MicroRNA-145 promotes the apoptosis of leukemic stem cells and enhances drug-resistant K562/ADM cell sensitivity to adriamycin via the regulation of ABCE1

ZHIJUN WUXIAO[1*], HUA WANG[2*], QUNHAO SU[1], HAIYAN ZHOU[1], MIN HU[1], SHI TAO[1], LU XU[1], YU CHEN[1] and XINBAO HAO[1]

[1]Department of Hematology, Lymphoma and Myeloma Center, HMC Cancer Institute, The First Affiliated Hospital of Hainan Medical University, Haikou, Hainan 570102; [2]Department of Hematological Oncology, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou, Guangdong 510060, P.R. China

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Abstract. Leukemia is a type of cancer which originates in blood-forming tissues. MicroRNAs (miRNAs or miRs) have been shown to be involved leukemogenesis. In the present study, following the gain- and loss-function of miR-145 and ATP-binding cassette sub-family E member 1 (ABCE1) in K562 cells and K562 adriamycin-resistant cells (K562/ADM cells), the levels of multidrug resistance protein 1 (MRP1) and P-glycoprotein (P-gp) were measured. The viability of the K562 cells and K562/ADM cells treated with various concentrations of ADM, and cell sensitivity to ADM were measured. The apoptosis of stem cells was detected. K562/ADM cells were transfected with miR-145 mimic or with miR-145 mimic together with ABCE1 overexpression plasmid to examine the effects of ABCE1 on the sensitivity of K562/ADM cells to ADM. The association between miR-145 and ABCE1/MRP1 was then verified. The dose- and time-dependent effects of ADM on the K562 cells and K562/ADM cells were examined. The K562/ADM cells exhibited a greater resistance to ADM, higher levels of MRP1 and P-gp, and a lower miR-145 expression. The K562/ADM cells and stem cells in which miR-145 was overexpressed exhibited a suppressed cell proliferation, decreased MRP1 and P-gp levels, and an increased apoptotic rate. However, K562 cells with a low expression of miR-145 exhibited an increased cell proliferation, increased levels of MRP1 and P-gp, and a suppressed apoptotic rate. Compared with the overexpression of miR-145, the combination of miR-145 and ABCE1 decreased the sensitivity of drug-resistant K562/ADM cells to ADM. The above-mentioned effects of miR-145 were achieved by targeting ABCE1. Taken together, the findings of the present study demonstrate that the overexpression of miR-145 promotes leukemic stem cell apoptosis and enhances the sensitivity of K562/ADM cells to ADM by inhibiting ABCE1.

Correspondence to: Dr Xinbao Hao, Department of Hematology, Lymphoma and Myeloma Center, HMC Cancer Institute, The First Affiliated Hospital of Hainan Medical University, 31 Longhua Road, Longhua, Haikou, Hainan 570102, P.R. China
E-mail: drhaoxinbao333@163.com

*Contributed equally

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Introduction

Leukemia is recognized as a fatal malignant clonal disorder of hematopoietic multi-potent stem cells and immature progenitors with increased leucocytes in human blood and bone marrow (1,2). Benzene is an industrial chemical and component of gasoline and it is commonly recognized that benzene exposure in either occupational or environmental conditions is an affirmative contributor to leukemia (3). Clonal hematopoiesis is confirmed as an important risk factor for hematological cancers, and acquired mutations may lead to the age distribution of acute myeloid leukemia, while physical abnormalities have a greater influence on acute lymphoblastic leukemia (1). It has also been determined that a younger age, the female sex and greater physical activities are associated with an evidently reduced risk of developing chronic myeloid leukemia; however, smoking and obesity are risk factors (4). Approximately 25% of patients suffering from chronic lymphocytic leukemia will undergo secondary autoimmune disorders, complicating the symptoms and treatment of leukemia, decreasing their quality of life, and even endangering their lives (5). Adriamycin (ADM) is an antibiotic and is a widely used chemotherapeutic drug for the treatment of solid tumors and hematological disorders (6). In particular, it is the most effective drug for the treatment of childhood acute lymphoblastic leukemia (7). Leukemic stem cells have the ability of limitless self-renewal and possess potent functions to maintain leukemia (8). Leukemic stem cells are being studied as potential therapeutic targets, and the signaling pathways that control their development and survival are of particular interest (9).

MicroRNAs (miRNAs or miRs) are a family of small non-coding RNAs, functioning as key post-transcriptional mediators of genes involved in multiple fundamental processes, such as differentiation, proliferation, apoptosis and cancer drug resistance (10). Several miRNAs participate in the differentiation of various hematopoietic cells, and their dysregulation is clearly associated with cancer development and particularly, with leukemia (11). The expression of miR-145 has been to be markedly increased in normal hematopoietic progenitor cells (12). A lower expression of miR-145 was identified as an independent risk factor by Xia *et al* and miR-145 overexpression was shown to suppress tumor cell growth in adult T-cell leukemia/lymphoma cell lines (13). In the present study, through bioinformatics prediction and dual-luciferase reporter gene assay, it was found that miR-145 targeted adenosine triphosphate (ATP)-binding cassette (ABC) transporter E1 (ABCE1) to inhibit its expression. ABCE1 is a less extensively studied member of the ABC multigene family and plays key roles in diverse biological events, such as viral infection, cell proliferation and anti-apoptosis (14). ABC transporters play important roles in numerous disorders, particularly in acute myeloid leukemia, while the overexpression of certain ABC members in leukemic cells has a strong link with the poor outcome of patients afflicted with acute myeloid leukemia (15). Based on the above-mentioned information, it was hypothesized that miR-145 and ABCE1 may play a role in the biological processes of leukemia and in cell sensitivity to ADM.

Materials and methods

Cells and cell culture. The human leukemia cell line, K562, and corresponding ADM-resistant cells, K562/ADM cells, were obtained from the Kunming Cell Bank of Chinese Academy of Sciences and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences) in an incubator (37˚C, 5% CO_2). Cells were passaged once for 2-3 days with a total of 3 passages. The corresponding K562/ADM cells were continuously cultured in the above-mentioned medium containing 1.0 μg/ml ADM, and then cultured in normal medium 2 weeks before the experiment.

Cell transfection and treatment. K562/ADM cells in good growing conditions (i.e., cells have good light transmittance, less granular matter in the cytoplasm, smaller volume of the cytoplasm, compact shape of the whole cell, and even cell growth without hypertrophy) were assigned into the K562/ADM group, mimic-negative control (NC) group (transfected with miR-145 mimic NC), miR-145 mimic group (transfected with miR-145 mimic) (from Shanghai GenePharma Co., Ltd.), miR-145 mimic + overexpression (EO)-NC group (transfected with miR-145 mimic and ABCE1 empty plasmid (Shanghai GenePharma Co., Ltd.) and miR-145 mimic + ABCE1 group (transfected with miR-145 mimic and ABCE1 overexpression plasmid). K562 cells in good growing conditions (i.e., cells have good light transmittance, less granular matter in the cytoplasm, smaller volume of the cytoplasm, compact shape of the whole cell, and even cell growth without hypertrophy) were assigned into the K562 group, inhibitor-NC group (transfected with miR-145 inhibitor NC), and miR-145 inhibitor group (transfected with miR-145 inhibitor). The inhibitor NC and miR-145 inhibitor were provided by Shanghai GenePharma Co., Ltd. Transfection was conducted using Lipofectamine® 2000 according to the instructions of the manufacturer (Invitrogen; Thermo Fisher Scientific, Inc.). The final concentration of the plasmid was 50 nM. miR-145 expression was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at 24 h following transfection to verify the transfection efficiency.

