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MicroRNA-204 Potentiates the Sensitivity of Acute Myeloid Leukemia Cells to Arsenic Trioxide

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Although arsenic trioxide (ATO) is a well-known antileukemic drug used for acute promyelocytic leukemia treatment, the development of ATO resistance is still a big challenge. We previously reported that microRNA-204 (miR-204) was involved in the regulation of acute myeloid leukemia (AML) cell apoptosis, but its role in chemoresistance is poorly understood. In the present study, we showed that miR-204 was significantly increased in AML cells after ATO treatment. Interestingly, the increased miR-204 level that was negatively correlated with ATO induced the decrease in cell viability and baculoviral inhibition of apoptosis protein repeat-containing 6 (BIRC6) expression. Overexpression of miR-204 potentiated ATO-induced AML cell growth inhibition and apoptosis. Furthermore, miR-204 directly targets to the 3'-UTR of BIRC6. Upregulation of miR-204 decreased BIRC6 luciferase activity and expression, which subsequently enhanced the expression of p53. Restoration of BIRC6 markedly reversed the effect of miR-204 on the regulation of AML cell sensitivity to ATO. Taken together, our study demonstrates that miR-204 decreases ATO chemoresistance in AML cells at least partially via promoting BIRC6/p53-mediated apoptosis. miR-204 represents a novel target of ATO, and upregulation of miR-204 may be a useful strategy to improve the efficacy of ATO in AML treatment.

Key words: Acute myeloid leukemia (AML); Arsenic trioxide (ATO); miR-204; Apoptosis; BIRC6

INTRODUCTION

Leukemia is a malignant disorder of hematopoietic precursor cells and stem cells¹. These cells escape into the blood and accumulate in a large number, leading to the clinical presentations of the disease². Acute myeloid leukemia (AML) is a highly heterogeneous clonal disorder, characterized by rapid proliferation of leukemia blasts and impaired differentiation of hematopoietic progenitor cells³. Since the advanced healthcare systems, the overall 5-year survival for leukemia patients has reached 70%⁴. However, in the case of AML, this is only 25%, just in the US, with more than 10,000 estimated deaths in 2016⁴. Although the World Health Organization categorization of AML mentioned that molecular analysis is of great importance to predict the survival rates of AML⁵, it is still difficult to estimate accurately the prognosis of

AML patients due to drug resistance^{6,7}. Thus, it is necessary to develop more effective treatment to increase the sensitivity of AML cells to chemotherapy.

Arsenic trioxide (ATO) is a common antileukemic drug widely used for acute promyelocytic leukemia (APL) treatment⁸. It effectively induces APL cell differentiation and apoptosis^{9,10}. However, a clinical study has shown that AML patients treated with ATO frequently acquire drug resistance^{11,12}. Although several factors have been reported to contribute to ATO resistance, such as PI3K/AKT pathway activation and specific chromosomal translocations^{13,14}, the mechanisms of ATO resistance are still not yet fully understood.

MicroRNAs (miRNAs) are a class of endogenous, small, and noncoding RNAs with around 22 nucleotides in length, which posttranscriptionally regulate the target

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genes expression by binding to the 3'-untranslated region (3'-UTR)^{15,16}. Increasing lines of evidence have reported that several miRNAs are important regulators in chemoresistance^{15,17}. miR-204 plays a key role in the development of several malignant tumors, including prostate cancer¹⁸, ovarian cancer¹⁹, gastric cancer²⁰, and colorectal cancer²¹. Our previous study reported that miR-204 level was decreased in clinical AML samples and regulated AML cell apoptosis¹⁶. However, the effect of miR-204 on chemoresistance of AML cells remains unknown. In this study, we found that miR-204 increases the sensitivity of AML cells to ATO treatment by targeting baculoviral inhibition of apoptosis protein repeat-containing 6 (BIRC6), a gene known to be involved in regulating cell apoptosis, suggesting that miR-204 is a promising target for chemoresistance treatment.

MATERIALS AND METHODS

Materials and Reagents

RPMI-1640 medium, fetal bovine serum (FBS), gentamicin, TRIzol reagent, and Lipofectamine 3000 were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against BIRC6, p53, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). miR-204 mimics, miRNA mimics negative control, and miR-204 and U6 primers were from Rio Biotechnology (Guangzhou, P.R. China). Stock solution of ATO prepared in 1 mol/L NaOH was obtained from Sigma-Aldrich (St. Louis, MO, USA) and diluted in phosphate-buffered saline (PBS) to a concentration of 10 mmol/L.

