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miR-181a down-regulates MAP2K1 to enhance adriamycin sensitivity in leukemia HL-60 cells

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Abstract. – OBJECTIVE: MAPK kinase 1 (MEK1), also known as MAP2K1, plays a role in activating extra-cellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway to regulate cell proliferation and apoptosis. The abnormal expression of MAP2K1 is associated with leukemia. Bioinformatics analysis showed the targeted relationship between microRNA-181a (miR-181a) and the 3'-UTR of MAP2K1. This study aimed to investigate the role of miR-181a in regulating MAP2K1 expression, the effects on leukemic cell proliferation, apoptosis, and adriamycin (ADM) resistance.

MATERIALS AND MATERIALS: Dual luciferase reporter gene assay was applied to confirm the targeted relationship between miR-181a and MAP2K1. ADM resistant cell line HL-60/ADM was established. MiR-181a and MAP2K1 expressions were detected. HL-60/ADM cells were cultured *in vitro* and divided into two groups including microRNA-Normal control (miR-NC) group and miR-181a mimic group. MAP2K1, phosphorylated MAP2K1 (p-MAP2K1), and phosphorylated (p-ERK) protein expressions were tested. Cell apoptosis was assessed with flow cytometry. Cell proliferation was determined using MTT staining.

RESULTS: The targeted regulatory relationship between miR-181a and MAP2K1 mRNA. miR-181a expression was significantly lower, while MAP2K1 mRNA and protein expressions were markedly higher in HL-60/ADM cells than in HL-60 cells ($p<0.05$). Transfection of miR-181a mimic markedly reduced expressions of MAP2K1, p-ERK in HL-60/ADM cells, elevated cell apoptosis, and weakened proliferation compared to miR-NC ($p<0.05$).

CONCLUSIONS: MiR-181a reduction and MAP2K1 elevation were related to ADM resistance in leukemia cells. Up-regulation of miR-181a expression inhibited leukemia cell proliferation, induced apoptosis, and reduced ADM resistance via targeting MAP2K1 expression and inactivating MAPK signaling pathway.

Key Words:
Leukemia, ADM, Drug resistance, miR-181a, MAP2K1.

Introduction

Leukemia is a group of heterogeneous hematopoietic stem cell malignant clonal diseases caused by differentiation block, apoptosis arrest, and abnormal proliferation at different stages of hematopoietic stem/progenitor cells. Chemotherapy is an important method for treating leukemia. However, the emergence of drug resistance is one of the key factors that limit the efficacy of chemotherapy, affect the survival and prognosis of patients. MAPK kinase 1 (MEK1), also known as MAP2K1, phosphorylates and activates extracellular signal regulated kinases (ERK) protein, thereby activating ERK/mitogen activated protein kinase (MAPK) signaling pathway. It was showed that the expression and functional activity of MAP2K1 are associated with the development, progression, and drug resistance of leukemia[3-5]. MicroRNAs are a type of endogenous small non-coding single-stranded RNA. They participate in the biological process, such as cell survival, proliferation, apoptosis, and migration, by complimentary binding with the 3'-UTR of target gene to promote target gene mRNA degradation or inhibit mRNA translation. The abnormal expression and function of miRNA in chemotherapy resistance have attracted more and more attention[6,7]. MiR-181a is a highly studied miRNA that is related to the occurrence, progression, and drug resistance of various tumors, such as lung cancer[8], thyroid cancer[9], cervical cancer[10], and prostate cancer[11]. It was found[12-15] that miR-181a is involved in the regulation of biological processes, such as proliferation, apoptosis, cycle and drug resistance of leukemia cells. Bioinformatics analysis showed the targeted relationship between miR-181a and 3'-UTR of MAP2K1. This study investigated the role of miR-181a in regulating MAP2K1 expression, affecting leukemic cell proliferation, apoptosis, and adriamycin (ADM) resistance.

