miRNA control (miR-control), pcDNA-HMGB1, and pcDNA empty control (vector) were purchased from Genepharma (Shanghai, China). Cells were seeded into six-well plates and incubated at  $37^{\circ}\text{C}$  for 24 hours to reach approximately 80% confluence. Subsequently, cell transfection with miR-142-3p, pcDNA-HMGB1, miR-142-3p + pcDNA-HMGB1, or matched controls was carried out by Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Cells were harvested for subsequent experiments 48 hours posttransfection.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from purified mononuclear cells and cultured cells using TriZol Reagent (Takara, Dalian, China). First-strand cDNA was synthesized from these RNA samples using Revert Ace kit (TOYOBO, Osaka, Japan). For the analyses of HMGB1 and miR-142-3p expressions, RT-PCR was performed using a SYBR Premix Ex Taq II (Takara) and TaqMan microRNA assay (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7000 Sequence Detection System (Applied Biosystems), respectively. The specific primers used were as follows: HMGB1 forward 5'-CTGTCCATTGGTGATGTTGC-3', reverse 5'-CTGATAGCCTGCTCCAGGTC-3'; miR-142-3p forward 5'-TGCGG TGTAGTGTTCCTACTT-3', reverse 5'-CCAGTGCAGGG TCCGAGGT-3'; GAPDH forward 5'-TCGGAGTCAACGGATT TGG-3', reverse 5'-CATGGGTGGAATC ATATTGGA-3'. The relative gene expressions of miR-142-3p and HMGB1 mRNA were calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method, and GAPDH level was used as the internal control.

### Western Blot Analysis

Total protein was isolated from transfected cells using RIPA lysis buffer (Thermo Fisher Scientific, Beyotime, China). Protein concentration from cell extracts was determined using BCA protein quantitation kit (CoWin Biotechnology, Beijing, China). Equal

amounts of protein (50 µg of each sample) were separated by 12% sodium dodecyl sulfate polyacrylamide gel and then electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After being blocked with 5% nonfat dry milk in TBST for 1 hour, the membranes were probed with specific primary antibodies against HMGB1, LC3-I, LC3-II, Atg5, and P-gp (1: 500; Abcam, Cambridge, MA) and GAPDH (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by incubating with corresponding horseradish peroxidase-conjugated secondary (1:5000; Santa Cruz Biotechnology) antibodies for 2 hours at room temperature. The blots were visualized by a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA). GAPDH was used as the internal control.

### MTT Assay

Cell viability and survival rates were estimated using MTT assay (Promega Corporation, Fitchburg, WI). Briefly, HL-60 cells were plated into the 96-well plates at a density of  $3 \times 10^3$  cells and incubated for 72 hours in medium containing horizontal dilutions of anticancer agents ATRA (0.5, 1, 2, 4, and 8 µM) or ADR (50, 100, 200, 300, and 400 nM). Subsequently, 20 µl of MTT (5 mg/ml; Sigma) was added to each well and incubated at 37°C for another 4 hours. Then the cultured medium was discarded, and 150 µl of dimethyl sulfoxide (DMSO) was then added to dissolve the formazan precipitate for 10 minutes. Absorbance at 490 nm was measured by an automatic microplate reader (Molecular Device Corp., Sunnyvale, CA). The IC<sub>50</sub> (inhibitory concentration) values, which referred to drug concentrations that demonstrate 50% of cell growth inhibition, were calculated using Graphpad Prism software 5.0 software (GraphPad Prism, San Diego, CA) according to the relationship of drug concentration and cell survival rate. In addition, the viability of transfected cells was detected using the method as above.

### Detection of Apoptosis by Flow Cytometry

For cell apoptosis analysis, the transfected cells were harvested, washed, and resuspended in 1 ml of binding buffer. The cells were then stained with 5 µl of fluorescein isothiocyanate-Annexin V (BD Biosciences, San Jose, CA) and 10 µl of propidium iodide (Sigma-Aldrich, Shanghai, China) in the dark for 15 minutes at room temperature and analyzed by flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences).

### Luciferase Reporter Assay

The potential miR-142-3p binding sites within HMGB1 sequence were predicted by [microRNA.org](http://www.microRNA.org)-target program ([www.microRNA.org](http://www.microRNA.org)). The wild type of HMGB1 containing the putative miR-142-3p target binding sites and its mutant were synthesized and inserted into 3'UTR of firefly luciferase gene in the pGL3 luciferase vector (Promega, Madison, WI). The constructed luciferase plasmids were defined as pGL3-HMGB1-3'UTR-WT and pGL3-HMGB1-3'UTR-MUT, respectively. HL-60/ATRA and HL-60/ADR cells ( $3 \times 10^4$  cells/well) were inoculated in 96-well plates and incubated for 48 hours. Then the cells were cotransfected with 400 ng of luciferase reporter plasmids (pGL3-HMGB1-MT and pGL3-HMGB1-MUT), along with 50 ng of pRL-TK plasmid (Promega), and 50 nM of miR-142-3p or miR-control by Lipofectamine 2000 (Invitrogen). The firefly and Renilla luciferase activity was measured 48 hours posttransfection using the Dual-Luciferase Reporter Assay System (Promega) and normalized against Renilla luciferase activity.