RT-qPCR. Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and ultraviolet analysis combined with formaldehyde denaturation electrophoresis then confirmed the high quality of the extracted RNA. RNA (1 μg) was reverse transcribed into cDNA using avian myeloblastosis virus reverse transcriptase (Beijing Solarbio Science & Technology Co., Ltd.). qPCR was conducted according to SYBR-Green method (Thermo Fisher Scientific, Inc.) with U6 as the internal reference of miR-145 and β-actin as the internal reference of ABCE1. A NC with PCR products, but no primers was set. PCR primers were devised and synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Table I). The PCR system included cDNA 1.0 μl, 2X SYBR-Green Mix 10 μl, Forward Primer (10 μM) 0.5 μl, Reverse Primer (10 μM) 0.5 μl, and was supplemented into 20 μl with the addition of RNase-free water. The reaction conditions were as follows: 5 min pre-denaturation at 94˚C, 40 cycles of 40 sec denaturation at 94˚C, 40 sec annealing at 60˚C, 60 sec extension at 72˚C, and finally 10 min extension at 72˚C. The products were tested by agarose gel electrophoresis. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (16), which indicated the multiple association between the experimental group and the control group. $\Delta\Delta Cq=[Cq\ (target\ gene)-Cq\ (reference\ gene)]_{experimental\ group}-[Cq\ (target\ gene)-Cq\ (reference\ gene)]_{control\ group}$. The normally cultured K562 and K562/ADM cells served as the controls.

Western blot analysis. The RIPA lysis buffer (Beyotime Institute of Biotechnology) was used to extract the proteins, and the extracted proteins was determined based on the instructions of bicinchoninic acid (BCA) kit (Wuhan Boster Biological Technology Co., Ltd.). The extracted proteins were placed in loading buffer, boiled at 95˚C for 10 min, and loaded on each well (each for 30 μg), and then separated by 6% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with the voltage changing from 80 to 120 v. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane by semi-dry transfer at 100 mv for 30-45 min. The membrane was then sealed for 1 h in 5% bovine serum albumin (BSA) at room temperature, followed by incubation with the following primary antibodies (all from Abcam): ABCE1 (1 μg/ml, ab32270), multidrug resistance protein 1 (MRP1) (1:500, ab32574), P-glycoprotein (P-gp) (1:500, ab216656), and β-actin (1:5,000, ab8227) at 4˚C overnight. Subsequently, the membrane was rinsed with Tris-buffered saline Tween (TBST) 3 times (5 min/time), and then incubated for 1 h with the secondary antibody [horseradish peroxidase labeled goat anti-rabbit IgG (H+L),

Table I. Primer sequences of RT-qPCR.

Gene	Sequences
miR-145	F: 5'-GGCACTGCTGAAGGCATCTC-3'
	R: 5'-CTGTTAAGCCATGACCTCAAGAAC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-AACGCTTCACGAATTTGCGT-3'
ABCE1	F: 5'-CCAGGTGAAGTTTTGGGATTAG-3'
	R: 5'-AGGTTTGATGATGGCTTTTAGG-3'
MRP1	F: 5'-CATTCAGCTCGTCTTGTCCTG-3'
	R: 5'-GGATTAGGGTCGTGGATGGTT-3'
β-actin	F: 5'-GGCATCACACTTTCTACAACG-3'
	R: 5'-GGCAGGAACATTAAAGGTTTC-3'

RT-qPCR, reverse-transcription quantitative polymerase chain reaction; F, forward; R, reverse; miR-145, microRNA-145; ABCE1, adenosine triphosphate (ATP)-binding cassette (ABC) E1.

ZB-2301, 1:2,500, unconjugated; or horseradish peroxidase labeled goat anti-mouse IgG (H+L), ZB-2305, 1:2,500, unconjugated; ZSGB-Bio Co., Ltd.] at room temperature. Finally, the membrane was washed 3 times (5 min/time) and developed by chemiluminescence reagent and bands were visualized using the Bio-Rad Gel Dol EZ imager (Bio-Rad Laboratories). The target band was analyzed using Image J software (National Institutes of Health) for gray value analysis.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. After counting the number, cells were adjusted into $5x10^4$ cells/l and 180 μl/well in a 96-well plate and cultured in an incubator (37˚C, 5% CO_2) for 6 h until cell adherence. K562 cells (concentrations of ADM used were 0.1, 0.2, 0.4, 0.6 and 0.8 μmol/ml) and K562/ADM cells (concentrations of ADM used were 1, 2, 4, 6 and 8 μmol/ml) were placed in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences), respectively for incubation in a 37˚C incubator (Heal Force Bio-Meditech Holdings Group) with 5% CO_2 for 48 h, with 3 duplicated wells set for each group. After discarding the supernatant, 90 μl fresh medium and 20 μl of 5 g/l MTT solution were added to the cells for 4 h of culture. Following the removal of the supernatant, 150 μl dimethyl sulphoxide (DMSO) solution (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well, followed by shaking for 10 min at a low speed on a shaking table. The optical density (OD) value was detected at 490 nm wavelength using a microplate reader (Shenzhen Rayto Life Science Co., Ltd.). The cell survival rate was calculated as follows: Cell survival rate=(OD value in the experiment group/mean OD value in the control group) x100% (17). The half maximal inhibitory concentration (IC_{50}) was calculated by mid-efficacy analysis (Logit method) with SPSS 11.0 software (SPSS Inc.) (18). The detection of the proliferation of K562 cells overexpressing miR-145 and of the K562/ADM cells with a low expression of miR-145 was performed as described above.

Colony formation assay. Following treatment of the K562 cells (concentrations of ADM used were 0.1, 0.2, 0.4, 0.6 and 0.8 μmol/ml) and K562/ADM cells (concentrations of ADM used were 1,2,4,6 and 8 μmol/ml), and culture medium removal, the adherent cells were then rinsed with phosphate-buffered saline (PBS) once, and detached with 0.25% trypsin [without ethylene diamine tetraacetic acid (EDTA)]. The cells were then triturated into a single cell suspension and suspended in complete medium, and 200 cells/well were inoculated into a 6-well plate. The plate was shaken gently, so that the cells were evenly dispersed and were cultured for 1-2 weeks. When cell clones (>50) were visible to the naked eye, following cultivation termination and culture medium removal, the cells were washed 3 times with PBS and fixed 10 min with anhydrous methanol. Subsequently, the cells were stained with Giemsa solution (Sigma-Aldrich; Merck KGaA) for 15 min, and the dying solution was washed away slowly with running water and the cells were dried and photographed under a microscope (Leica, AF6000, Leica Microsystems GmbH). The experiment was repeated 3 times. Under the microscope (low power microscope) (AF6000, Leica Microsystems GmbH), the colonies with >10 cells were counted. The data are expressed as the means ± standard deviation.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. The ADM-treated K562 cells and K562/ADM cells, and the K562 cells in which miR-145 was intervened with, and the K562/ADM cells were detached with trypsin and collected to form a single cell suspension. Following centrifugation (4˚C, 300 x g, 5 min) and supernatant removal, cells were washed by pre-cooled PBS twice, resuspended in the binding buffer and reacted with 5 μl Annexin V working fluid and 1 μl PI working fluid (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min. Subsequently, the cells were mixed with 300 μl of binding buffer gently. Approximately 10,000 cells were measured using a flow cytometer (MoFloAstrios EQ, Beckman Coulter, Inc.), and the percentage of AV^+ (apoptotic cells) was then calculated. The experiment was repeated 3 times, The apoptotic rate was calculated by flow cytometry (FACS420, BD Biosciences). The data are expressed as the mean ± standard deviation.

