European Review for Medical and Pharmacological Sciences 2018; 22: 3333-3341

MiR-143 regulates proliferation and apoptosis of myelocytic leukemia cell HL-60 via modulating ERK1

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Abstract. – OBJECTIVE: Extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathway is widely involved in cell proliferation and invasion regulation. Enhanced expression or function of ERK1 is important for leukemia. Abnormal down-regulation of microRNA (miR) correlated with leukemia pathogenesis, ing possible tumor-suppressing role. Bioinformatics analysis showed the existence of plementary binding sites between miR-143 ERK1. This study aims to investigate whether the miR-143 plays a role in me ERK1 expression and proliferation an is of leukemia cells.

PATIENTS AND METH: Dual reporter gene assay co lation between miR-143 a quantita RT-PCR (qRT-PCR) use asure and compare the peri l miR-143 RK1 expression betwee thy and acut myelocytic leukemia nts to analyze the effect of miR-1 d survival and prognosis. Cultured HL-60 re treated with miR-143 c or small g RNA (siRNA)-ER ollowed by qRT R to measure miR-1 xpres Western blot quantified expres of E and p-ERK1, flow cytometry mea osis, EdU staining measured ion.

RESUL rgeted and modulated APL presented lower miR-143 gher ERK eripheral blood. Those with mi down-regulation displayed worse prog- with high miR-143 expression 0.039). Patients with ERK1 mRlow-expression presented better prognon those a having higher expression (Log-r $\chi^2 = 5.873$, $p = 0.028$). Transfection of miR mimic or siRNA-ERK1 remarkably suppressed ERK1 and p-ERK1 expression in HLells, inhibited proliferation and induced poptosis.

CONCLUSIONS: MiR-143 down-regulation and lation are correlated with APL pathog Their expression level affected patient's prognosis. MiR-143 targeted and inhibERK1 expression, weakened proliferation HL-60 cells, and induced apoptosis.

Key Words:
MiR-143, ERK1, Acute proteolytic leukemia, Cell proliferation, Apoptosis.

Introduction

Leukemia is one group of heterogenous malignant clonal disease of hematopoietic stem cells caused by impeded differentiation/apoptosis or malignant proliferation of hematopoietic stem/progenitor cells during a certain stage of differentiation. Acute myeloid leukemia (AML) refers to the sub-group of diseases derived from myeloid hematopoietic cells[1,2].

Extracellular signal-regulated kinase (ERK) induced mitogen-activated protein kinase (MAPK) pathway is one classical MAPK signaling transduction pathway. Meanwhile, the MAPK pathway is also the major transducing pathway for MAPK signal pathway to exert its roles. Ras/Raf/MEK/ERK is the major transducing module of ERK/MAPK signal pathway[3,4]. As one important sub-type of ERK protein, ERK1 over-expression or over-activation is correlated

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with onset, progression or metastasis of various tumors including colorectal carcinoma[5], prostate cancer6, and breast cancer[7]. Furthermore, the ERK1 is one factor with potent oncogenic functions. Various studies[8-10] showed that ERK1 enhancement was correlated with pathogenesis, drug resistance, and unfavorable prognosis, and inhibition of ERK1 alleviated drug resistance, inhibited proliferation of leukemia cells and induced apoptosis, indicating the oncogenic role of ERK1 in leukemia. MicroRNA (miR) is one type of newly discovered non-coding single-stranded RNA with 22-25 nucleotides length in eukaryotes. Meanwhile, the miR can also modulate more than one-third of human gene expression via targeted degradation of mRNA or suppressing target mRNA translation. It is thus involved in the regulation of various biological processes including organ/tissue development, cell proliferation, apoptosis or differentiation. The abnormal expression or dysfunction of miR was found to play important roles in tumor occurrence, progression, and resistance[11]. MiR-143 has been found to be abnormally down-regulated in leukemia, indicating its potential tumor-suppr roles in leukemia as shown by various stu Prediction by microRNA.org showed t is- tence of complementary binding sites be n miR-143 and 3'-UTR of ERK1 mRNA. A one common subtype of AML with unfavora prognosis. This study investig xpressio of miR-143 and ERK1 in per d of APL patients to analyze its co ion w nt's survival and prognosis. A w *vitro* cell model to in stig -143 pla d a role in regulating l exp nd modulating proliferatio apoptosis c cells.