### Statistical Analysis

Results were expressed as mean ± standard deviation (S.D.) from at least three independent experiments. Statistical analyses of experimental groups were carried out by Student's *t* test or analysis of variance tests using SPSS version 11.5 software (SPSS Inc., Chicago, IL). A *P* value less than .05 was considered to be statistically significant.

### Results

#### *miR-142-3p Was Downregulated and HMGB1 Was Upregulated in PBMC from Pediatric AML Patients*

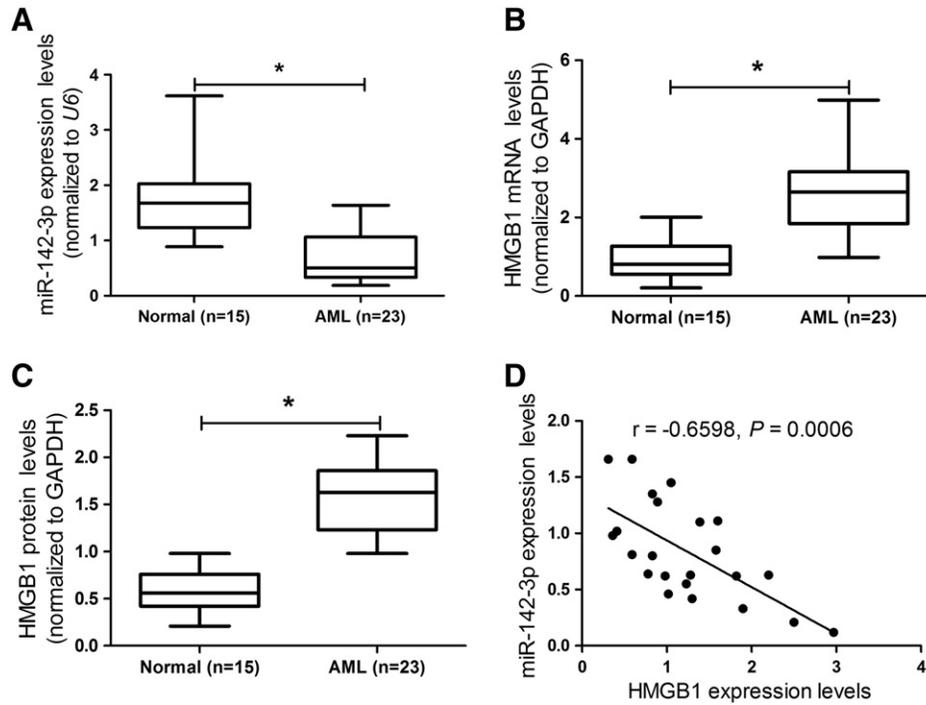
To evaluate the functional roles of miR-142-3p and HMGB1 in AML progression, the expressions of miR-142-3p and HMGB1 were firstly confirmed in 23 pediatric AML PBMCs and 15 normal PBMCs by qRT-PCR analysis. The results showed that the expression level of miR-142-3p was significantly reduced in pediatric AML PBMCs in comparison with those from normal children (Figure 1A). Meanwhile, the qRT-PCR and Western blot analyses revealed that HMGB1 was conspicuously highly expressed at both mRNA and protein levels (Figure 1, B and C). Notably, there was a significantly negative correlation between miR-142-3p and HMGB1 expression in pediatric AML PBMCs (Figure 1D). Taken together, these results demonstrated that miR-142-3p was negatively correlated with HMGB1 expression.

#### *miR-142-3p Level Was Reduced, HMGB1 and P-gp Expression Were Improved, and Autophagy Was Promoted in AML and Drug-Resistant AML Cells*

To explore the effects of miR-142-3p and HMGB1 on chemoresistance in AML cells, the expression levels of miR-142-3p and HMGB1 in drug-resistant AML cells were firstly investigated. As shown in Figure 2, A–D, qRT-PCR and Western blot results exhibited that miR-142-3p was strikingly downregulated and HMGB1 was evidently upregulated in AML HL-60 cells compared with the human bone marrow stromal cell line HS-5 cells. Moreover, a lower expression of miR-142-3p and a higher expression in human drug-resistant AML cell lines HL-60/ATRA and HL-60/ADR were observed with respect to their parental cell line HL-60. In addition, Western blot analysis confirmed that P-gp level was remarkably increased in HL-60 cells compared with HS-5 cells and in HL-60/ATRA and HL-60/ADR cells as opposed to HL-60 cells (Figure 2, E and F). Furthermore, the levels of autophagy-related proteins Atg5 and microtubule-associated protein 1 light chain 3 (LC3) conversion from LC3-I to LC3-II were substantially improved in HL-60 cells compared with HS-5 cells and in HL-60/ATRA and HL-60/ADR cells in comparison with HL-60 cells (Figure 2, G and H). Taken together, these findings indicated that miR-142-3p, HMGB1, and autophagy may be implicated in the development of drug resistance in AML.