Patients and Specimen Preparation

The primary AML samples were collected from patients with AML before and after ATO treatment between 2010 and 2015 in the Department of Hematology of the First Affiliated Hospital of Xi'an University. The samples from 48 cases of AML were selected based on the follow-up visits: 1) subjects with a good response to ATO (10 mg daily until remission then 10 mg for 2 weeks every 2 months), who were given as a prolonged outpatient maintenance course over 2 years; 2) subjects received no other chemotherapies. The research protocols were approved by the ethics committee of Xi'an University and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from all individual participants included in the study.

Cell Culture

Human AML cell lines, AML-5 and HL-60, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium containing 10% FBS and 50 µg/ml gentamicin in a humidified incubator with 5% CO₂ at 37°C.

Cell Viability Assay

The viability of AML-5 or HL-60 cells was measured by Cell Counting Kit-8 (CCK-8; Yiyuan Biotechnology, Guangzhou, P.R. China) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates (2×10^3 cells/well) and rendered quiescent by replacing the medium with 0.2% FBS for 24 h. After indicated treatment according to the experimental design, the cells were incubated with fresh medium containing 10 µl of CCK-8 reagent for 4 h at 37°C, 5% CO₂. The absorbance was read at 450–540 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Quantitative Real-Time PCR

Total RNA from AML cells was isolated using TRIzol reagent according to manufacturer's instructions and was reversed transcribed using the miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) or SuperScript III First-Strand Synthesis system (Qiagen, Valencia, CA, USA). For the determination of miR-204 level, cDNA was mixed with TaqMan[®] Micro Assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and amplified using 7500 Fast Real-Time PCR Systems (Applied Biosystems). To examine the mRNA expression of BIRC6, PCR reaction was performed using Fast SYBR[®] Green Master Mix kit (Applied Biosystems). The specific primer sequences of BIRC6 and GAPDH were synthesized and provided by the Shanghai Biological Engineering Technology Services Co. Ltd. (Shanghai, P.R. China): BIRC6 sense 5'-CCTGACTTCACTCCGGCTA-3' and antisense 5'-GAGCTGCTGTGCCTCTGTAA-3'; GAPDH sense 5'-GCCATCGTCACCAACTGGGAC-3' and antisense 5'-CGATTTCCCGCTCGGCCGTGG-3'. The mRNA level of target genes was normalized by U6 or GAPDH, and calculated using the $2^{-\Delta\Delta CT}$ method.

Cell Transfection

Human BIRC6 cDNA was purchased from Open BioSystems (Huntsville, AL, USA) and cloned into pSMCV expression vector (OpenBioSystems), which was confirmed by sequencing. AML-5 or HL-60 cells were transfected with miR-204 mimics (20 nmol/L), miRNA mimics negative control (20 nmol/L), BIRC6 plasmid or empty vector for 48 h using Lipofectamine 3000 according to the manufacturer's instruction.

Apoptosis Detection Assay

The apoptosis of AML cells was measured with the FITC-Annexin-V Apoptosis Detection kit (Beyotime Institute of Biotechnology, Shanghai, P.R. China) using FACS Caliber flow cytometry (Becton Dickinson, San Diego, CA, USA) according to the manufacturer's instruction. In brief, the cells were washed with PBS, centrifuged

at 1,000×g for 10 min, and then the cell pellets were resuspended and incubated with annexin V-FITC and propidium iodide (PI) for 20 min at room temperature. The stained cells were counted with flow cytometry and analyzed using WinMDI software (The Scripps Research Institute, La Jolla, CA, USA).

Cell Cycle Analysis

Distribution of cell cycle in AML cells was evaluated by flow cytometry. The cells were harvested by centrifugation at 1,000×g for 10 min at 4°C. The pellets were washed with cold PBS twice, and then fixed with 70% ethanol for 30 min on ice. Before flow cytometry analysis, the samples were incubated with 50 µg/ml of PI dissolved in PBS for 30 min at 37°C. Cellular DNA contents were analyzed using a Becton Dickinson FACScan flow cytometer. The subG₁, G₁ and S/G₂/M populations were quantified with the ModFit software program (Verity Software House, Topsham, ME, USA).