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Materials and methods

Main Reagents and Materials

Human normal peripheral blood mononuclear cell (PBMC) and promyelocytic leukemia cell HL-60 were purchased from Beijing Beina Biotechnology Co., Ltd. (Beijing, China). HEK293T cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640), optional essential medium (Opti-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). TRIzol and Lipofectamine 2000 were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). MiR-181a mimic, miR-181a inhibitor, and miR-NC were purchased from RiboBio (Guangzhou, China). Rabbit anti-human MAP2K1 and p- MAP2K1 polyclonal antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). Rabbit anti-human β-actin antibody was purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). Goat anti-Rabbit IgG (H+L) secondary antibody was purchased from Biotech. Co. Ltd. (Shanghai, China). Cell Counting Kit-8 (CCK-8), Annexin V/ propidium iodide (PI) cell apoptosis detection kit, and bicinchoninic acid (BCA) quantification kit were purchased from Beyotime Biotech (Jiangsu, China). EdU Flow Cytometry Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dual-Glo Luciferase Assay System and pGL3-basic vector were purchased from Promega (Madison, WI, USA). Forma 3131 cell incubator was purchased from Thermo Electron (Waltham, MA, USA).

Cell Culture

PBMC and HL-60 cells were maintained in RPMI-1640 (Roswell Park Memorial Institute 1640) medium containing 10% fetal bovine serum (FBS) cultured in 37°C and 5% CO_2. The cells in logarithmic phase were used for experiments. The study was approved by the Ethics Committee of Nuclear Industry 215 Hospital of Shaanxi Province (Xianyang, Shaanxi, China).

ADM Drug Resistant Cell Line Establishment

HL-60 cells in logarithmic phase were treated by ADM from 0.1 μg/ml for 2 weeks. Then, cells kept growing and were treated by increased concentration of ADM up to 1.6 μg/ml when the cells can stably grow in ADM. At last, the cells can be stable passaged in ADM to obtain ADM resistant leukemia cell line HL-60/ADM. HL-60 and HL-60/ADM cells were treated by different concentrations (0, 0.1, 1, 10, 100, and 1[illegible] of ADM for 48 h. Next, the cells were added with CCK-8 to measure the absorbance value (A450). Inhibition rate = (1-A450 in drug group/A450 in control × 100%. IC50 was calculated using SPSS software. Resistance index (RI) = IC50 of resistant cell/IC50 of parental cell.

Flow Cytometry Detection of Cell Proliferation

The cells were added with [illegible]tion at 10 μM in logarithmic phase. After incubated for 2 h, the cells were [illegible] for 48 h and digested by trypsin. After fixed [illegible] paraformaldehyde (Sigma-[illegible], St. Louis, [illegible]USA), the cells were incubated in 100 μl Triton X-100 (Beyotime Biotech, Shanghai, China) at room temperature and [illegible]00 μl reaction liquid at room temperature avoid [illegible]t for 30 [illegible]. At last, the cell was tested on [illegible] MCL flow cytometry (BD Biosciences, San Jose, [illegible]SA).

Luciferase Reporter Gene Assay

[illegible]CR product of the MAP2K1 3'-UTR full-length fragment or mutant fragment was double-digested and then ligated into the pGL3 vector. After sequencing, the plasmid was designated as pGL3-MAP2K1-WT and pGL3-MAP2K1-MUT. The HEK293T cells were transfected with pGL3-MAP2K1-WT (or pGL3-MAP2K1-MUT) together with miR-181a mimic (or miR-181a inhibitor, miR-NC) by Lipofectamine 2000. After incubated for 48 h, luciferase activity was detected by Dual-Glo Luciferase Assay System kit according to the manual.

Cell Transfection and Grouping

HL-60/ADM cells were divided into miR-NC group and miR-181a mimic group. A total of 10 μl of Lip2000, 50 nmol miR-NC, and 50 nmol miR-181a mimic were diluted with 100 μl serum-free Opti-MEM medium, and incubated for 5 min at room temperature, respectively. The mixture was added to the cell culture medium for 72 h. At last, the cells were collected for detection. The cells were seeded in 6-well plate and treated by 1.6 μg/ml ADM for 48 h. The cells were added with EdU solution at 10 μM in logarithmic phase. After incubated for 2 h, the cells were seeded for 48 h and digested by trypsin. After fixed in para-

Table I. Primers for the RT-PCR assay.