#### *miR-142-3p Overexpression Improved Drug Sensitivity of AML Cells*

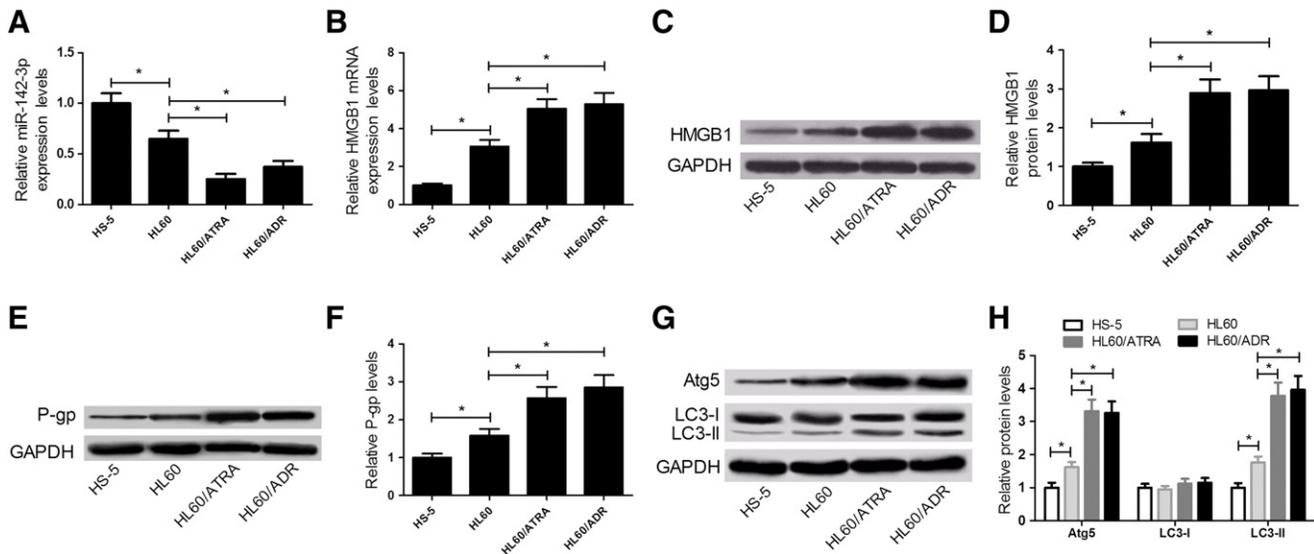
To identify the differential sensitivity of HL-60/ATRA, HL-60/ADR and their parental HL-60 cells to chemodrugs, MTT assay was employed to determine the cytotoxicity of chemotherapeutic drugs, including ATRA and ADR, which were frequently used for AML treatment [26]. As compared with HL-60 cells, HL-60/ATRA and HL-60/ADR cells were resistant to ATRA and ADR in a dose-dependent manner with higher IC<sub>50</sub> values (ATRA:  $4.0 \pm 0.33$  µM, ADR:  $300.0 \pm 12.31$  nM) (Figure 3, A and B). Thus, 4 µM ATRA and 300 nM ADR were chosen for subsequent



**Figure 1.** Expression levels of miR-142-3p and HMGB1 in PBMCs from pediatric AML patients. qRT-PCR assay was performed to examine the expressions of miR-142-3p (A) and HMGB1 mRNA (B) in 23 pediatric AML PBMCs and 15 normal PBMCs. (C) The level of HMGB1 in 23 pediatric AML PBMCs and 15 normal PBMCs was determined by Western blot. (D) A negative correlation between miR-142-3p and HMGB1 expression. \* $P < .05$ .

experiments. The biological role of miR-142-3p on chemoresistance in AML cells was further estimated. Cell viability and apoptosis in HL-60/ATRA and HL-60/ADR cells transfected with miR-142-3p or miR-control were assessed by MTT assay and flow cytometry with or without ATRA or ADR. As demonstrated by MTT assay,

miR-142-3p overexpression led to a marked decrease of cell viability in both the cell lines compared to miR-control groups (Figure 3, C and D). Moreover, miR-142-3p overexpression enhanced ATRA or ADR-induced cell viability inhibition. Furthermore, miR-142-2p overexpression remarkably induced apoptosis of both HL-60/ATRA