Western Blotting Analysis

Cells were washed with PBS and lysed in a lysis buffer containing 50 mM HEPES (Promocell, Heidelberg, Germany), 1% Triton X-100, and protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA). Cellular protein concentration from total cell lysates (20 µg) was quantified using a bicinchoninic acid kit (Bio-Rad, Hercules, CA, USA). Samples containing equal amounts of protein were electrophoresed on 6%–8% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in TBST (in mmol/L, 10 Tris-HCl, 150 NaCl, 0.05% Tween-20, pH 7.6) and probed with the indicated primary antibodies (1:1,000) at 4°C overnight. Then the membranes were washed with TBST three times and incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, Billerica, MA, USA) for 1 h. The membranes were exposed to enhanced chemiluminescence kit according the manufacturer's instructions (Beyotime Institute of Biotechnology). Image quantification was performed by ImageJ software (Version 1.41; NIH, Maryland, MD, USA).

Luciferase Assay

The 3'-UTR of BIRC6 (GenBank ID: NM_016252), which is predicted to contain the binding site for miR-204, was cloned into the pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). The mutant BIRC6 3'-UTR was constructed by substitution of 4 bp from the seed region of miR-204. The cells (1 × 10⁵) were cotransfected with BIRC6 3'-UTR or mutant BIRC6 3'-UTR and miR-204 mimics or miRNA mimics negative control. Forty-eight hours later, the cells were harvested, and the luciferase activity was assessed

using a dual-luciferase reporter system (Promega) according to the manufacturer's protocols.

Statistical Analysis

All data were given as mean value ± SEM; *n* value represents the number of independent experiments. The regression analysis between miR-204 level and cell viability or BIRC6 expression was determined by the Pearson correlation test. The statistical significance was analyzed by two-tailed Student *t*-test or one-way ANOVA, followed by the Bonferroni multiple comparison test using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

miR-204 Level Was Negatively Correlated With AML Cell Viability and BIRC6 Expression After ATO Treatment

To investigate the involvement of miR-204 in ATO sensitivity, we first examined the effect of ATO on miR-204 level. The result showed that ATO induced the level of miR-204 in a dose-dependent manner in AML-5 and HL-60 cells, respectively (Fig. 1A). On the contrary, the viability of AML-5 and HL-60 cells was gradually inhibited by ATO treatment (Fig. 1B). Moreover, ATO also decreased the mRNA expression of BIRC6 (Fig. 1C). Importantly, the increased miR-204 level was negatively correlated with cell viability and BIRC6 expression (Fig. 1D and E). Similar tendencies were also observed in primary AML samples from AML patients. After ATO treatment, miR-204 level was dramatically increased, but BIRC6 expression was inhibited (Fig. 1F). These results suggest that the increased miR-204 level may be involved in ATO-mediated inhibition of AML cell viability and BIRC6 expression.

miR-204 Upregulation Promoted ATO-Induced AML Cell Apoptosis

To further explore the functional role of miR-204 in regulating chemosensitivity of AML cells, we next tested the effects of miR-204 overexpression on cellular functions. Quantitative real-time PCR confirmed that miR-204 was successfully overexpressed after transfection with miR-204 mimics (Fig. 2A). miR-204 upregulation significantly enhanced ATO-induced decrease in AML-5 and HL-60 cell viability. Compared with the ATO group, the cell viability after miR-204 overexpression was decreased from 42.5% to 18.7% in AML-5 cells and from 49.2% to 23.3% in HL-60 cells, respectively (Fig. 2B). The Annexin V/PI double staining by flow cytometry showed that overexpression of miR-204 markedly potentiated ATO-induced apoptosis in AML-5 and HL-60 cells compared with the ATO group with an increase in the percentage of apoptotic cells from 19.9% to 38.3% and 17.8% to 30.8%, respectively (Fig. 2C and D). Furthermore, the effect of miR-204 overexpression