Patients a thods

Major gent and Equip ent

Hu APL line HL-60 was purchased fro ric ssue c lture collection (ATCC Cell assas , USA). Roswell park memoria e (P I) 1640 culture medium, ovine BS) and streptomycin-pen- were pu ed from Gibco (Grand Isla USA). RNA extraction reagent TRIzol, tr gent Lipofectamine 2000 were hased om Invitrogen/Life Technologies bad, CA, USA). Fluorescent quantitative nd SYBR dye were purchased from TaKa alian, China). Mouse anti-human monoclonal antibody against ERK1 and phosphorylated ERK1 (p-ERK1) were purch Abcam Biotechnology (Cambridge Rabbit anti-human polyclonal a dy agains beta-actin, and horse radish p dase (HRP) conjugated secondary antibody urchased from Sangon Biotechnolo (Shan hina) Annexin V/propidium io (PI) apopt kit was purchased fro olege l (San CA, USA). siRNA-E and NA-NC w re purchased from San B chnolo (Santa Cruz, CA, USA) EdU ferati ow cytometry assay as pur olecular Probes (Eu OR, USA). ase activity assay Glo Lucifer Assay System, pM RE uciferase plasmid were purcha d from Y Bio. (Wuhan, China). Hu peripheral mo ear cell separation b was purchased fro Haoyang Bio. (Tianj hina). Bio-safe cabinet and culture chamber purchased fr Thermo Scientific Pierce ford, IL, U). Sodium dodecyl sulfate p electrophoresis (SDS-PAGE) elect apparatus, fluorescent quantitative PCR were purchased from Bio-Rad Laboories (Hercules, CA, USA). Flow cytometry sed from Beckman Coulter Inc. (Brea,). The inverted fluorescent microscope as purchased from Nikon (Mode: ECLIPSE TE2000-S, Nikon, Japan).

Clinical Information

A total of 38 APL patients who received Affiliated Taihe Hospital of Xi'an Jiaotong University Health Science Center between June 2014 and January 2015 were recruited in this study. Among the patient cohort, there were 18 males and 20 females, aging between 12 and 58 years. Another cohort of 38 healthy individuals was recruited meantime, including 19 males and 19 females, aging between 15 and 55 years. The present investigation was approved by the Ethics Committee of Affiliated Taihe Hospital of Xi'an Jiaotong University Health Science Center. All of the patients gave the signed informed consent and approved this study.

Separation of Peripheral Blood Mononuclear Cell (PBMC) and RNA Extraction

Total of 5 ml venous blood samples were collected from fasted patients and were treated with heparin sodium for anti-coagulation. PBMC was separated following the manual instruction and

was rinsed by centrifuging in phosphate-buffered saline (PBS). 1 ml TRIzol was mixed with each 5 million PBMC for 5 min complete lysis. 0.2 ml chloroform was then added for a vigorous shaking and 5 min room temperature incubation. RNA in the supernatant was transferred to a new tube, and 1 ml isopropanol was added to precipitate RNA by 10000 ×g centrifugation for 10 min. RNA pellet was washed in 1 ml 75% ethanol, followed by 10000 ×g centrifugation for 10 min. RNA was dissolved in diethyl pyrocarbonate (DEPC) water.

Cell Culture

HL-60 cells were incubated in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin, and were placed in a 37°C chamber with 5% CO_2. Culture medium was changed every 2 days, and cells at log-growth phase with satisfactory growth status were used for assays.

Dual Luciferase Gene Reporter for Construction of Recombinant Plasmid

Using RNA of HEK283T cell as the tem
3'UTR of ERK1 mRNA containing
binding sites or its mutant form was amp by
PCR. Products were then digested using Sa d
Hind III dual enzymes, along with pMIR e
plasmid at 37°C for 4 h. Products were then
rified at 1.5% agarose gel el esis, an
purified PCR products were plasmid
at 16°C overnight. Ligate oduce used
to transform DH5α com ell
inoculated onto a pen illi g plate r
37°C overnight inc on. T positive
clone was picked °C overnig g and
were extracted id. Targete quence
was sequence tion and was named
as pMIR-ERK1-wt or p K1-mut.