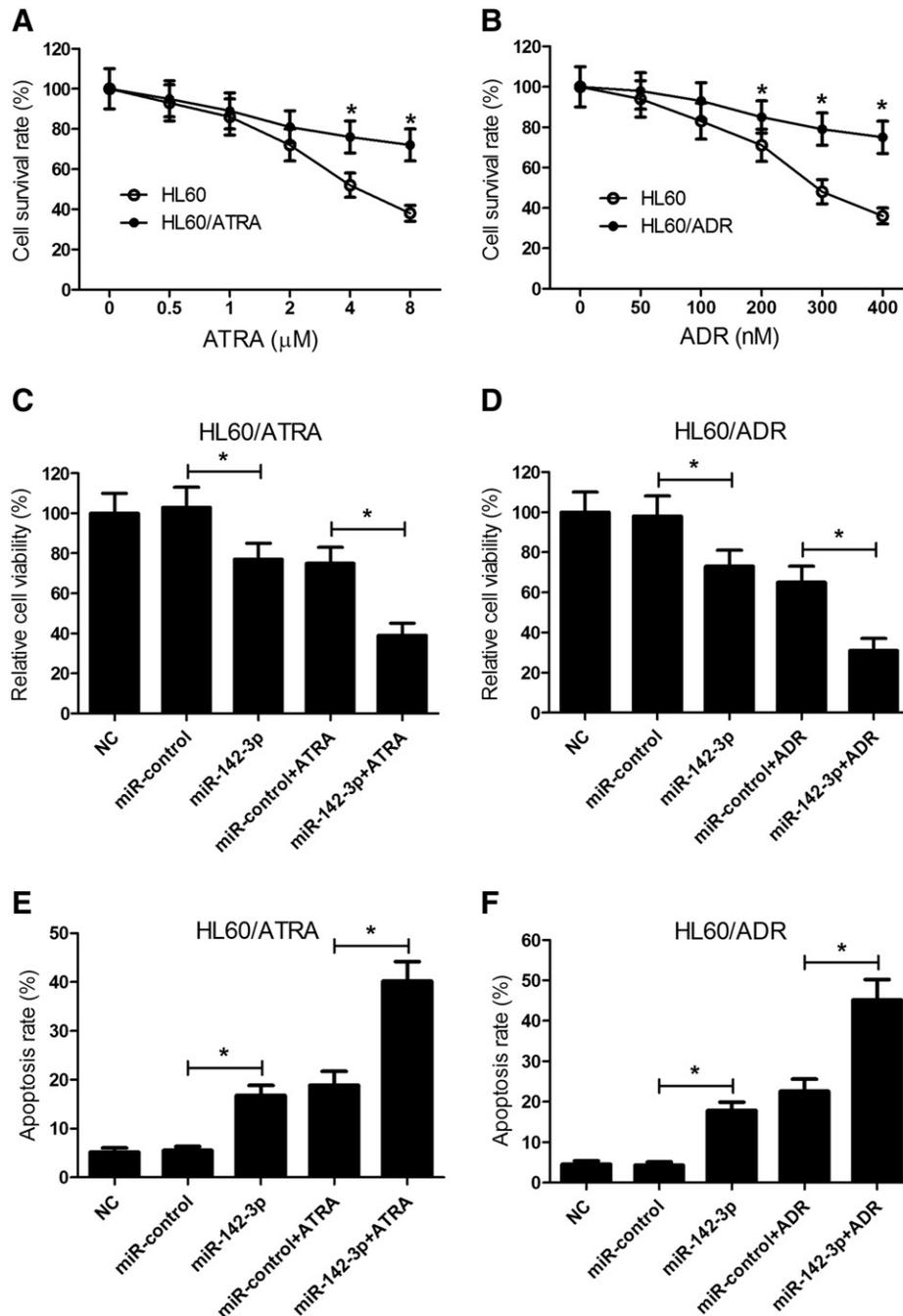


**Figure 2.** Expression levels of miR-142-3p, HMGB1, P-gp, and autophagy-related proteins in human drug-resistant AML cell lines HL-60/ATRA and HL-60/ADR and their parental cell line HL-60. The expressions of miR-142-3p (A) and HMGB1 mRNA (B) in the human bone marrow stromal cell line HS-5 cells, AML cell line HL-60, and drug-resistant AML cell lines HL-60/ATRA and HL-60/ADR were evaluated by qRT-PCR. The levels of HMGB1 (C and D), P-gp (E and F), and autophagy-related proteins (Atg5, LC3-I, and LC3-II) (G and H) in HS-5 cells, AML cell line HL-60, and drug-resistant AML cell lines HL-60/ATRA and HL-60/ADR were detected by Western blot. \* $P < .05$ .