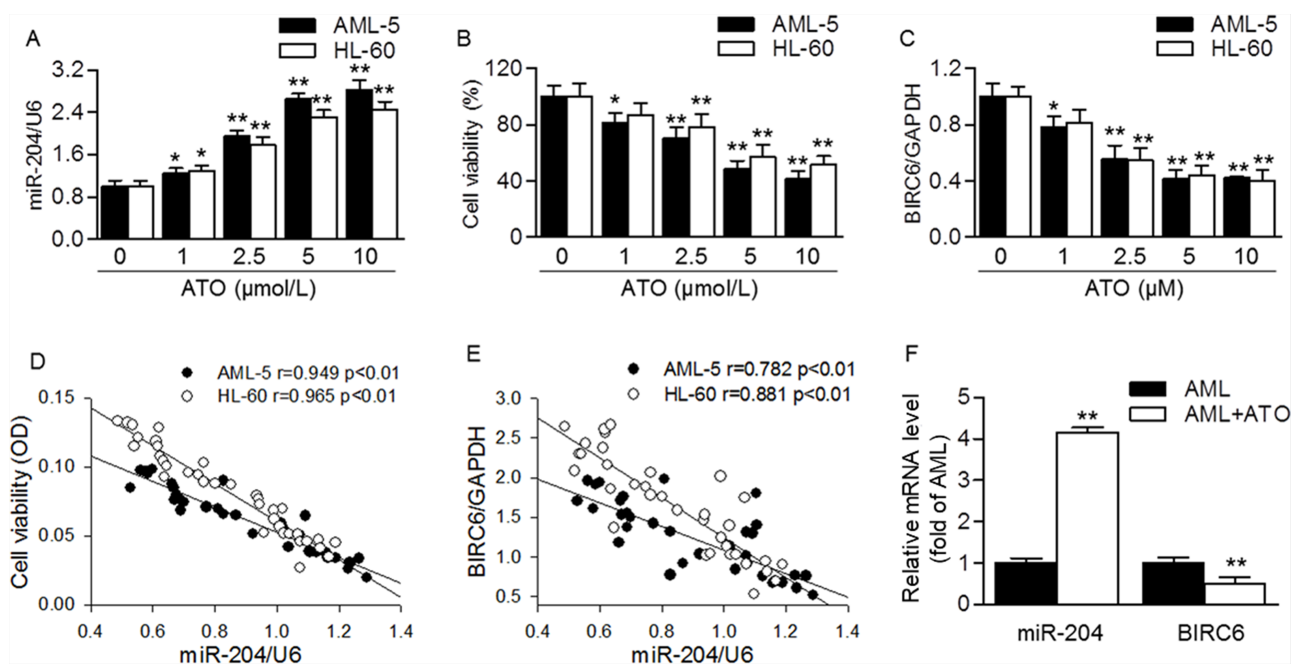


Figure 1. MicroRNA-204 (miR-204) level was negatively correlated with arsenic trioxide (ATO)-induced decrease in cell viability and baculoviral inhibition of apoptosis protein repeat-containing 6 (BIRC6) expression in acute myeloid leukemia (AML) cells. (A) AML-5 or HL-60 cells were treated with different concentrations of ATO (0, 1, 2.5, 5, or 10 $\mu\text{mol/L}$) for 48 h. The miR-204 level was determined by quantitative real-time PCR. (B) Cell viability was assessed by cell counting kit (CCK-8) assay. (C) The mRNA expression of BIRC6 was examined by quantitative real-time PCR. * $p < 0.05$; ** $p < 0.01$ versus 0 $\mu\text{mol/L}$, $n = 6-8$. (D, E) The cell viability (D) and BIRC6 expression (E) were negatively correlated with miR-204 level, respectively. (F) Forty-eight cases of AML patients with a good response to ATO were selected after 2-year outpatient maintenance, and then the RNA of AML samples was harvested. Quantitative real-time PCR analysis of miR-204 and BIRC6 mRNA levels in primary AML samples before and after ATO treatment. ** $p < 0.01$ versus AML (before ATO treatment).

on cell cycle distribution was also examined. The results revealed that the subG₁ population induced by ATO was further augmented in cells treated with miR-204 mimics, indicating the increase in apoptotic cells. In AML-5 and HL-60 cells, the subG₁ percentages in ATO-treated cells before or after miR-204 mimic transfection were 27.0% versus 40.5%, and 16.8% versus 38.2%, respectively (Fig. 2E and F). The data indicate that miR-204 increases ATO chemosensitivity via promoting cell apoptosis rather than inhibiting cell proliferation.

miR-204 Targeted to BIRC6 and Enhanced p53 Expression

Computational mRNA target analysis by bioinformatic software Targetscan shows that miR-204 contains a potential binding site in BIRC6. A 6-bp fragment of BIRC6 3'-UTR is complementary to the miR-204 seed sequence (Fig. 3A). To confirm whether miR-204 directly binds to BIRC6 and inhibits its translation, we upregulated miR-204 level and measured its effect on BIRC6 3'-UTR luciferase activity. By cotransfection with miR-204 mimics and BIRC6 3'-UTR luciferase reporter into AML-5 or HL-60 cells, the luciferase assay showed