Dual L erase Reporter e Assay

Ab × 10 K-293T cells were inoculated
in or 24 h incubation. Lipofect-
amine used ansfect 100 ng pMIR-
ERK1-wt -mut), 900 ng miR-143
(or mi R-143 inhibitor), and 50 ng
p ull renilla iferase into HEK293T cells,
fol 48 h incubation. Dual-Glo Luciferase
A st kit was used to measure dual
erase activity. In brief, 100 µl Passive Lysis
was added to each well of 24-well plate for
haking at room temperature. Total of 20
µl c ysate was added to 100 µl luciferase test kit (LARII). After mixture, luciferase illuminator was used to measure luciferase activity
nilla luciferase reagent was added f
renilla luciferase activity. Relati tivity wa
measured by the ratio of firefly rase activity
against renilla luciferase activity

Cell Transfection and ouping

In vitro cultured H cell were
into four groups: mi gro iR-143 mimic
group, siRNA-NC A-ERK group.
All transfected c ls w ated fo h, and
cells were coll for fu

qRT-PCR Expressio

Prime pt agent kit was used to
prepar cDNA fro by reverse transcrip-
tion g cDNA as mplate, qPCR was
p med to measure ge expression. Reverse
t cription system included 0.5 µl oligdT prim-
µM), 0.5 µl r om 6 mers (100 µM), 0.5 µl
Script RT me Mix, 1.0 µg RNA, 2 µl
5 fer, and RNase Free water up
to 1 e transcription conditions were:
37°C 15 min, and 85°C 5s. qPCR reaction system
sisted of 10.0 µl SYBR Fast qPCR, 0.8 µl for-
er (10 µM), 0.8 µl reverse primer (10
µl cDNA and 6.4 µl RNase Free water.
-PCR conditions were listed as the followings:
95°C pre-denature for 10 min, followed by 40 cycles each containing 95°C denature for 10 s, 60°C annealing for 20 s and 72°C elongation for 15 s. PCR was performed on Bio-Rad Real Time PCR Detection System (Mode: CFX96, Bio-Rad Laboratories (Hercules, CA, USA).

Western Blot

Radioimmunoprecipitation assay (RIPA) buffer was used to lyse all transfected cells. The supernatant was extracted for measuring protein concentration. Total of 40 µg samples were loaded and were separated using 8%-10% SDS-PAGE separating gel and 5% condensing gel (50 V, 180 min). Proteins were then transferred to PVDF membrane (300 mA, 100 min), which was blocked in 5% defatted milk powder for 60 min. Primary antibody (ERK1 at 1:23000, p-ERK1 at 1:1000, beta-actin at 1:10000) was used for 4°C overnight incubation. The membrane was rinsed in PBST for three times. HRP conjugated secondary antibody (1:20000) was used for 60 min room temperature incubation, followed by three times of PBST washing. ECL approach was used to measure protein expression.

Flow Cytometry for Cell Proliferation

1640 complete medium containing 10% FBS was used to re-suspend all transfected cells. After 2 h incubation in 10 μM EdU for 2 h, cells were incubated for 48 h. After digestion by trypsin, cells were collected for PBS centrifugation. 100 μl fixation buffer was added for 15 min incubation. With PBS centrifugation and rinsing, 100 μl permeabilization buffer was added for 15 min processing. 500 μl test buffer was added for 30 min dark incubation at room temperature. 3 ml washing buffer was then for rinsing. Cells were re-suspended in 500 μl rinsing buffer, and Beckman-Coulter MCL/MPL flow cytometry (Mode: FC500, Beckman Coulter Inc., Brea, CA, USA).