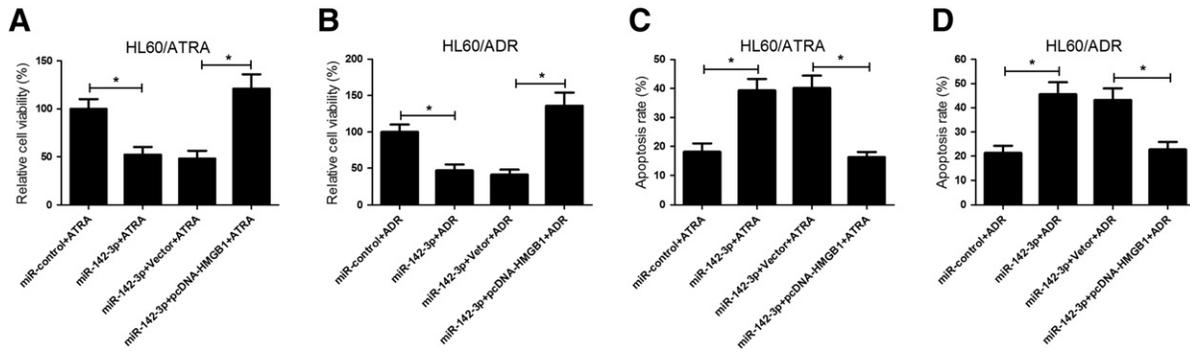
and HL-60/ADR cells compared with miR-control-transfected cells (Figure 3, E and F). More importantly, combination of ATRA and miR-142-3p or ADR and miR-142-3p treatment both dramatically reinforced ATRA or ADR-induced apoptosis. Therefore, we concluded that miR-142-3p overexpression conspicuously improved drug sensitivity in AML cells via inhibiting cell viability and promoting apoptosis.

### HMGB1 Overexpression Attenuated miR-142-3p-Induced Drug Sensitivity in Drug-Resistant AML Cells

The effect of HMGB1 overexpression on miR-142-3p overexpression-induced drug sensitivity in AML cells was explored by transfecting with miR-142-3p or combined with pcDNA-HMGB1 into HL-60/ATRA and HL-60/ADR cells. As displayed in Figure 4, A and B, under the ATRA or ADR condition, miR-142-3p overexpression resulted in an



**Figure 3.** Effect of miR-142-3p on drug sensitivity of resistant AML cells. (A) Cell survival rates were detected by MTT assay in HL-60 and HL-60/ATRA treated with various concentrations (0.5, 1, 2, 4, and 8 μM) of ATRA for 72 hours. (B) Cell survival rates were detected by MTT assay in HL-60 and HL-60/ADR incubated with various concentrations of ADR (50, 100, 200, 300, and 400 nM) for 72 hours. Cell viability in HL-60/ATRA (C) and HL-60/ADR (D) cells transfected with miR-142-3p or miR-control or along with 4 μM ATRA or 300 nM ADR was evaluated by MTT assay. Apoptosis in HL-60/ATRA (E) and HL-60/ADR (F) cells transfected with miR-142-3p or miR-control or along with 4 μM ATRA (E) or 300 nM ADR (F) was assessed by flow cytometry. \**P* < .05.