Genes		Sequences
miR-181a	Forwards	5'-TGCGCAACATTCAACGCTGTCG-3'
	Reverse	5'-CTCAAGTGTCGTGGAGTCGGCA
MAP2K1	Forwards	5'-ATCTTCGGGAGAAGCACAAG
	Reverse	5'-CGAAGGAGTTGGCCATAGA
β-actin	Forwards	5'-TACCACATCCAAGAAGGC
	Reverse	5'-TGCCCTCCAATGGATCCTC-3

formaldehyde, the cells were incubated in 100 µl Triton X-100 at room temperature and in 500 µl reaction fluid at room temperature avoid of light for 30 min. At last, the cell was tested on FC500 MCL flow cytometry (BD Biosciences, San Jose, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

The total RNAs from the cells were extracted by using the TRIzol regents, which purchased from Beyotime Biotech. (Shanghai, China). Next, the complementary DNA (cDNA) was synthesized by using the SuperScript III first-strand synthesis system (Cat. No. 18080051, Invitrogen/Life Technologies, Carlsbad, CA, USA). QuantiTect S... Green RT-PCR Kit (Cat. No. 204243, ... Hilden, Germany) was used to amplify the ...ting genes based on the synthesized cDNAs. ... PCR reaction system was composed of 2×Qu... tiTect SYBR Green RT-PCR M...ix 10.0 µ... forward primer (0.5 µM) 1.0... primer (0.5 µM) 1.0 µl, Template ...A 2.0 ... Quanti-Tect RT Mix 0.5 µl, and ... The ... performed on Bio-Ra... Mo... 6, Bio... d Laboratories, Herc... CA, U... t 45°C for 5 min and 94°C fo... followed ... cycles of 95°C for 5 s an... 30 s. The pr...s for the RT-PCR assay ...ere lis... Table I.

Weste... Blot

To... protein... s extracted from the cells by ra... mun... ipitation assay (RIPA). After quant... CA m... d, a total of 40 µg pro- was ...rated ... sodium dodecyl sulpha- ...Acry... el electrophoresis (SDS-PA-GE... d trans... d to polyvinylidene difluoride (P... embrane at 250 mA for 100 min. After ...Rec... % skim milk at room temperature, ...e membrane was incubated in primary antibody ...C overnight (MAP2K1 1: 2000, p-MAP2K1 1:... and β-actin 1: 10000). After washed by phosphorylate-buffered saline Tween-20 (PBST, Beyotime Biotech. Shanghai, China), the membrane was further incubated in horseradish peroxidase (HRP) conjuga... econd... antibo... room temperature fo... min (1:... 00). At last, the membrane was ...trea... ...anced ...ilu-minescence (EC... Amer... Biosci... es (Piscataway, NJ, ...) reagent a... ...ed.

Cell Ape...sis ...ction

The cells were di... d by enzyme and collect... r resuspend... 100 µl binding buffe... e cells were added w...n 5 µl Annexin V-FI-T... nd 5 µl PI at ...om temperature avoid of light ...5 min. Nex...e cells were tested on flow cy...etry.

Statis... alysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, ...as applied for data analysis. Measurement ...ta ...e presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups. $p<0.05$ represents a significant difference.

Results

The Targeted Regulatory Relationship between miR-181a and MAP2K1

Bioinformatics analysis showed the complementary binding site between miR-181a and the 3'-UTR of MAP2K1 mRNA (Figure 1A). Dual luciferase reporter gene assay exhibited that miR-181a mimic transfection significantly reduced the relative luciferase activity in HEK293T cells transfected by pGL3-MAP2K1-WT but not by pGL3-MAP2K1-MUT, confirming that MAP2K1 was the target gene of miR-181a (Figure 1B, $p<0.05$).

Drug Resistant Leukemia Cells Exhibited Strong ADM Resistance

CCK-8 assay showed that the proliferative activities of parental HL-60 cells were significantly lower than those of HL-60/ADM cells under the