Flow Cytometry for Cell Apoptosis

All transfected cells were digested by trypsin and were collected by centrifugation. After twice rinsing in PBS, 100 μl binding buffer was added for complete mixture. 5 μl Annexin V-FITC and 5 μl PI dye were added for 10 min dark incubation. 400 μl Binding Buffer were then added for re-suspending cells. Beckman-Coulter FC500 MCL/MPL flow cytometry was used for c[illegible] fication.

Statistical Analysis

SPSS 18.0 was used for data analysis (SP[illegible] Inc., Chicago, IL, USA). Meas[illegible] data wer[illegible] presented as mean ± stand[illegible]de[illegible]on (SD). Comparison of measureme[illegible]ta bet[illegible]oups was performed by the St[illegible]test[illegible] ney U test was emplo[illegible]ed [illegible]ng expr[illegible]- sion level of miR-1[illegible]ERK1 [illegible]in human lymph tissues. Pa[illegible] survival c[illegible]s plotted by Kaplan[illegible]pproach. Lo[illegible]nk test was used to c[illegible]al[illegible]al rate. A statistical significance wa[illegible] defined[illegible] < 0.05.

Results

Targ[illegible]ulatio[illegible]etween miR-143[illegible]ERK

[illegible]line pr[illegible]y microRNA.org showed t[illegible]istence c[illegible]mplementary binding sites bet[illegible]iR-143 and 3'-UTR of ERK1 mRNA ([illegible]ual luciferase gene reporter assay found t[illegible]t transfection of miR-143 mimic [illegible]cantly suppressed relative luciferase activity in HEK293T cells transfected with pMIR-ERK[illegible]t. The transfection of miR-143 inhibitor significantly elevated relative luciferase activity in HEK293T cells transfected with pM[illegible] wt, but not in those HEK293T cel[illegible] with pMIR-ERK1-mut (Figure 1B[illegible]ese results showed the targeted regulatory[illegible]tionship between miR-143 and ERK1 mRN[illegible]

MiR-143 Down-Regul[illegible]n and ERK1 Up-Regulatio[illegible] Peri[illegible]heral Blood of APL Pati[illegible]

qRT-PCR results[illegible]d[illegible]t, com[illegible]ed to healthy controlle[illegible] indi[illegible]APL p[illegible]ts presented a signi[illegible]ly lowe[illegible]pression in peripheral[illegible]d (Mann-W[illegible] = 41, $p < 0.001$, Fig[illegible]Comparing to[illegible]ontrol group, DLBCL[illegible]ent[illegible]ted a significantly higher ME[illegible]1 mRNA[illegible]ion in tumor tissues (Ma[illegible]nitney U=114[illegible]001, Figure 2B).

[illegible]ression Level of miR-143, ERK1 is [illegible]elated with[illegible]L Patient Survival

[illegible]g the med[illegible]evel of miR-143 and ERK1 m[illegible]the[illegible]dary, we sub-divided APL patient[illegible]43 or ERK1 high-expression and low-expression groups, to analyze the correlation

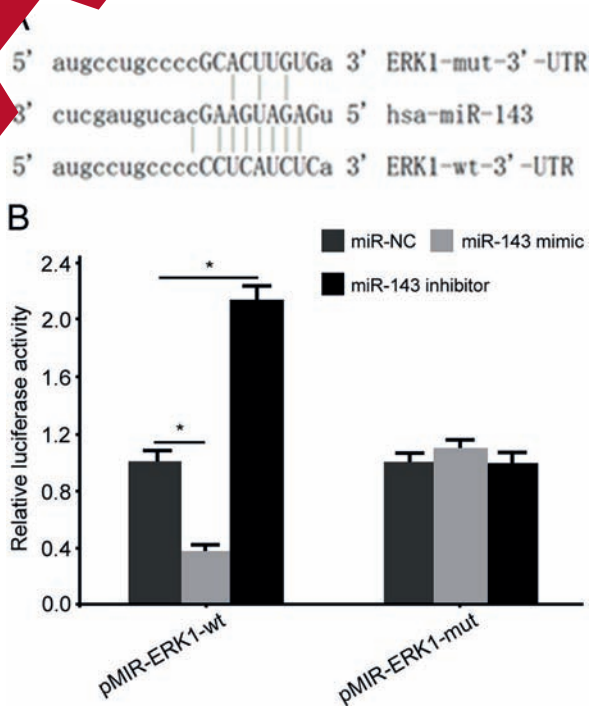


Figure 1. Targeted regulatory relationship between miR-143 and ERK1 mRNA. **A,** Binding sites between miR-143 and ERK1 mRNA. **B,** Dual luciferase gene reporter assay. *$p < 0.05$ comparing between two groups.