that miR-204 overexpression markedly decreased the luciferase activity of BIRC6 3'-UTR (Fig. 3B). In addition, the effect of miR-204 mimics on BIRC6 expression was determined. Quantitative real-time PCR showed that BIRC6 mRNA expression was reduced in cells treated with miR-204 mimics (Fig. 3C). Consistently, the protein expression of BIRC6 after miR-204 mimic transfection was also decreased (Fig. 3D and E). Since p53 is an important anticarcinogenic factor that can be regulated by BIRC6^{22,23}, we investigated the effect of miR-204 on p53 expression in the presence of ATO. The results showed that ATO-induced increase in p53 expression was significantly enhanced after miR-204 overexpression (Fig. 3F and G). These data suggest that miR-204 increases the sensitivity of AML cells to ATO at least partially via BIRC6/p53-dependent apoptotic pathway.

Rescued BIRC6 Expression Abolished the Effect of miR-204 on AML Cell Apoptosis Induced by ATO

To confirm the critical role of BIRC6 in miR-204-mediated ATO sensitivity, BIRC6 expression was restored in AML cells, and its effect on p53-mediated apoptosis was measured. Expectedly, transfection with BIRC6

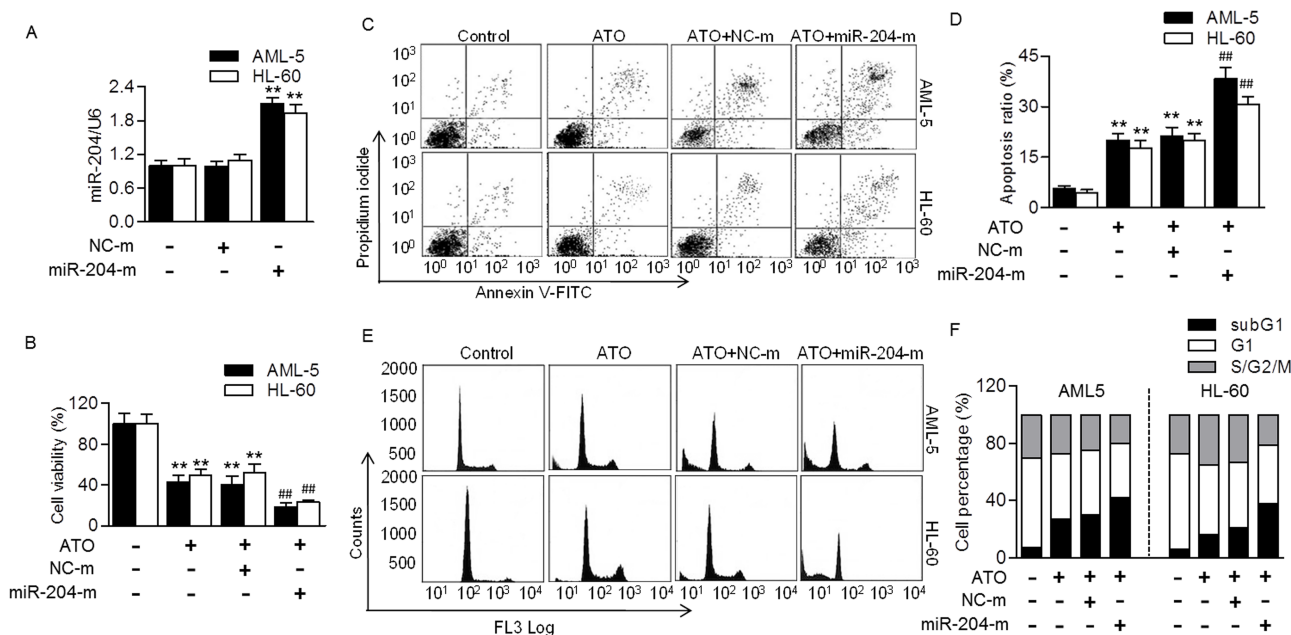


Figure 2. Upregulation of miR-204 promoted ATO-induced AML cell apoptosis. (A) Human AML cell lines, AML-5 and HL-60, were transfected with miR-204 mimics (miR-204-m; 20 nmol/L) or mimic negative control (NC-m) for 48 h. Quantitative real-time PCR was performed to test the level of miR-204. $**p < 0.01$ versus control, $n = 4$. (B) The cells were transfected with miR-204 mimics or mimic negative control for 48 h, and then treated with ATO (5 $\mu\text{mol/L}$) for another 48 h. Cell viability was examined. $**p < 0.01$ versus control; $##p < 0.01$ versus ATO, $n = 6$. (C) Cell apoptosis was examined by annexin V/propidium iodide (PI) staining using flow cytometry. (D) The percentage of apoptosis ratio was quantified. (E) Cell cycle was quantified by flow cytometry. (F) Graphs correspond to the distribution of cell population in different phases. $n = 6$.