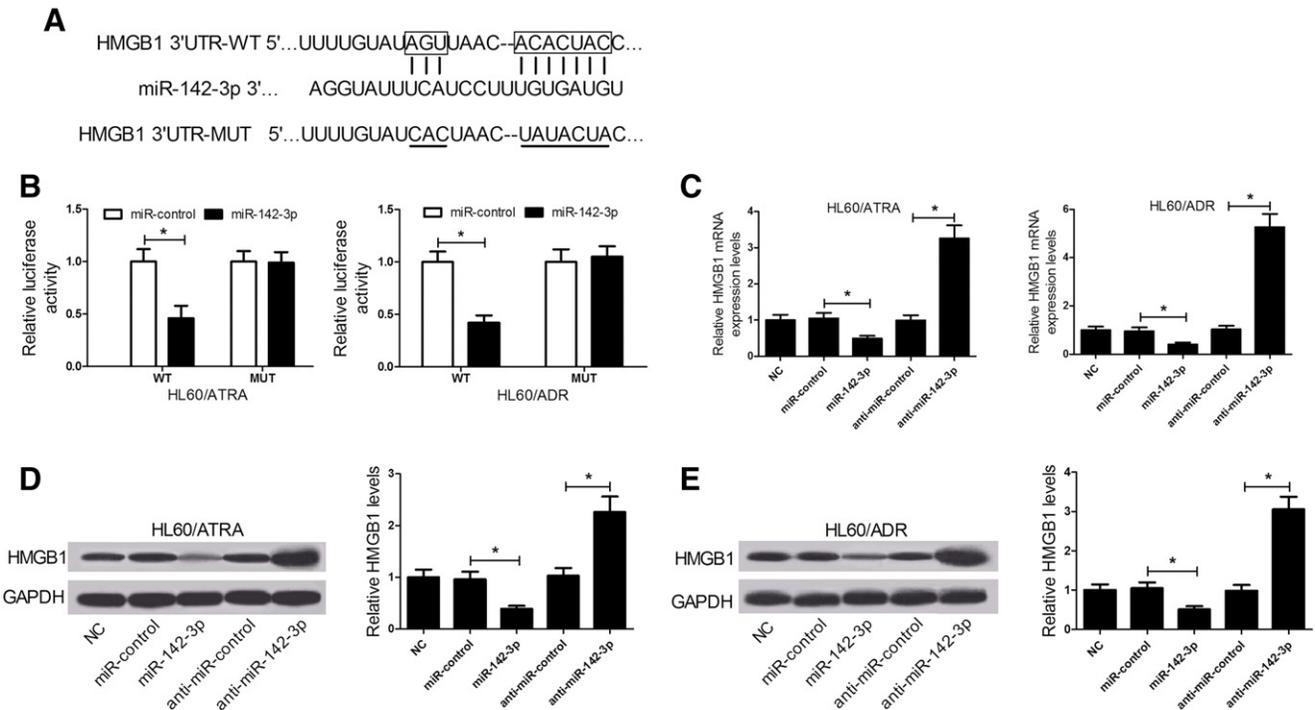


**Figure 4.** HMGB1 reversed miR-142-3p–induced drug sensitivity in HL-60/ATRA and HL-60/ADR cells. (A and B) After cells treated with ATRA or ADR were transfected with miR-142-3p, miR-control or along with pcDNA-HMGB1 or vector, cell viability was determined by MTT assay. (C and D) After cells treated with ATRA or ADR were transfected with miR-142-3p, miR-control or along with pcDNA-HMGB1 or vector, apoptosis was examined by flow cytometry. \**P* < .05.

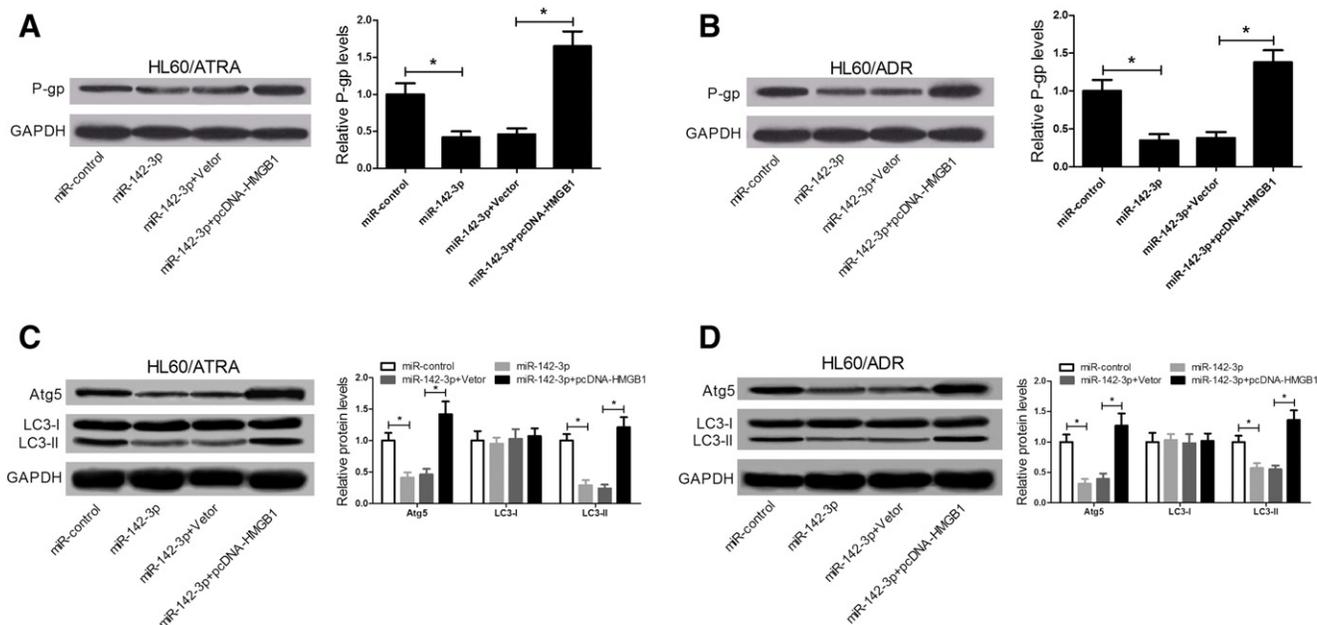
bvious inhibition on cell viability compared with miR-control groups, whereas HMGB1 overexpression significantly reversed this effect. Additionally, under the ATRA or ADR condition, miR-142-3p overexpression markedly promoted apoptosis in HL-60/ATRA and HL-60/ADR cells with respect to miR-control groups (Figure 4, C and D). However, HMBG1 overexpression strikingly abolished miR-142-3p overexpression-induced apoptosis. Taken together, HMGB1 overexpression dramatically relieved miR-142-3p overexpression-induced drug sensitivity in AML cells.