To investigate the effects of the overexpression of miR-145 on the apoptosis of K562/ADM stem cells, approximately $2x10^8$ K562/ADM cells were resuspended in 600 μl magnetically activated cell separation (MACS) solution according to the requirements of magnetic bead sorting. The cells were then incubated with 200 μl Fc receptor (FcR) blocker to block FcR on the cell surface, and 200 μl $CD34^+$ Multisort MicroBeads (Miltenyi Biotec) and 10 μl CD38-FITC antibodies were incubated with the cells in a dark room at 4-8˚C for 10 min. Following centrifugation and supernatant removal, the cells were resuspended in 500 μl MACS solution, and stem cells were sorted and collected by magnetic bead separation column in a magnetic field. Subsequently, the cells were resuspended with 1 ml MACS solution, and cultured with 20 μl Multisort releasing agent at 4˚C for 10 min. Following centrifugation (300 x g, 4˚C for 5 min) and supernatant removal, the cells were suspended again in 40 μl MACS solution, and cultivated with 60 μl MACS Multisort terminating agent and 100 μl anti-FITC MicroBeads at 4˚C for 30 min. $CD34^+CD38$ stem cells were obtained by sorting with magnetic bead separation column in magnetic field. The obtained $CD34^+CD38$ stem cells were

transfected with miR-145 mimic for 24 h as described above, followed by apoptosis detection. Following centrifugation removal (4˚C, 400 x g, 5 min) and supernatant, the cells were incubated with 100 μl binding buffer, 1 μl Annexin V-FITC, and 2 μl PI at 4˚C for 30 min. The cell apoptotic rate was measured using a flow cytometer (FACS420, BD Biosciences). The experiment was repeated 3 times. The apoptotic rate was calculated by flow cytometry (FACS420, BD Biosciences). The data are expressed as the means ± standard deviation.

Acridine orange/ethidium bromide (AO/EB) double fluorescence staining. K562 cells and K562/ADM cells in each group were mixed with 2 μl AO/EB working fluid (Beijing Leagene Biotechnology Co., Ltd.) gently in each group. Following centrifugation at 4˚C, 500 x g for 3 min with the supernatant removed, the cells were resuspended with AO/EB buffer, and adjusted to (0.5-5)x10^6 cells/ml. The cells were then fully mixed with 1 μl AO/EB working fluid. Clean slides were taken and dripped with 5 μl cell suspension. The slides were then lightly covered and cells were examined under a fluorescence microscope [TS100 Nikon Instruments (Shanghai) Co., Ltd.]. The experiment was repeated 3 times.

Dual luciferase reporter gene assay. Bioinformatics software and the http://www.microrna.org/ website were utilized to predict the binding site of miR-145 to ABCE1, and the binding site of miR-145 to MRP1 was examined through the website, http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs. The 3'UTR sequences of ABCE1 and MRP1 containing the binding site of miR-145 were synthesized and the wild-type (WT) plasmid of ABCE1 and MRP1 3'UTR (ABCE1-WT and MRP1-WT) was constructed. On the basis of ABCE1-WT and MRP1-WT, the mutant type (MUT) plasmid of ABCE1 3'UTR (ABCE1-MUT) and MRP1 3'UTR (MRP1-MUT) was constructed by the mutation binding site. The constructed plasmids were then mixed with miR-145 mimic NC and miR-145 mimic plasmids, respectively, and transfected into 293T cells (Cell Bank, Shanghai Institute of Life Sciences, Chinese Academy of Sciences), cultured in DMEM containing 10% FBS, 1% Glutamax and 1% penicillin-streptomycin at 37˚C with 3% CO_2 and 95% relative humidity. The cells were collected and lysed using Passive lysis buffer (E1941, Promega Corp.) at 48 h following transfection. Luciferase activity was measured using a luciferase detection kit (BioVision, Inc.) and Glomax 20/20 luminometer fluorescence detector (Promega Corp.). The experiment was repeated 3 times. The relative fluorescence expression was calculated by taking the dual luciferase value of the control group as 1 and the other groups as multiple of the control group.

Statistical analysis. SPSS 21.0 (IBM Corp.) was used for data analysis. The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed. The results are presented as the means ± standard deviation. Comparisons between 2 groups were analyzed using a t-test, and comparisons among multiple groups were analyzed by one- or two-way analysis of variance (ANOVA); pairwise comparisons after ANOVA were conducted with Tukey's multiple comparisons test. The P-values obtained were two-tailed and a value of P<0.05 was considered to indicate a statistically significant difference.

Table II. IC_{50} values of ADM in K562 cells and K562/ADM cells.

Time (h)	K562 cells (μmol/l)	K562/ADM cells (μmol/l)
12	2.48±0.34	56.34±0.51
24	1.13±0.29	48.92±4.79
48	0.21±0.03	14.98±1.22

IC_{50}, half maximal inhibitory concentration; ADM, adriamycin.

Results

K562/ADM cells exhibit potent drug resistance. The drug resistance of K562/ADM cells was examined with K562 cells as the reference. K562 and K562/ADM cells were treated with increasing concentrations of ADM for different periods of time. The results revealed that ADM suppressed the growth of K562/ADM and K562 cells in varying degrees. The cytotoxicity induced by ADM was dose- and time-dependent in both cell lines. Compared with the K562 cells, the K562/ADM cells were more resistant to ADM (P<0.05) (Fig. 1A and Table II).

The results revealed that the number of cell colonies of K562/ADM cells significantly decreased with the increasing ADM concentration (Fig. 1B), and the levels of MRP1 and P-gp in the K562/ADM cells were notably higher than those in the parental drug-sensitive K562 cells (Fig. 1C) (all P<0.05).

miR-145 is downregulated in K562/ADM cells. RT-qPCR was used to detect miR-145 expression in K562/ADM and K562 cells, and it was demonstrated that miR-145 expression was at a lower level in the K562/ADM cells than in the K562 cells (P<0.05) (Fig. 2A). To further examine the effect of miR-145 on K562/ADM cells, mimic NC and miR-145 mimic were transfected into the K562/ADM cells, respectively. miR-145 expression was detected following transfection, and the results revealed a significant increase in miR-145 expression in the miR-145 mimic group as compared with the NC group (P<0.05) (Fig. 2B), indicating successful transfection.

Overexpression of miR-145 enhances the sensitivity of K562/ADM cells to ADM. Following transfection, the K562/ADM cells further treated with various concentrations of ADM. The results demonstrated that the cells in each group exhibited a dose dependence on ADM. Compared with the K562/ADM group, there was no notable difference in cell proliferation in the mimic NC group (P>0.05); however, the miR-145 mimic group exhibited a decreased cell proliferation (P<0.05). In addition, when the ADM concentration was 6 μmol/l, cell viability was >50%, and cell viability was <50% when the ADM concentration was 8 μmol/l (Fig. 3A). Therefore, the ADM concentration of 6 μmol/l was selected for data determination in subsequent experiments.