plasmid upregulated BIRC6 expression in AML-5 and HL-60 cells (Fig. 4A and B). Importantly, miR-204 mimics that mediated the enhanced effect on p53 expression were markedly reversed after restoration of BIRC6 (Fig. 4C). CCK-8 assay also showed that the effect of miR-204 mimics on ATO-mediated cell growth and apoptosis was significantly attenuated in cells transfected with BIRC6 plasmid (Fig. 4D and E). Similarly, the enhanced population of subG₁ phase induced by miR-204 overexpression was inhibited after BIRC6 upregulation (Fig. 4F), further supporting that downregulation of BIRC6 contributes to the effect of miR-204 on ATO sensitivity in AML cells.

DISCUSSION

In the past decade, miRNA regulation has been suggested as an important mechanism that is involved in a wide range of cancer development^{1,17}. Because they are more stable than many mRNA moieties and even some proteins, many investigators have thought that this stability can be exploited to function as a novel gene therapeutic approach in some diseases, including leukemia, such as AML and chronic myeloid leukemia^{3,24}. The alteration of miRNAs may indicate important clinicopathological significance of AML²⁵⁻²⁷. For example, miR-192 level was reduced in the specimens of AML patients, and this miRNA caused G₀/G₁ cell cycle arrest and inhibited cell

proliferation by targeting CCNT2²⁵. In addition, a study of miR-22 found that it was downregulated in primary AML samples, suggesting that miR-22 may play an antitumor role with therapeutic potential in AML²⁶. Our recent work also reported that miR-204 was downregulated in blood samples of AML patients and different AML cell lines. Overexpression of miR-204 inhibited the growth of AML cells¹⁶. In this study, we used two different APL cell lines: AML-5 and HL-60 cells. The results showed that ATO, a well-recognized antileukemic drug, significantly increased miR-204 level, suggesting that the antileukemic effects of ATO may be associated with the changes in miR-204 level. Indeed, enforcing miR-204 markedly potentiated ATO-induced apoptosis of AML cells. Moreover, we selected AML patients with a good response to ATO that represented normal recovery marrow after ATO treatment. The data clearly showed that the levels of miR-204 and BIRC6 were dramatically altered after ATO treatment. Although the sample quantity was low, it could still indicate that higher miR-204 and lower BIRC6 may be associated with favorable prognosis of AML.

Inhibitor of apoptosis proteins (IAPs) family plays a critical role in regulating cell survival and death, which is closely associated with apoptosis resistance in a variety of cancer cells^{28,29}. BIRC6 is the largest member of IAP