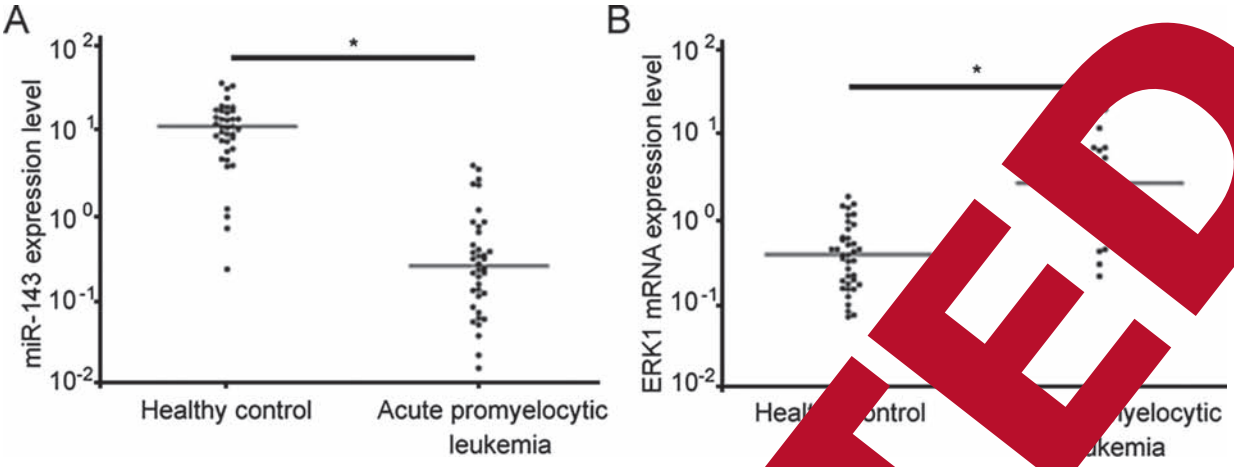


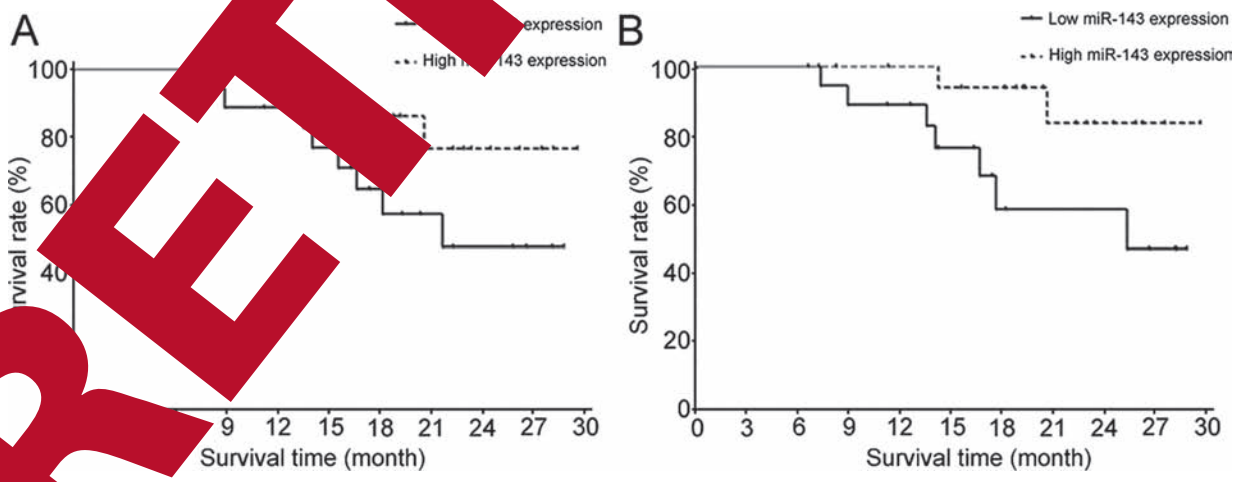
Figure 2. Down-regulation of miR-143 and up-regulation of ERK1 in APL [illegible] PCR for miR-143 expression in peripheral blood of two groups of people. **B,** ERK1 mRNA expression in [illegible] T-PCR. *$p<0.05$ comparing between two groups.