Following treatment with ADM at a concentration of 6 μmol/l, no significant difference was observed between the

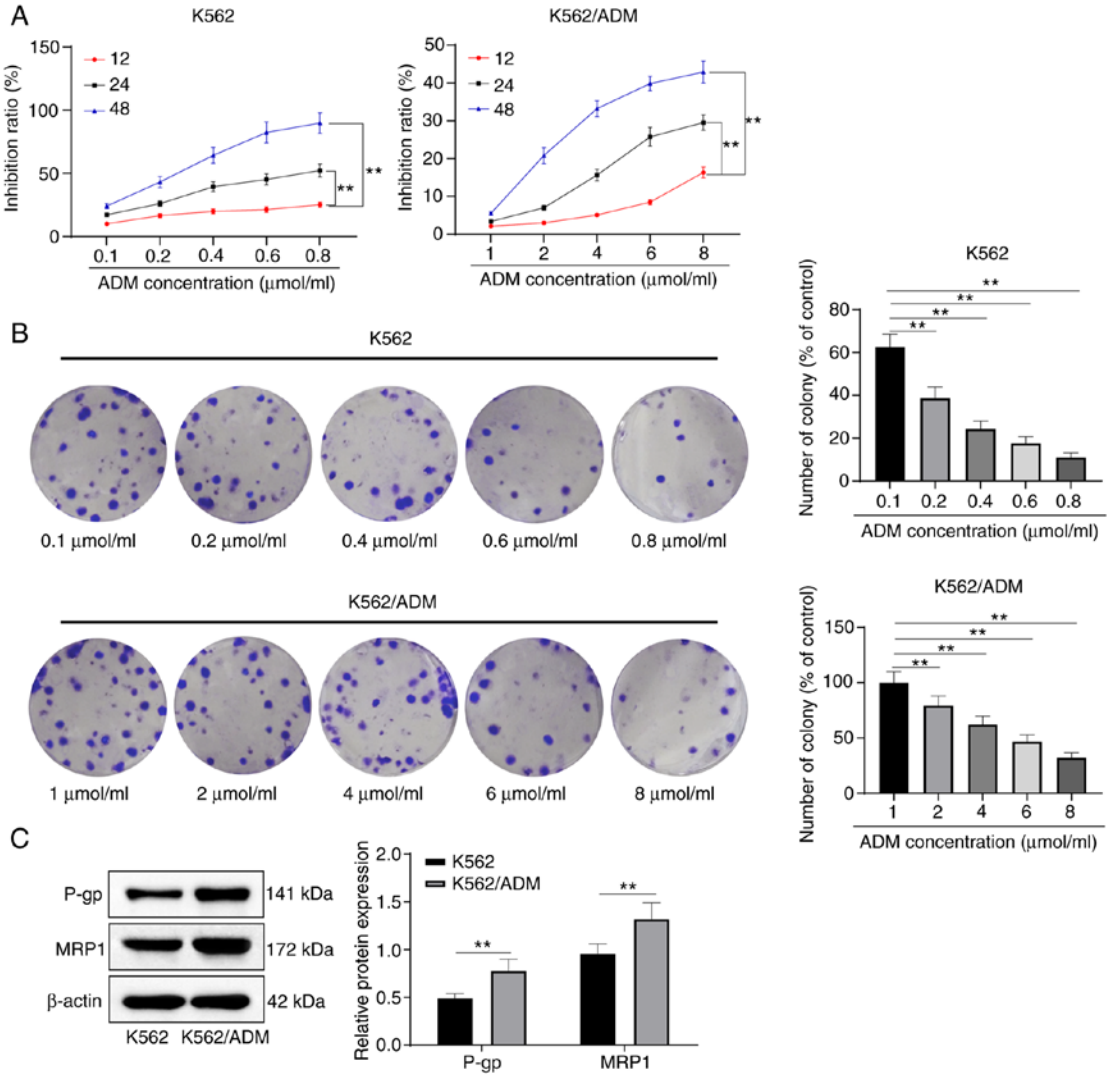

Figure 1. K562/ADM cells exhibit potent drug resistance. (A) Inhibition ratio of K562 cells and K562/ADM cells with various concentrations of ADM at 12, 24 and 48 h detected by MTT assay. (B) Maps of cell colonies of K562 and K562/ADM cells treated with various concentrations of ADM detected by colony formation assay. (C) Levels of MRP1 and P-gp in K562 cells and K562/ADM cells detected by western blot analysis; n=3. **P<0.01. Data in (A and C) were analyzed by two-way ANOVA, while data in (B) were analyzed with one-way ANOVA. Tukey's multiple comparisons test was applied as a post hoc test. ADM, adriamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; ANOVA, analysis of variance.

K562/ADM group and the NC group as regards the number of cell colonies, apoptosis rate and protein levels of MRP1 and P-gp (all P>0.05). Compared with the NC group, the number of cell colonies and the levels of MRP1 and P-gp in the miR-145 mimic group were decreased, while the apoptotic rate increased significantly (all P<0.05), and the majority of nuclear chromatins of the cells were orange-red in color with a solid or bead-like shape (Fig. 3B-E). This suggested that the overexpression of miR-145 enhanced the sensitivity of drug-resistant K562/ADM cells to ADM.

Overexpression of miR-145 promotes the apoptosis of K562/ADM stem cells. $CD34^+CD38^-$K562/ADM stem cells were sorted from K562/ADM stem cells using magnetic bead sorting methods. Flow cytometry verified that the purity of the $CD34^+CD38^-$K562/ADM stem cells was 94.69±1.93% (this can be seen from $CD34^+$ $CD38^-$ in the lower right quadrant of the flow chart after sorting) (Fig. 4A). To examine the effects of the overexpression of miR-145 on K562/ADM stem cells, flow cytometry was utilized to detect the apoptotic rate of the K562/ADM stem cells. It was revealed that the overexpression of miR-145 decreased the apoptotic rate of the K562/ADM stem cells (P<0.05) (Fig. 4B). The results demonstrated that the overexpression of miR-145 promoted the apoptosis of stem cells.

Low miR-145 expression reduces the sensitivity of K562 cells to ADM. To examine the effects of miR-145 on K562 cells, miR-145 inhibitor and inhibitor NC were transfected into the K562 cells, respectively. RT-qPCR was performed to detect miR-145 expression in each group and it was found miR-145 expression in the miR-145 inhibitor group was notably decreased as compared to the inhibitor NC group (P<0.05) (Fig. 5A). To verify the effects of a low miR-145 expression on the sensitivity of K562 cells to ADM, the cells in each group were treated with various concentrations of ADM. It was

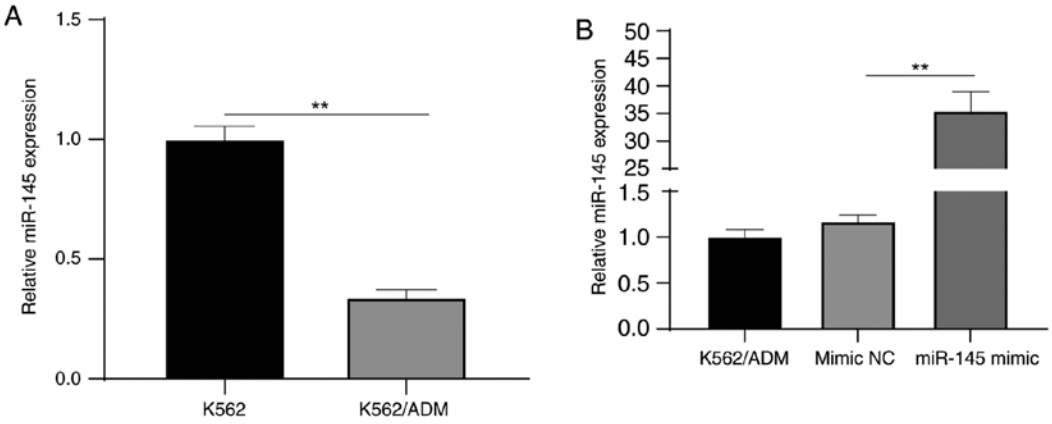

Figure 2. miR-145 expression is downregulated in K562/ADM cells. (A) Relative miR-145 expression in K562/ADM cells and K562 cells detected by RT-qPCR. (B) Relative miR-145 expression following transfection detected by RT-qPCR. **P<0.01; n=3. Data in (A) were analyzed by an independent t-test, while data in (B) were analyzed with one-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ANOVA, analysis of variance.