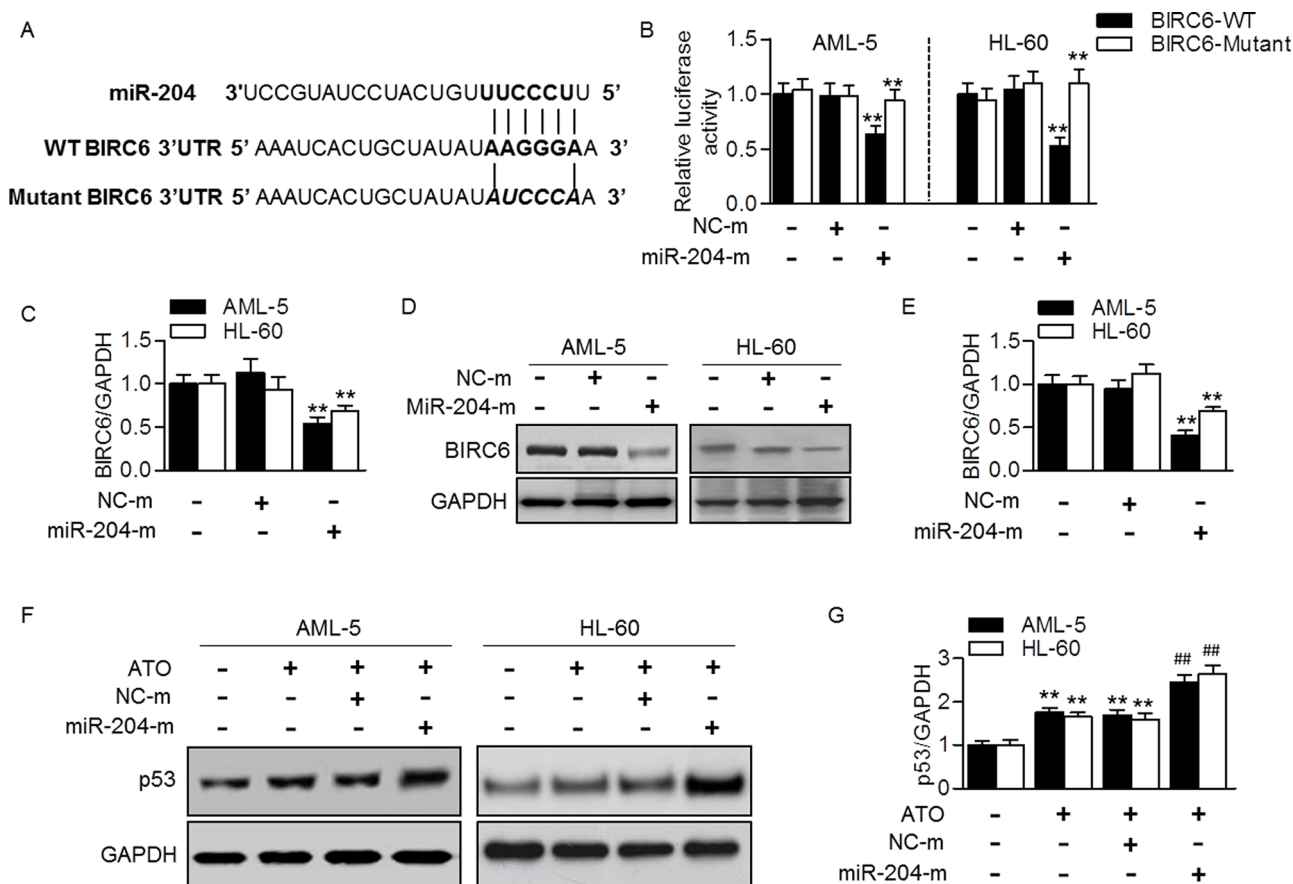


Figure 3. miR-204 targeted at BIRC6 and increased p53 expression. (A) The human miR-204 with BIRC6 3'-untranslated region (3'-UTR) with seed sequence. (B) Luciferase activity of BIRC6 3'-UTR was examined in AML-5 or HL-60 cells treated with miR-204 mimics (miR-204-m, 20 nmol/L) or mimic negative control (NC-m) for 48 h. $**p < 0.01$ versus control, $n = 6$. (C) BIRC6 mRNA expression was determined by quantitative real-time PCR. $**p < 0.01$ versus control, $n = 5$. (D) Western blotting analysis of BIRC6. Representative images were shown. (E) Densitometric analysis of BIRC6 was performed. $**p < 0.01$ versus control, $n = 6$. (F) The cells were treated with miR-204 mimics for 48 h before ATO incubation (5 $\mu\text{mol/L}$). p53 expression was tested. (G) Densitometric analysis of BIRC6 was performed. $**p < 0.01$ versus control; $##p < 0.01$ versus ATO, $n = 5$.

family with a unique ubiquitin-conjugating domain^{23,30}. The role of BIRC6 in AML has been well documented. For example, upregulation of BIRC6 was associated with unfavorable response to therapy in childhood AML, indicating BIRC6 may contribute to carcinogenic effects in AML^{31,32}. p53, a well-known anticarcinogenic regulator, has been demonstrated to act as a substrate of BIRC6 E3 ubiquitin ligase²³. BIRC6 can interact with p53 and facilitates its degradation²². Therefore, the feedback loop between BIRC6 and p53 is significant for the regulation of cancer cell apoptosis and drug resistance.