between miR-143 or ERK1 expression level and patients' survival or prognosis. Survival curve analysis showed significantly worse survival rate and prognosis in miR-143 down-regulated patients comparing to those with miR-143 over-expression (Log-rank test $\chi^2 = 5.873$, $p =$ [illegible] Figure 3B).

MiR-143 Over-Expression Significant[ly] Suppressed ERK1 Expression, Inhibited HL-60 Cell Proliferation and Induced Cell Apoptosis

qRT-PCR results showed [illegible] pared to miR-NC group, miR-143 [illegible] c tran[illegible] HL-60 cells presented signif[illegible]

[illegible]ession, whilst ERK1 mRNA expression level [illegible]emarkably de[illegible]ased (Figure 4A). Compar[illegible] siRNA-NC [illegible]up, siRNA-ERK1 transfect[illegible] [illegible]owed remarkably decreased ERK[illegible] [illegible]xpression (Figure 4A). Western blot test showed that transfection of miR-143 [illegible]mic or siRNA-ERK1 all significantly sup[illegible]RK1 and p-ERK1 protein expression [illegible]0 cells (Figure 4B). Flow cytometry results showed that comparing to miR-NC transfection group, miR-143 mimic transfected HL-60 cells presented significantly lower proliferation potency (Figure 4C) whilst cell apoptosis was remarkably enhanced (Figure 4D). Comparing to siRNA-NC transfection group, siRNA-ERK1



[illegible]3. Correlation between miR-143, ERK1 expression, and APL patient survival and prognosis. **A,** Comparison of s[illegible]e among patients with miR-143 over-expression and low-expression. **B,** Survival curves in patients with ERK1 mRN[illegible]er-expression and low-expression.

transfected HL-60 cells showed remarkably lower proliferation potency (Figure 4C) whilst cell apoptosis was enhanced (Figure 4D).

Discussion

APL is one special type of acute myelocytic leukemia (AML) with severe conditions. APL pathogenesis is mainly related to abnormality in cytogenetics. Such kinds of leukemia have high risk of hemorrhage, and may lead to various symptoms including anemia, bleeding, infection, fever, and infiltration into skin, liver, lymph node. Most patients presente... ditions and unfavorable prognosi... ne investi... gation of signal molecules with ...rmal change during APL pathogenesis and pr... n is thus of critical importance for diagnos... men... efficiency and improving ...gnosis[1,2].

MAPK signaling tra... action ...athway ... important system w... distri... d in euka...y-otes. It can activate ... cripti... factors and regulate mu... le p... ...nase v... ...tracel-