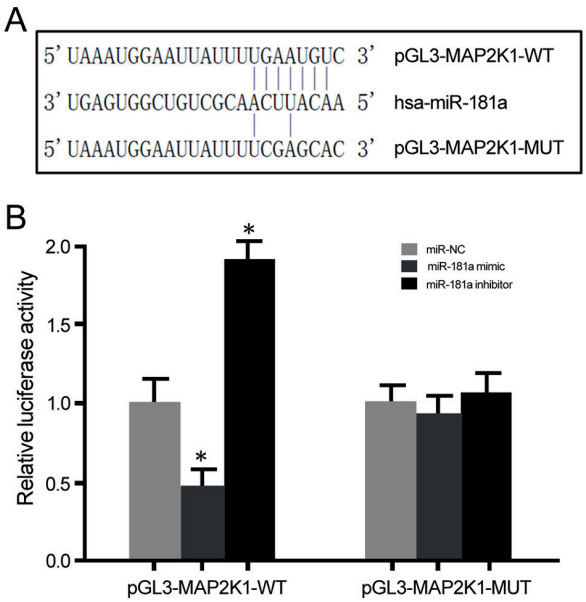

Figure 1. The targeted regulatory relationship between miR-181a and MAP2K1 mRNA. **(A)** The complementary binding site between miR-181a and the 3'-UTR of MAP2K1 mRNA. **(B)** Dual luciferase reporter assay. $^{*}p<0.05$, compared with miR-NC.

same dose treatment of ADM. The IC_{50} of [illegible]60 cells was 1.36±0.11 μg/ml, while it was 18.79±[illegible] μg/ml in the drug-resistant HL-60/ADM cells, leading the RI of HL-60/ADM [illegible]13.82 (Figure 2A). Under the ADM t[illegible]1.36 μg/ml, the apoptotic rate of [illegible]-60 cells reached 25.17%±3.75%, while it [illegible]nly 2[illegible]% in HL-60/ADM cells ([illegible]ur[illegible]

MiR-181a Reduced, while MAP2K1 Up-Regulated in Drug Resistant Cells

qRT-PCR demonstrated that compared with human normal PBMC cells, miR-181a exp[illegible]n was significantly decreased in HL-60 [illegible] its level was obviously lower in [illegible]resistant HL-60/ADM cells than that of [illegible]tal HL-60 cells (Figure 3A, $p<0.05$). qRT-PC[illegible]led that the MAP2K1 mRNA expre[illegible]n in [illegible] cells was markedly higher th[illegible]at of PB[illegible] ($p<0.05$), and it was an[illegible]ntly h[illegible]er in [illegible] mia resistant HL-60/[illegible]l cell[illegible]mpared with the parental HL-60 c[illegible] 3A). [illegible]stern blot exhibited th[illegible]ompa[illegible]ith PB[illegible] cells, MAP2K1 pro[illegible]expression [illegible] cells was significantl[illegible]lated ($p<0.$[illegible]nd its level in leuken[illegible]rug-[illegible]ant HL-60/ADM cells obviously enhanced (Fi[illegible]B).

M[illegible]81a Over-Expres[illegible]on Promoted [illegible]kemia Cell [illegible]poptosis and Reduced [illegible]M Resistan[illegible]

[illegible]T-PCR sho[illegible]d that miR-181a mimic transfec[illegible]sig[illegible]ntly up-regulated miR-181a expres[illegible] declined MAP2K1 mRNA level in HL-60/ADM cells compared with miR-NC (Figure 4A, $p<0.05$). Western blot de[illegible]ed that miR-181a mimic transfection significantly reduced MAP2K1, p-MAP2K1, and p-ERK1 protein levels in HL-60/ADM cells (Figure 4B, $p<0.05$). Flow cytometry revealed that transfection of miR-181a mimic markedly enhanced cell apoptosis, while inhibited cell proliferation in HL-60/ADM cells (Figure 4C, D, $p<0.05$).