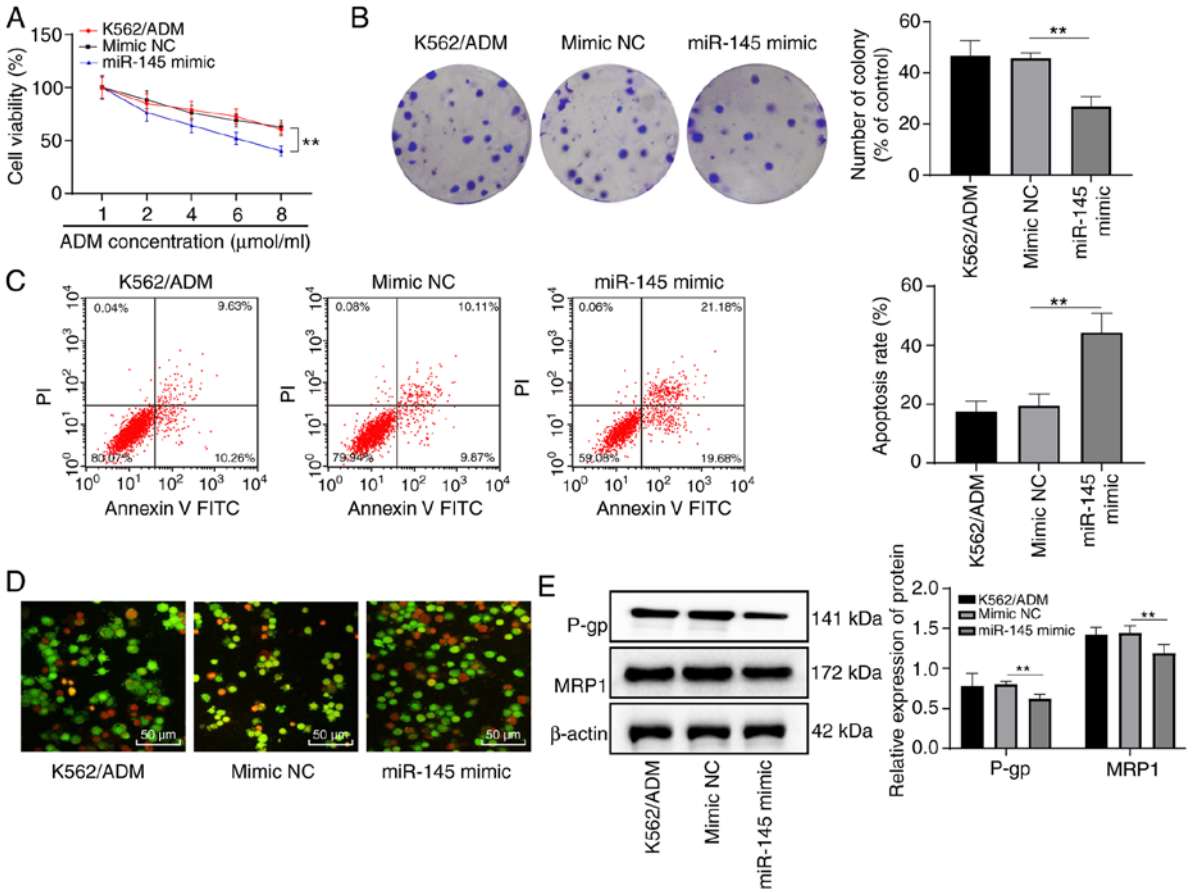

Figure 3. Overexpression of miR-145 enhances the sensitivity of K562/ADM cells to ADM. (A) Relative cell viability in each group treated with various concentrations of ADM detected by MTT assay. (B) Relative cell colonies in K562/ADM cells with 6 μmol/l ADM measured using colony formation assay. (C) Relative apoptosis of K562/ADM cells treated with 6 μmol/l ADM measured by flow cytometry. (D) The morphology and apoptosis of K562/ADM cells treated with 6 μmol/l ADM observed under a fluorescence microscope. (E) Levels of MRP1 and P-gp of K562/ADM cells treated with 6 μmol/l ADM measured by western blot analysis. **P<0.01; n=3. Data in (A and E) were analyzed by two-way ANOVA, while data in (B and C) were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; ANOVA, analysis of variance.

revealed that with the increase in the ADM concentration, cell proliferation in each group was inhibited (P<0.05), although no significant difference was observed in cell viability at each concentration between the K562 group and the NC group (P>0.05). Compared with the NC group, K562 cell proliferation was significantly increased with the low expression of miR-145 (P<0.05). In addition, when the ADM concentration was 0.6 μmol/l, cell viability was >50% (Fig. 5B). Therefore,

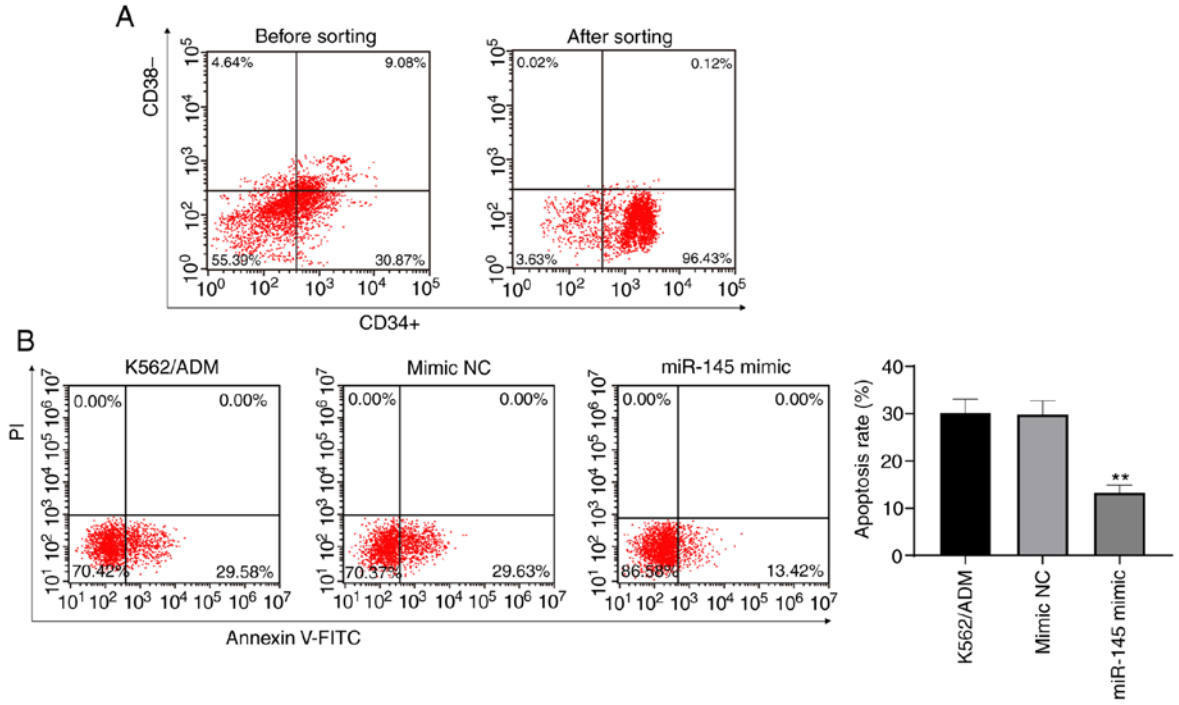

Figure 4. Overexpression of miR-145 promotes the apoptosis of stem cells. (A) $CD34^{+}CD38^{-}$K562/ADM stem cells were sorted from K562/ADM stem cells using magnetic bead sorting methods. (B) Cell apoptosis was detected by flow cytometry. $^{**}P<0.01$; n=3. Data were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145.

the ADM concentration of 0.6 μmol/l was selected for use in subsequent experiments.