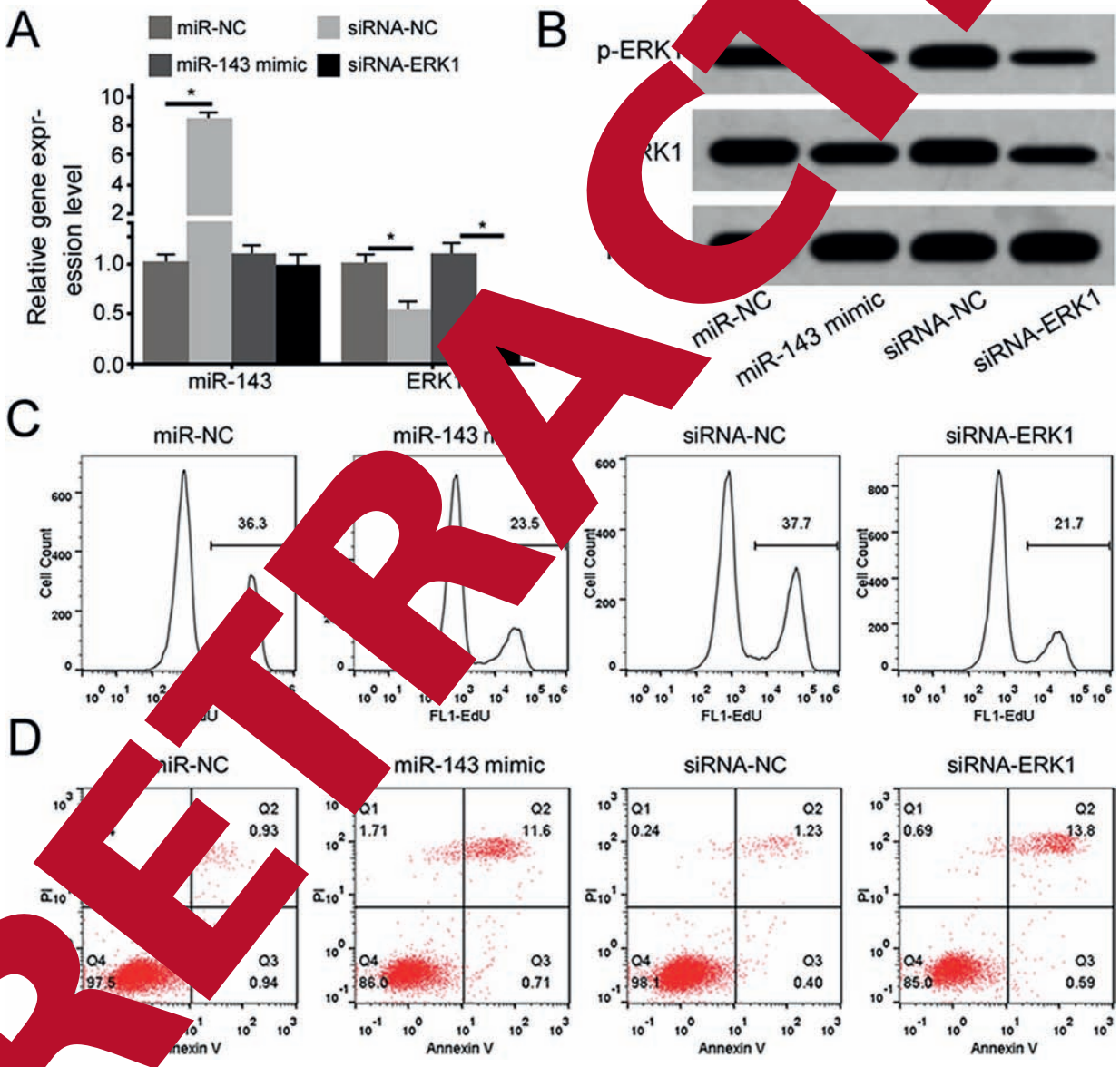


Figure 4. Over-expression of miR-143 significantly suppressed ERK1 expression, inhibited HL-60 cell proliferation and in... d apoptosis. **A,** qRT-PCR for gene expression. **B,** Western blot for protein expression. **C,** EdU staining for cell proli... ion. **D,** Flow cytometry for cell apoptosis. *$p < 0.05$ comparing between two groups.

lular receptor tyrosine kinase, G protein coupled receptor (GPCR), and cytokine receptor activation under various extracellular stimuli including cytokines, growth factors, neurotransmitters, and GPCR ligands, eventually modulating multiple physiological and biological processes including cell survival, proliferation, migration, and apoptosis[15,16]. Over-activation of MAPK signal pathway plays a crucial role in facilitating onset and progression of breast cancer[17], gastric carcinoma1[8-20]. MAPK signal pathway family mainly consists of four transducing pathways: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and ERK5/big MAP kinase 1 (BMK1). Among those ERK1-induced MAPK signal transducing pathway is the classical MAPK pathway, and the major route by which MAPK exerts its effects. This study quantified peripheral expression of miR-143 and ERK1 in APL patients to analyze its correlation with patient survival and prognosis, and utilized *in vitro* cultured cells to investigate if miR-143 played a role in modulating ERK1 expression and affecting proliferation or apoptosis of HL-60 cell