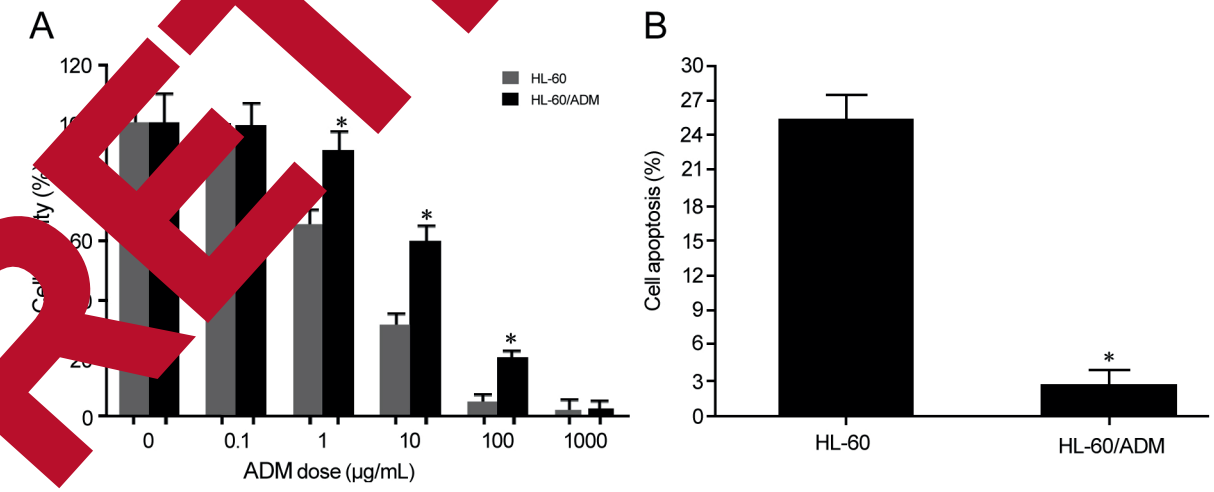

Figure 2. Drug resistant cells exhibited strong drug resistance. (A) CCK-8 detection of cell viability. (B) Flow cytometry detection of cell apoptosis. $^{*}p<0.05$.

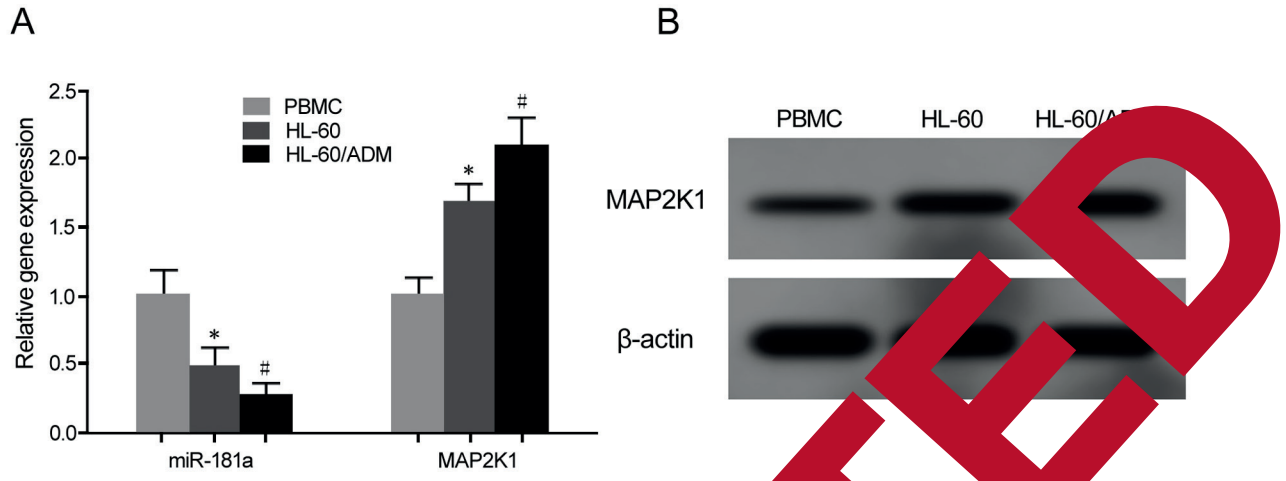

Figure 3. MiR-181a reduced, while MAP2K1 upregulated in drug resistant ce… T-PCR detecti… RNA expression. **(B)** Western blot detection of MAP2K1 protein expression. *p<0.05, …ared … BMC, #p<0.0…, compared with HL-60 cells.