Previous studies have reported that BIRC6 is negatively regulated by several miRNAs by binding to specific sequence in the 3'-UTR region, such as miR-181a, miR-342, and miR-446h^{23,33,34}. In this study, we evidenced that miR-204 level was negatively correlated with BIRC6 expression during ATO treatment. Luciferase assay further showed that BIRC6 was also a potential target of

miR-204. Upregulation of miR-204 suppressed BIRC6 expression and luciferase activity and subsequently increased p53 expression. This suggests that miR-204 inhibits BIRC6 expression and in turn activates p53-dependent apoptotic pathway. Given that miR-204 enhances ATO sensitivity and targets to BIRC6, we speculated that the increased expression of BIRC6 may contribute to the drug resistance of AML cells. Indeed, higher BIRC6 expression was detected in childhood AML^{31,32}. Similar to the previous observations that BIRC6 upregulation was also resistant to various anticancer drug in different cancer cells^{31,35-37}, the effect of miR-204 on apoptosis through BIRC6 was further confirmed in our study using BIRC6 plasmid in AML cells. However, a previous study reported that reduced BIRC6 expression was associated with an immature myeloid phenotype of AML samples rather than immature myeloid cells³⁸. These apparent discrepancies may be related to different

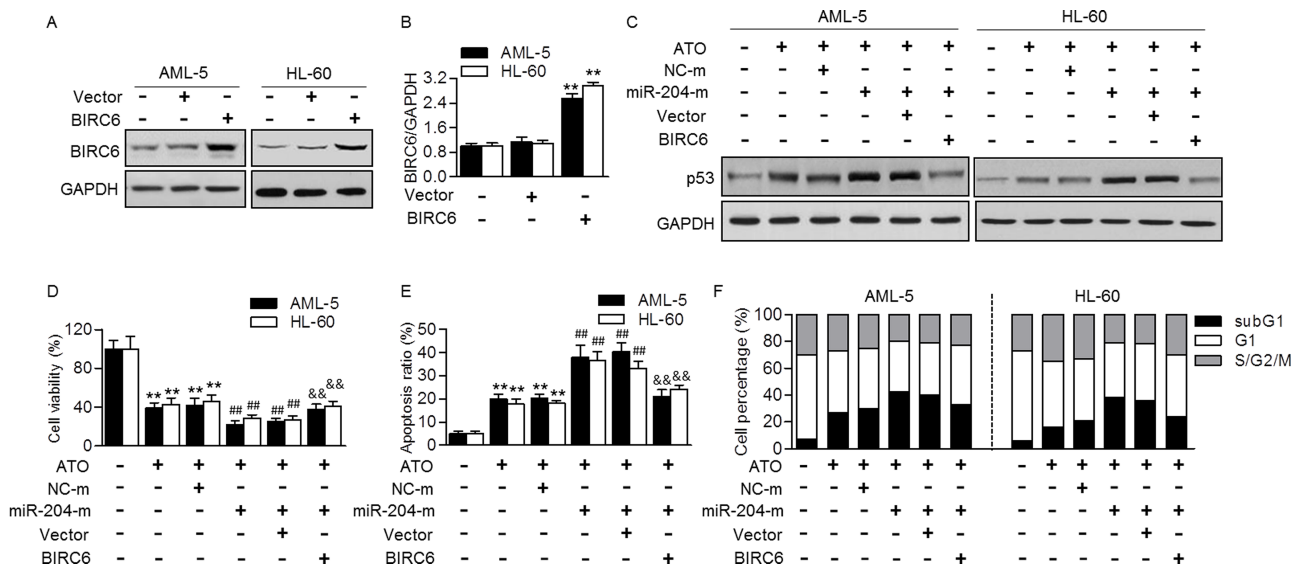


Figure 4. Restoration of BIRC6 reversed the effect of miR-204 on AML cell apoptosis induced by ATO. (A) Western blotting analysis of BIRC6 expression in AML-5 or HL-60 cells transfected with BIRC6 plasmid for 48 h. Representative images are shown. (B) Densitometric analysis of BIRC6 was performed. $**p < 0.01$ versus control, $n = 4$. (C) The cells were cotransfected with miR-204 mimics (miR-204-m, 20 nmol/L) and BIRC6 plasmid for 48 h and then treated with ATO (5 $\mu\text{mol/L}$) for 48 h. p53 protein expression was determined by Western blotting analysis. (D, E) After the treatment mentioned in (C), cell viability (D) and apoptosis (E) were examined. $**p < 0.01$ versus control; $##p < 0.01$ versus ATO; $\&\&p < 0.01$ versus ATO + miR-204-m, $n = 6$. (F) Cell cycle was quantified by flow cytometry, and the graphs correspond to the distribution of cell population in different phases. $n = 6$.

cell subtypes in distinct leukemic entities and must be reconciled by additional studies.

In conclusion, this study is the first to our knowledge to demonstrate the important role of miR-204 in regulating ATO sensitivity in AML cells, suggesting that miR-204 could be a potential target for overcoming chemoresistance in AML.

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