*HMGB1 Was a Direct Target of miR-142-3p*

To explore the underlying mechanism responsible for drug sensitivity enhancement in AML by miR-142-3p upregulation, the potential target genes of miR-142-3p were predicted by several bioinformatics programs (miRTarBase and TargetScan database). HMGB1 was identified to be a potential target of miR-142-3p and to contain the putative binding sites for the seed region of miR-142-3p. To confirm whether miR-142-3p could directly target HMGB1, we constructed luciferase reporter vectors containing wild-type or mutated



**Figure 5.** miR-142-3p directly targeted HMGB1 and negatively regulated its expression. (A) Diagram of putative miR-142-3p binding sequence in HMGB1 3'UTR and its mutant in luciferase reporter assay. (B) Luciferase reporter assay was performed to measure luciferase activity in HL-60/ATRA or HL-60/ADR cells cotransfected with luciferase reporter vectors (pGL3-HMGB1-3'UTR-WT or pGL3-HMGB1-3'UTR-MUT) with miR-142-3p or miR-control. (C) qRT-PCR was carried out to assess the expression of HMGB1 mRNA in HL-60/ATRA and HL-60/ADR cells transfected with miR-142-3p, anti-miR-142-3p, or respective controls. Western blot was used to determine the level of HMGB1 in HL-60/ATRA (D) and HL-60/ADR (E) cells transfected with miR-142-3p, anti-miR-142-3p, or respective controls. \**P* < .05.



**Figure 6.** miR-142-3p upregulation enhanced drug sensitivity of AML cells through reducing P-gp and suppressing autophagy by targeting HMGB1. The level of P-gp in HL-60/ATRA (A) and HL-60/ADR (B) cells transfected with miR-142-3p or miR-control, or together with pcDNA-HMGB1 or vector were detected by Western blot. The levels of autophagy related proteins Atg5, LC3-I, and LC3-II were analyzed by Western blot in HL-60/ATRA (C) and HL-60/ADR (D) cells transfected with miR-142-3p or miR-control, or together with pcDNA-HMGB1 or vector. \* $P < .05$ .

HMGB1 3'UTR binding sites for miR-142-3p (Figure 5A) to cotransfect with miR-142-3p or miR-control into HL-60/ATRA and HL-60/ADR cells. Luciferase reporter assay results demonstrated that miR-142-3p overexpression remarkably reduced the luciferase activity of pGL3-HMGB1-3'UTR-WT but had no obvious inhibitory effect on pGL3-HMGB1-3'UTR-MUT (Figure 5B). To explore whether HMGB1 expression was regulated by miR-142-3p in drug-resistant AML cells, we further examined the expression level of HMGB1 at mRNA and protein levels by qRT-PCR and Western blot in HL-60/ATRA and HL-60/ADR cells transfected with miR-142-3p, anti-miR-142-2p, or matched controls. qRT-PCR and Western blotting analyses revealed that miR-142-3p overexpression markedly inhibited HMGB1 expression at mRNA and protein levels in both HL-60/ATRA and HL-60/ADR cells. In contrast, in HL-60/ATRA and HL-60/ADR cells, anti-miR-142-3p dramatically increased the mRNA and protein levels of HMGB1 (Figure 5, C-E). These data demonstrated that miR-142-3p directly targeted HMGB1 and negatively regulated its expression.

#### *miR-142-3p Overexpression Improved Drug Sensitivity of AML Cells through Reducing P-gp and Suppressing Autophagy by Targeting HMGB1*

To investigate the underlying mechanism of interaction between miR-142-3p and HMGB1 regulating drug sensitivity of AML cells, the P-gp level and autophagy in HL-60/ATRA and HL-60/ADR cells transfected with miR-142-3p or miR-control, or together with pcDNA-HMGB1 or vector were estimated by Western blot. As presented in Figure 6, A and B, miR-142-3p overexpression strikingly reduced P-gp level in HL-60/ATRA and HL-60/ADR cells, while HMGB1 overexpression effectively recuperated miR-142-3p-induced decrease of P-gp level. Additionally, a significant decrease of the levels of autophagy-related proteins Atg5 and LC3-II was observed

in miR-142-3p-transfected HL-60/ATRA and HL-60/ADR cells (Figure 6, C and D), suggesting that miR-142-3p overexpression remarkably restrained autophagy in drug-resistant AML cells. Conversely, HMGB1 overexpression conspicuously overturned miR-142-3p-induced autophagy repression in HL-60/ATRA and HL-60/ADR cells. Taken together, these results indicated that miR-142-3p upregulation enhanced drug sensitivity of AML cells through reducing P-gp and suppressing autophagy by targeting HMGB1.

#### **Discussion**

Over the past few years, functional and prognostic studies have demonstrated that aberrantly expressed miRNAs are ultimately involved in tumorigenesis and the progression of various tumors, including certain subtypes of leukemia [27,28]. Emerging evidence suggests that the development of drug resistance is closely associated with altered expression of miRNAs in cancer cells [29,30]. Here, we identified for the first time that miR-142-3p/HMGB1 axis in human AML cells may be responsible for the chemosensitivity of AML cells. In the present study, we demonstrated that miR-142-3p was downregulated in pediatric AML PBMCs and cells and drug-resistant AML cells and negatively correlated with HMGB1 expression. Gain-of-function approach revealed that overexpression of miR-142-3p enhanced drug sensitivity of AML cells through reducing P-gp and suppressing autophagy, whereas HMGB1 overexpression markedly overturned this effect. Further mechanistic analyses indicated that upregulation of miR-142-3p improved drug sensitivity of AML cells through reducing P-gp and repressing autophagy by targeting HMGB1.