Discussion

ERK/MAPK signaling pathway is widely expressed in various tissues and cells, and can regulate a variety of biological processes …h as cell proliferation, cycle, apoptosis, mi… and invasion[16,17]. Over-activation of ERK/M…K signaling pathway is closely related to the …currence, progression, and metastasis of vari… tumors, such as oral cancer, e…al cance… and lung cancer[18-20]. MAP2K … tyr…ne/thre-onine (Tyr/Thr) dual-spec… protein …ase that specifically phosphoryla…K pr…ti… vate ERK/MAPK si…ling …s showed that the exp…on and …onal activity of MAP2K1 are …ated with t… elopment, progression, a… …istance of le…mia[3-5]. It was found tha… miR-18… involved in the regulation of …gical proce… such as proliferation, a…sis, cycle and d… esistance of leukemi… lls[12-15]. …informatics analysis showed th… ated …ionship between miR-181a and the 3… MAP… This study investigated the … of miR…a in regulating MAP2K1 …sion, … leukemic cell proliferation, apo…sis, and …DM resistance. In this study, d… …ase reporter gene assay exhibited that …ic transfection significantly reduced the relative luciferase activity in HEK293T … transfected by pGL3-MAP2K1-WT but … pGL3-MAP2K1-MUT, confirming that MAP2K1 was the target gene of miR-181a. CCK-8 assay showed that the proliferative activities of parental HL-60 cells were significantly lower … those of HL…0/ADM cells under the same … treatment …ADM. Compared with hu… normal PB… cells, miR-181a expression was …ifica… decreased, while MAP2K1 level was …ly enhanced in HL-60 cells, and their changes amplitude was significantly higher … resistant HL-60/ADM cells than that of … HL-60 cells, suggesting that miR-181a down-regulation was associated with MAP2K1 over-expression and participated in the regulation of leukemia cell ADM resistance. Schwind et al[23] showed that the treatment response, overall survival rate, and disease-free survival rate of patients with higher expression of miR-181a were significantly better than those with lower expression of miR-181a. Zhu et al[14] adopted gene expression profiling assay and found that the expression of miR-181a in peripheral blood of leukemia patients was abnormally decreased compared with healthy controls, which was related to poor survival and prognosis. Li et al[15] revealed that the expression of miR-181a in drug-resistant leukemia cells was significantly lower than that of the parental leukemia cells. All of the above studies indicated that miR-181a functions as a tumor suppressor gene in leukemia, which was similar to our results.

This study further explored whether miR-181a is involved in the regulation of drug resistance in leukemia HL-60 cells. Transfection of miR-181a mimic markedly reduced the expressions of MAP2K1, p-MAP2K1, and p-ERK in HL-60/ADM cells, enhanced cell apoptosis, and weakened cell proliferation. It was showed that the down-regulation of miR-181a was involved in