Following treatment with ADM at a concentration of 0.6 μmol/l, no significant difference was observed between the K562 group and the NC group as regards the number of cell colonies, apoptotic rate, and the protein levels of MRP1 and P-gp (all P>0.05). Compared with the NC group, the number of cell colonies, and the levels of MRP1 and P-gp in the miR-145 inhibitor group were increased, while the apoptotic rate decreased significantly (all P<0.05), and cell morphology was normal (Fig. 5C-E). These results suggested that the low expression of miR-145 reduced the sensitivity of K562 cells to ADM.

miR-145 targets and inhibits the expression of ABCE1. As demonstrated above, the levels of the drug-resistant gene, MRP1, were altered following the intervention of miR-145 expression. Through website prediction, it was found that there was a binding sequence between miR-145 and MRP1, and their targeting association was verified by a dual luciferase reporter gene assay (Fig. 6A). In addition, the results of RT-qPCR revealed that the overexpression of miR-145 inhibited the mRNA expression of MRP1 (P<0.05) (Fig. 6B).

The binding of miR-145 to ABCE1 was predicted through http://www.microrna.org/. It was noted that there was a binding site between the 3' UTR of miR-145 and ABCE1. The results of dual-luciferase reporter gene assay verified the targeting association between miR-145 and ABCE1 (P<0.05) (Fig. 6A). The ABCE1 mRNA and protein levels were significantly decreased in the K562/ADM cells overexpressing miR-145, but were substantially increased following transfection with miR-145 inhibitor (all P<0.05) (Fig. 6B and C), indicating that miR-145 targeted ABCE1.

Activation of ABCE1 reduces the promoting effect of miR-145 on the sensitivity of K562/ADM cells to ADM. Compared with miR-145 overexpression alone, the combination of ABCE1 activation and miR-145 overexpression significantly increased K56/ADM cell viability, the number of colonies, the levels of MRP1 and P-gp, and reduced the apoptotic rate (all P<0.05) (Fig. 7), suggesting that ABCE1 activation reversed the promoting effects of miR-145 overexpression on K562/ADM cell sensitivity to ADM.

Discussion

There is only one feasible option, namely allogeneic hematopoietic stem cell transplant for patients afflicted with recurrent or refractory leukemia whose cure rates are almost 10% with current standard chemotherapeutic regimens (19). Recently, it has been shown that a majority of therapeutic failures are due to cell resistance to leukemic therapies (20). miRNAs have been confirmed to be involved in normal hematopoiesis, suggesting that the dysregulation of miRNAs may be a contributing factor to leukemogenesis (21). In the present study, the mechanisms of miR-145 and ABCE1 in the biological processes of drug resistance to leukemia were examined. Consequently, it was found that the overexpression of miR-145 promoted leukemic stem cell apoptosis and K562/ADM cell sensitivity to ADM by inhibiting ABCE1.

To select an appropriate ADM concentration, a pre-experiment was performed. According to a previous study (22), following ADM treatment (0.1, 0.2, 0.4, 0.6 and 0.8 μmol/ml), the activity of K562 cells was significantly inhibited (Fig. 1A, left panel). The K562/ADM cells were also treated with ADM (0.1, 0.2, 0.4, 0.6, and 0.8 μmol/ml). It was found that ADM at

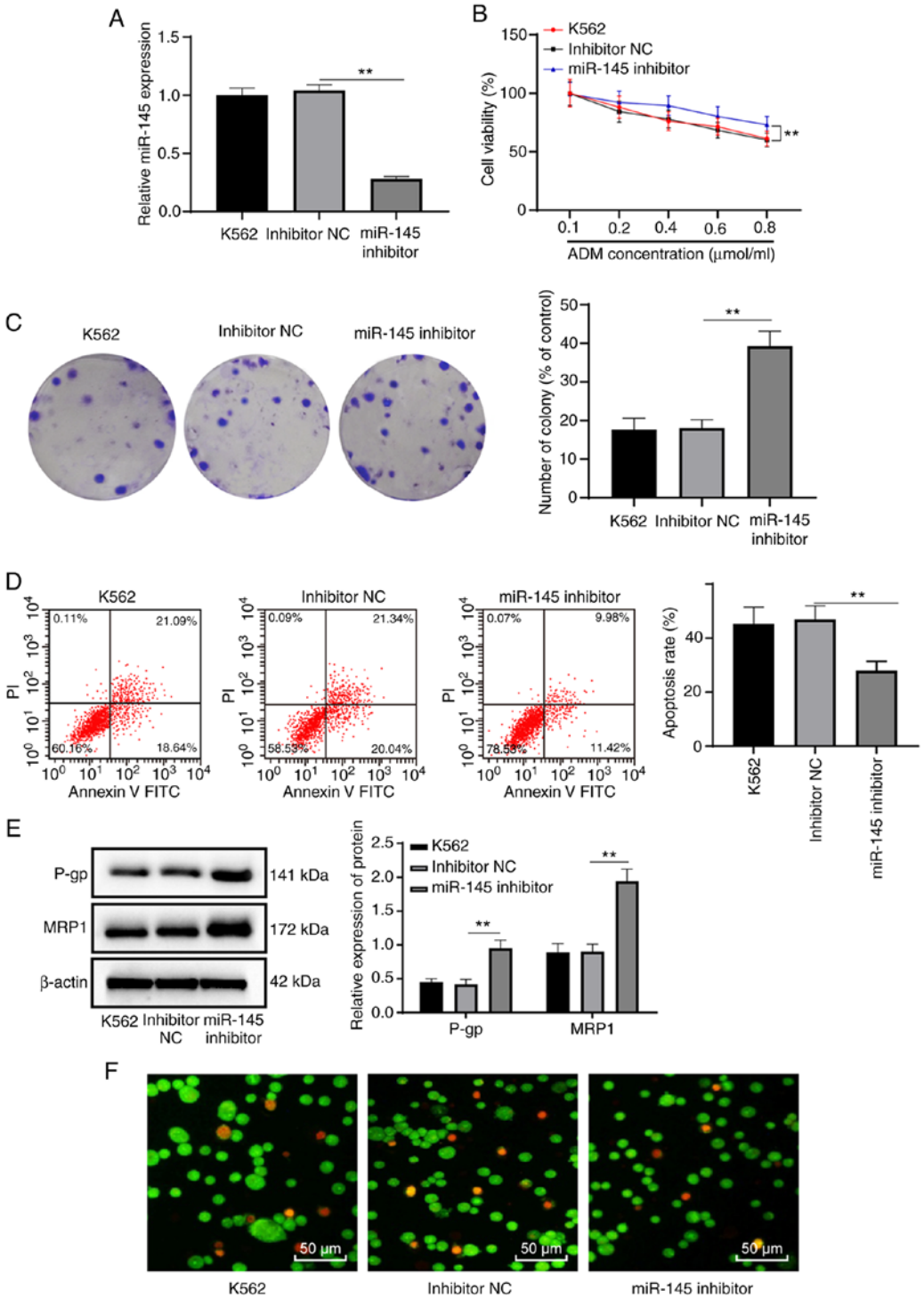

Figure 5. Low expression of miR-145 reduces the sensitivity of K562 cells to ADM. (A) Relative miR-145 expression in K562 cells detected by RT-qPCR. (B) Relative viability of K562 cells treated with various concentrations of ADM measured by MTT assay. (C) Relative cell colonies of K562 cells treated with 0.6 μmol/l ADM measured by colony formation assay. (D) Relative cell apoptosis of K562 cells treated with 0.6 μmol/l ADM measured by flow cytometry. (E) Levels of MRP1 and P-gp of K562 cells treated with 0.6 μmol/l ADM measured by western blot analysis. (F) The morphology and apoptosis of K562 cells treated with 0.6 μmol/l ADM were observed under a fluorescence microscope. **P<0.01; n=3. Data in (A, C and D) were analyzed by one-way ANOVA, while data in (B and E) were analyzed by two-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145; RT-qPCR, reverse transcription quantitative polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; ANOVA, analysis of variance.

this concentration gradient exerted minimal inhibitory effects on the K562/ADM cells (Fig. S1). Thus, according to a previous study (23), it was found that the difference between the ADM treatment concentration of the K562 and K562/ADM cells was approximately 10-fold. Therefore, the present study used (1, 2, 4, 6, 8 μmol/ml) ADM to treat the K562/ADM cells, and it was found that the activity of the K562/ADM cells was significantly inhibited (Fig. 1A, right panel). Finally, ADM was used at 0.1, 0.2, 0.4, 0.6 and 0.8 μmol/ml to treat the K562 cells, and ADM was at 1, 2, 4, 6 and 8 μmol/ml to treat the K562/ADM cells.