Recent studies reported that miRNAs play a crucial role in chemoresistance of AML [31]. For example, Shibayama et al. reported that higher expression of miR-126-5p reduced sensitivity to cytarabine and resulted in a poorer prognosis in AML [32]. Chen et al.

found that let-7a overexpression enhanced chemosensitivity by downregulating CXCR4 to promote YY1-mediated transcriptional activation of MYC and BCLXL in AML cells [33]. Zhang et al. exhibited that miR-125b contributed to pediatric AML development by promoting leukemic cell proliferation and inhibiting apoptosis and increased drug resistance of AML cells [34]. miR-142-3p was reported to be downregulated and played a tumor suppressive role in various tumors including leukemia [35]. For example, Duo et al. revealed that the downregulated miR-142-3p functioned as a growth suppressor in acute lymphoblastic leukemia by repressing MLL-AF4 expression [36]. Wang et al. indicated that miR-142-3p was a key regulator of normal myeloid differentiation and its reduced expression was related to AML development [37], therefore serving as a candidates for AML prognosis and therapeutic strategy [25]. Grassilli et al. demonstrated that the existence of Vav1, PU.1 which regulated ATRA-induced gene expression, as well as miR-142-3p, supported ATRA-induced differentiation in APL-derived NB4 cells [38]. Our study confirmed that miR-142-3p was downregulated in AML PBMC and cells, as well as AML/ATRA and AML/ADR cells. Furthermore, forced miR-142-3p expression resulted in a significant decrease of cell viability and a marked increase of apoptosis rates in AML cells. Notably, combination of ATRA and miR-142-3p or ADR and miR-142-3p treatment both remarkably augmented miR-142-3p-induced cell viability inhibition and apoptosis promotion in HL-60 cells, suggesting that miR-142-3p overexpression enhanced drug resistance of AML cells.

HMGB1, a well-known regulator of autophagy and apoptosis, functions as a highly conserved chromatin-associated nuclear protein which regulates nucleosome dynamics, chromosome stability, and gene transcription and also an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor progression [39,40]. HMGB1 release, along with other intracellular factors from tumor cells in response to chemotherapy or radiotherapy, is an important feature of the disordered tumor microenvironment [41]. Recent reports have revealed that autophagy induced by HMGB1 contributed to drug resistance of tumors [14]. For example, Liu et al. reported that HMGB1-mediated autophagy regulated sensitivity of colorectal cancer cells to oxaliplatin via MEK/ERK signaling pathway [41]. Zhang et al. found that interference with endogenous HMGB1 by shRNA improved the sensitivity to chemotherapy drugs in non-small-cell lung carcinoma cells by inhibiting HMGB1-mediated autophagy and increasing cell apoptosis [42]. Pan et al. showed that HMGB1-regulated autophagy contributed to docetaxel resistance in lung adenocarcinoma cells [43]. Yang et al. exhibited that HMGB1 expression was positively correlated with clinical status in childhood leukemia and endogenous HMGB1 increased starvation-induced autophagy, which enhanced leukemia cell chemoresistance likely through the PI3K/Akt/mTORC1 pathway [43]. In the current study, HMGB1 was highly expressed at both mRNA and protein levels in AML PBMCs and cells, as well as AML/ATRA and AML/ADR cells. Function studies indicated that HMGB1 overexpression significantly overturned miR-142-3p-induced cell viability inhibition and apoptosis promotion on AML cells treated with chemotherapy drugs ATRA or ADR. Furthermore, luciferase reporter assay and RT-PCR analyses suggested that HMGB1 was a direct target of miR-142-3p and miR-142-3p could negatively regulate HMGB1 expression. HMGB1 has been demonstrated to be a direct target of miR-142-3p in non-small-cell lung carcinoma [44], cardiomyocytes [45], and osteoar-

thritis [46]. In addition, we noticed that P-gp level and autophagy were both enhanced in drug-resistant AML cells. More notably, miR-142-3p overexpression led to a significant inhibition of P-gp level and autophagy, while HMGB1 overexpression dramatically abolished this effect. These results suggested that the miR-142-3p overexpression improved drug sensitivity of AML cells through reducing P-gp and repressing autophagy by suppressing HMGB1 expression.

In conclusion, we demonstrated that miR-142-3p was downregulated in AML PBMC samples and cell lines, as well as drug-resistant AML cells. Furthermore, upregulation of miR-142-3p enhanced drug sensitivity of AML cells through reducing P-gp and repressing autophagy by targeting HMGB1, contributing to better understanding the molecular mechanism of drug resistance in AML.

## Competing Interests

The authors declare no competing or financial interests.

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