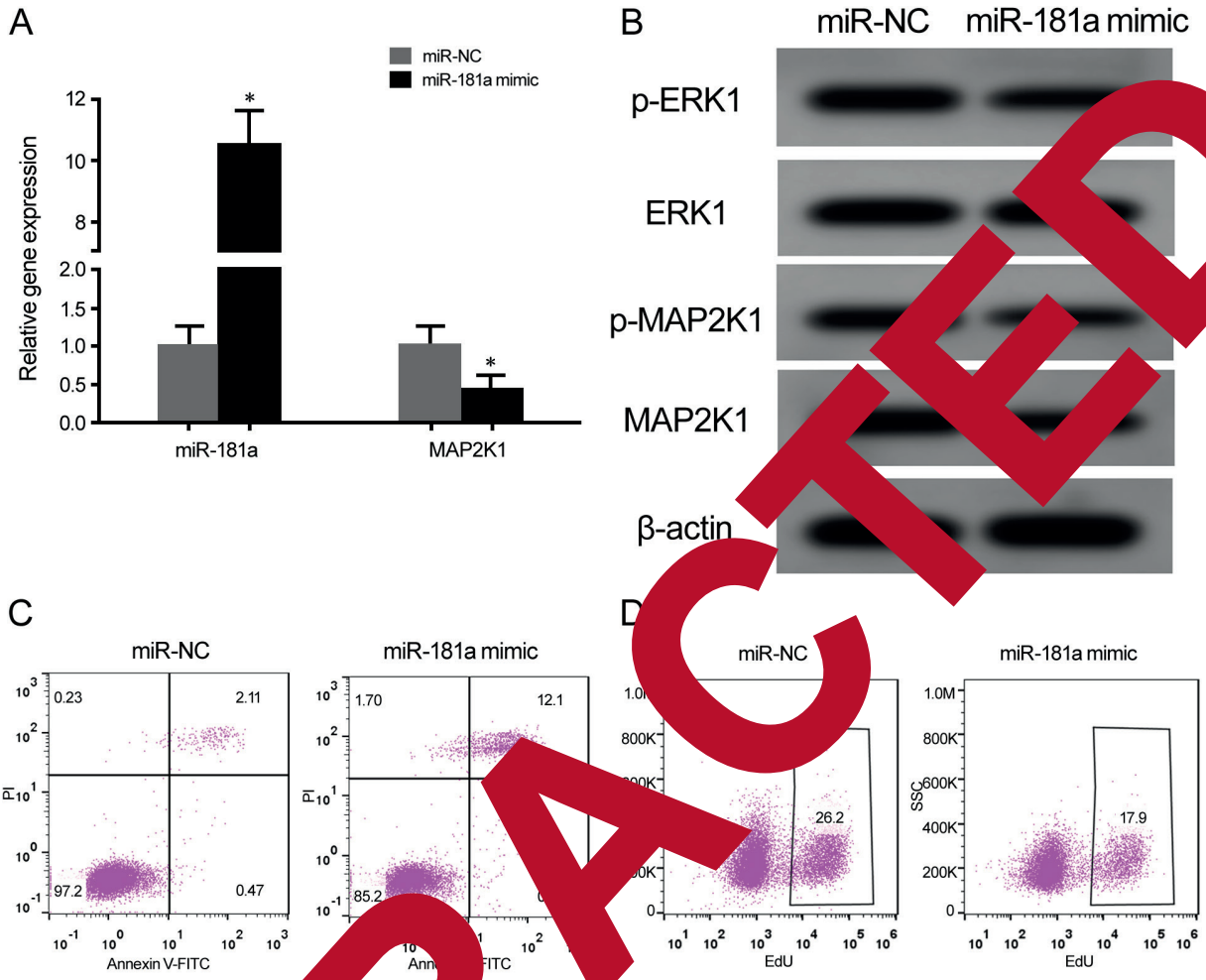

Figure 4. MiR-181a over-expl[illegible]arom[illegible]s and reduced ADM resistance. **(A)** qRT-PCR detection of mRNA expression. **(B)** Western b[illegible]dete[illegible]ein expr[illegible]on. **(C)** Flow cytometry detection of cell apoptosis. **(D)** Flow cytometry detection of cell [illegible]eration.

the regulation of drug [illegible]nce in leukemia cells, while [illegible]increase of [illegible]181a inhibited the expres[illegible]of MAP2K1, decr[illegible]d the activity of ERK[illegible]PK p[illegible]way, restrained cell proliferation, promot[illegible]poptosis, and reduced drug resistance. Li et al[24] f[illegible]d that abnormal expression of [illegible]81a w[illegible]ssociated with leukemia, and over-exp[illegible]f miR-181a in leukemia cells significantly in[illegible]ted cell proliferation, attenuated [illegible]formation ability, promoted apoptosis, [illegible]origenicity in animals, and reduced drug resistance mainly through targeted inhibition of KRAS, NRAS, and MAPK1 expressions. Zhu et al observed that increasing the expression of miR-181a obviously enhanced the sensitivity of leukemia cells to the anti-tumor drug fludarabine, resulting in a significant apoptosis enhancement through targeting BCL-2, MCL-1, and XIAP. Li et al[15] demonstrated that transfection of miR-181a inhibitor in leukemia cells markedly promoted cell proliferation and survival, and reduced the sensitivity to the anti-tumor drug daunorubicin (DNR). Transfection of miR-181a mimic apparently facilitated the apoptosis of drug-resistant leukemia cells, weakened cell proliferative capacity, and decreased DNR resistance by targeting Bcl-2 gene expression. Fei et al[25] revealed that miR-181a upregulation in leukemia cells can inhibited cell proliferation, arrested cell cycle in G2 phase, and promoted apoptosis by targeting Ras-related protein Ral-A (RalA). All of the above studies reported the role of miR-181a in the regulation of drug sensitivity in leukemia cells. This work combined the targeting regulation between miR-181a

and MAP2K1, revealing that miR-181a targeted inhibited MAP2K1 expression, decreased ERK/MAPK pathway activity, promoted leukemia cell apoptosis, and reduced ADM resistance. However, whether miR-181a regulating MAP2K1 is related to the drug resistance of leukemia patients is still unclear.

Conclusions

We revealed that MiR-181a reduction and MAP2K1 elevation were related to ADM resistance in leukemia cells. Up-regulation of miR-181a expression inhibited leukemia cell proliferation, induced apoptosis, and reduced ADM resistance via targeting MAP2K1 expression and ERK/MAPK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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