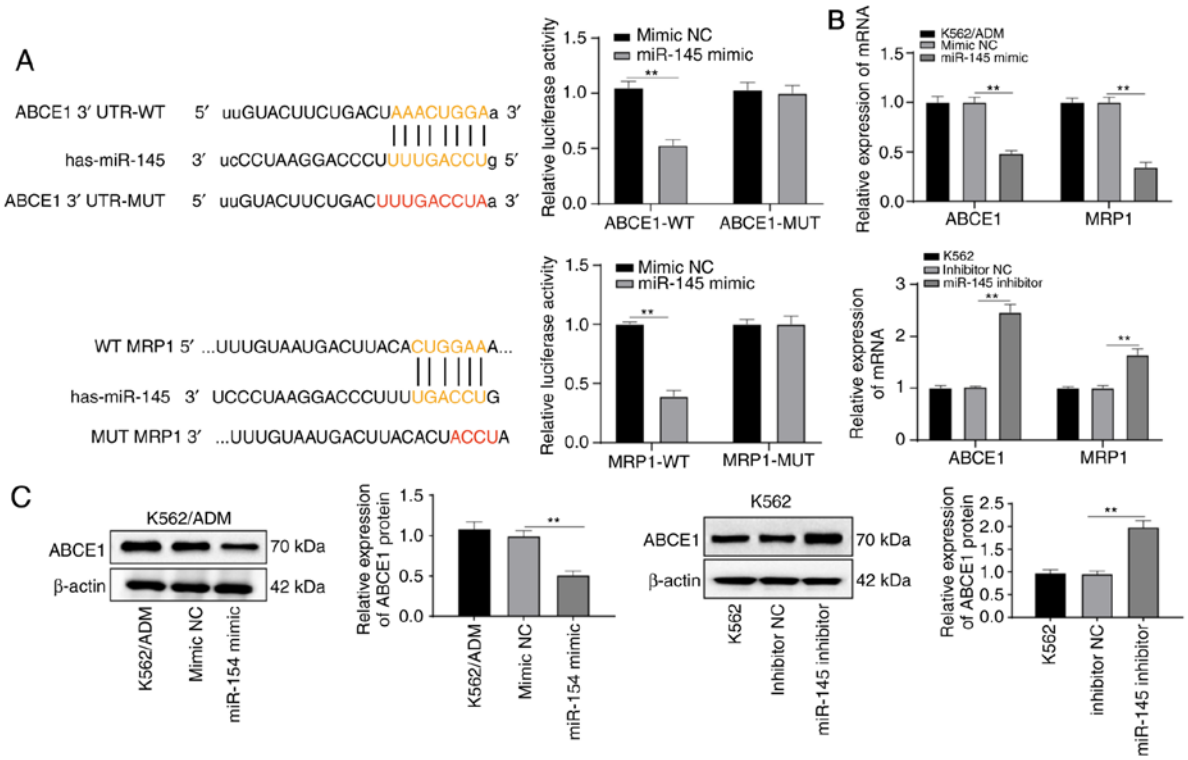

Figure 6. miR-145 targets MRP1 and ABCE1. (A) Binding sites between miR-145 and ABCE1, and between miR-145 and MRP1 predicted by bioinformatics software and website, and relative ABCE1 and MRP1 luciferase activity measured by dual-luciferase reporter gene assay. (B) Relative mRNA expression of ABCE1 and MRP1 detected by RT-qPCR. (C) Relative protein expression of ABCE1 detected by western blot analysis. **P<0.01; n=3. Data in (A and B) were analyzed by two-way ANOVA, and data in (C) were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145; MRP1, multidrug resistance protein 1; ABCE1, adenosine triphosphate (ATP)-binding cassette (ABC) transporter E1.

ADM, also known as doxorubicin, is considered a potent drug for the treatment of childhood with acute lymphoblastic leukemia (7). P-gp (also known as ABCB1) and MRP1 (also known as ABCC1), two major ABC transportation proteins grant resistance to a number of anticancer agents, lead to multidrug resistance (24). The first major result if the present study was that K562/ADM cells were more resistant to ADM, presenting with less cell colonies and cell growth inhibition with the increasing ADM concentrations, and markedly higher levels of MRP1 and P-gp. As an important chemotherapeutic drug with a variety of cellular targets, ADM is frequently used in combination therapies for leukemia, multiple myeloma and solid tumors, and it can also stimulate the differentiation of K562 cells (23). A previous study revealed that high levels of MDR1 and P-gp were associated with chemotherapy sensitivity, high-grade tumors, lymph node metastasis, a poor response and a shorter survival (25). Ge *et al* stated that ADM induced the overexpression of P-gp in breast cancer cells, which, in turn, increased the intracellular efflux of ADM (26). The present study further highlighted that K562/ADM cells were more resistant to ADM, which may provide new insight into leukemic therapies.

A previous study found that miRNAs are important for the drug resistance of leukemia cells (K562/ADM) (18). It was then found miR-145 was downregulated in K562/ADM cells. miR-145 was identified as a tumor-suppressor and to be downregulated in several types of cancer, such as glioma, lung cancer, colon cancer, breast cancer and gastric cancer (27). Similarly, miR-145 expression has been shown to be significantly decreased in A549/cisplatin cells when compared with A549 cells (28). The decreased expression of miR-145 in hematopoietic stem cells contributes to an increased platelet count in blood and the abnormal development of megakaryocytes (12). Additionally, the present study indicated that the overexpression of miR-145 suppressed proliferation and accelerated the apoptosis of K562/ADM cells, markedly decreasing the levels of MRP1 and P-gp, and enhancing the sensitivity of K562/ADM cells to ADM. miR-145 overexpression has also been shown to suppress cell proliferation and facilitate the apoptosis of human esophageal carcinomas cells (29). Xia *et al* found that the overexpression of miR-145 inhibited adult T-cell leukemia/lymphoma cell proliferation and growth (13). Similarly, a high expression of miR-145 has been shown to enhance breast cancer cell sensitivity to ADM via intracellular ADM accumulation and MRP1 inhibition (30). CD38, an antigen present on the surface of human cells, is a type II multifunctional transmembrane glycoprotein broadly distributed in hematopoietic cells, and its expression is used as a phenotypic marker for the proliferation and activation of T and B lymphocytes (31). Furthermore, non-thorough chemotherapeutic obliteration of $CD34^{+}CD38^{-}$ stem cells is prone to leukemia relapse (32). In the present study, the number of $CD34^{+}CD38^{-}$ subsets decreased markedly and the apoptosis of leukemic stem cells was promoted following the overexpression of miR-145. Yalçintepe *et al* considered that CD38