Dual luciferase gene reporter assay that transfection of miR-143 mimic rem bly decrease relative luciferase activity in HE T cells transfected with pMIR-ERK1-wt, but ha effect on HEK293T cells transfected with pM ERK1-mut, suggesting the t egulatio between miR-143 and ERK1 e further analyzed the expression l e of and ERK1 in peripheral blo C f tients and found decr ed xpressio peripheral blood of patie mpanied with elevated ER xpression, gges- ing the targeted n between i 43 and ERK1. In an st rding miR-143 and leukemia, Bat er et a d that AML patients ha nificantly lo R-143 expression in pheral blood neutr il granulocytes comp g to h y controlled cohorts. Elham- ma al[21] ormed a population study and show R-143 ession in plasma from AML pa dic g better clinical diagnolue of xpression for AML. Shen found t mpared to healthy control pe kemia patients presented significantly l expression in bone marrow tis- . Kovar a et al[22] found that comparing to y control people, leukemia patients present- miR-143 expression in peripheral blood. All e studies found significant depression of miR-143 expression in leukemia patients, indicating possible role of miR-143 as the t pressor gene in leukemia pathogene with our observation. However, w er miR-14 modulated leukemia pathogene still unclear. This study thus further investig ible regulatory mechanism of miR 43.

Survival curve anal showed lower miR-143 expre n wa accomp with higher ERK1 e ssion unfavora le prognosis of pati nd p nosis, indicating pote ial t ppres le of miR-143 whi RK1 p r facilitating role ther *in vitr* e of HL-60 cells t transfecti of miR-143 or siRN RK kably decreased ERK1 and p RK1 expr n HL-60 cells, inhibi cell prolifera nd enhanced cell a osis. Dou et al[13] sho ed that in MLL-AF4 n protein B lymphocyte leukemia cells, 143 promote gion displayed prominent lation, aco anied with lower miR-143 e . T -expression of miR-143 in ML n protein B lymphocyte leukemia cells remarkably weakened cell prolif- tion potency and induced cell apoptosis. l[23] found that during leukemia Jur- apoptosis, miR-143 expression was emarkably elevated, and over-expression of miR-143 significantly suppressed Jurkat cell proliferation and induced cell apoptosis. Shen et al[14] showed that over-expression of miR-143 could significantly suppressed chronic myeloid leukemia K562 cell proliferation, and weaken its proliferation or clonal formation potency via targeted inhibition on DNMTA3A expression, eventually arresting cell cycle progress and inducing cell apoptosis. Batliner et al[12] found that in using all-trans retinoic acid (ARTA) to induce granulocyte differentiation of APL cells NB4 and HT93, miR-143 expression was remarkably elevated, indicating possible involvement of miR-143 in differentiation regulation of leukemia cells, and the potential involvement of abnormally decreased miR-143 expression in myeloid cell differentiation dysfunction or leukemia pathogenesis. All these studies revealed the tumor suppressing role of miR-143 in regulating various biological behaviors of leukemia cells including proliferation and apoptosis, as similar with this study. We also analyzed abnormal expression profile of miR-143 and ERK1 in APL patient's peripheral blood samples, and combined miR-143 with

ERK1 targeted regulation, and found the role of miR-143 in mediating ERK1 expression and affecting proliferation and apoptosis of HL-60 cells, which have not been reported previously. However, the *in vivo* regulation on ERK1 expression or biological behaviors of leukemia by miR-143 is still unclear and requires further investigation and substantiation.

Conclusions

We showed that miR-143 down-regulation can induce ERK1 up-regulation, and is correlated with APL pathogenesis. The expression of miR-143 affects patient prognosis. miR-143 targets and inhibits ERK1 expression, significantly weakens proliferation potency of HL-60 cells, and induces cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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