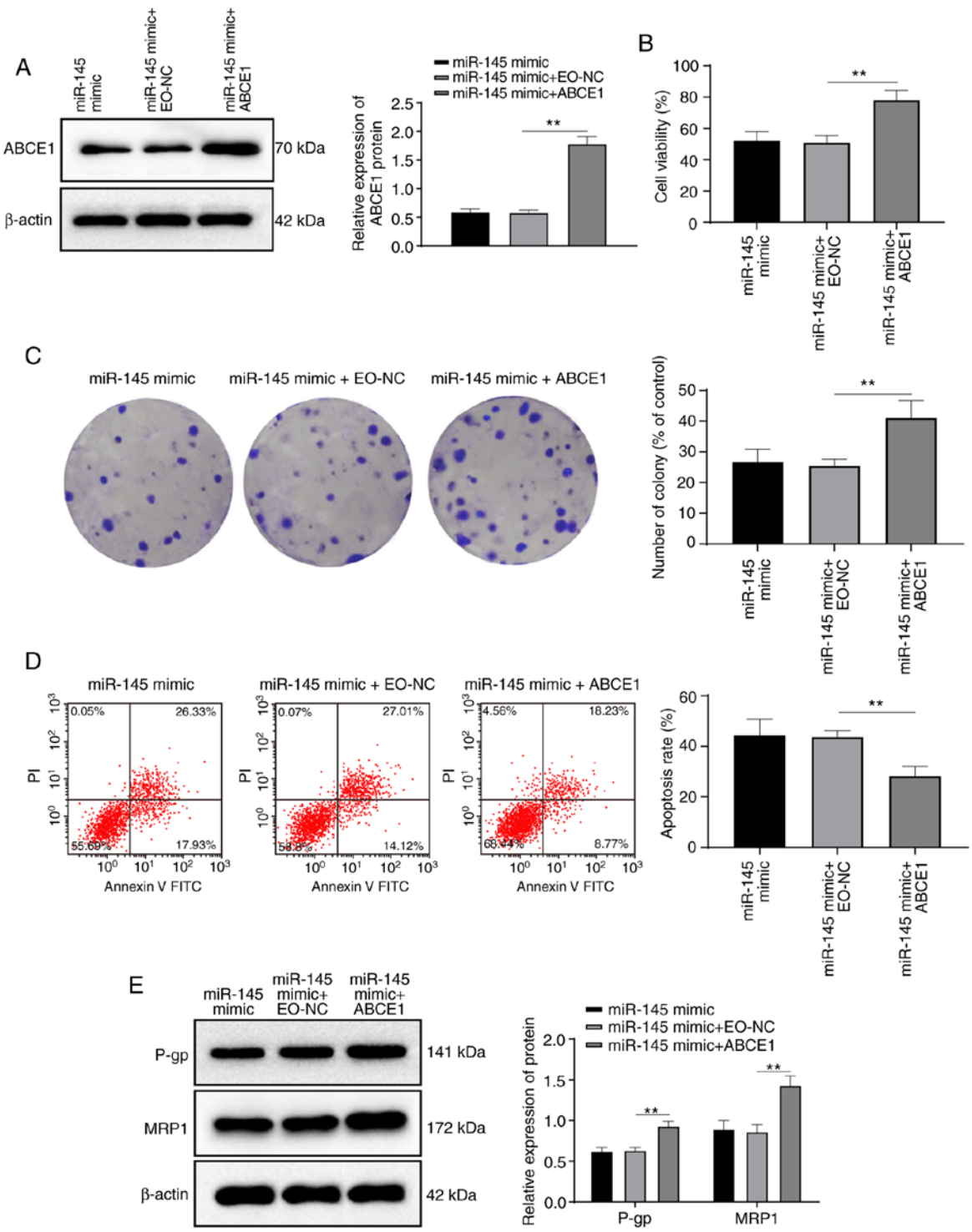

Figure 7. ABCE1 activation reverses the promoting effects of miR-145 overexpression on K562/ADM cell sensitivity to ADM. (A) Relative protein expression of ABCE1 detected by western blot analysis. (B) Relative cell viability in each group detected by MTT assay. (C) Relative cell colonies in K562/ADM cells treated with 6 μmol/l ADM measured by colony formation assay. (D) Relative apoptosis of K562/ADM cells treated with 6 μmol/l ADM measured by flow cytometry. (E) Levels of MRP1 and P-gp of K562/ADM cells with 6 μmol/l ADM measured by western blot analysis. **P<0.01; n=3. Data in (A-D) were analyzed by one-way ANOVA, and data in (E) were analyzed by two-way ANOVA. Tukey's multiple comparisons test was used as a post hoc test. ADM, adriamycin; miR-145, microRNA-145; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein.

may play an essential role in the process of drug resistance to ADM in K562 cells (33).

Moreover, in the present study, experiments using the K562 cells revealed that a low expression of miR-145 increased cell proliferation, decreased cell apoptosis, and increased the levels of MRP1 and P-gp, thus reducing the sensitivity of K562 cells to ADM. A previous study conducted by Ikemura *et al* found that miR-145 negatively regulated the expression and functions of P-gp by direct interaction with MRP1, and the downregulated miR-145 elevated P-gp expression following liver ischemia-reperfusion injury (34). miR-145 has also been shown to increase the sensitivity of esophageal squamous cell

carcinoma to cisplatin, and to facilitate ccisplatin-induced apoptosis to decrease MRP1 and P-gp expression (35). MRP1 protein expression in leukemic stem cells is a negative prognostic marker in acute myeloid leukemia patients (36). Through bioinformatics prediction and dual-luciferase reporter gene assay, in the present study, it was verified that miR-145 targeted ABCE1 and MRP1. miR-145 suppressed MRP1 expression by targeting MRP1 3'-UTR, and miR-145 overexpression sensitized breast cancer cells to ADM by inducing intracellular ADM accumulation via MRP1 inhibition (30). ABC transportation genes exhibit a high expression in hematopoietic stem cells; thus, they may play roles in the pathogenesis of stem cell-derived leukemia (37). The overexpression of ABC family members may lead to chemotherapy failure (33). Additionally, as observed in the present study, the activation of ABCE1 reduced the promoting effects of miR-145 on the sensitivity of drug-resistant K562/ADM cells to ADM. ABCE1 has been found to be overexpressed in drug-resistant cancer cells, and ABCE1 silencing enhances the sensitivity of lung cancer A549 cells to 5-Fluorouracil (38). Thus, the effects of a high miR-145 expression on ADM sensitivity in leukemia cells may be achieved via the inhibition of ABCE1 and MRP1.

In conclusion, the present study demonstrated that the overexpression of miR-145 promoted leukemic stem cell apoptosis and enhanced the sensitivity of K562/ADM cells to ADM by inhibiting ABCE1. These results reveal a potential target for cell resistance to leukemic therapies. Due to the limitations of the experimental conditions and costs, the present study did not explore the other effects of miR-145 on leukemic stem cells, apart from apoptosis. Another limitation of the present study is that normal, non-cancerous cells were not used as a negative control. If the experimental conditions permit in the future, the authors aim to explore the effects of miR-145 on other characteristics of leukemic stem cells. Although the present findings provide insight into the treatment of leukemia, the experimental results and effective application into clinic practice warrant further in-depth validation.

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Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Authors' contributions

ZW and HW are the guarantees of the integrity of the entire study and all authors (ZW, HW, QS, HZ, MH, ST, LX, YC and XH) contributed to the study concept and the design and definition of the intellectual content of this study. HZ, LX, YC and MH contributed to the experimental studies, data acquisition and statistical analysis. ZW contributed to the preparation of the manuscript. XH contributed to the manuscript review. All authors read and approved the final 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.

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