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MicroRNA 34b inhibits cell proliferation in pediatric acute myeloid leukemia *via* regulating LDHA

H.-X. QI, Q. CAO, G.-P. ZHOU, X.-Z. SUN, W.-D. ZHOU, Z. HONG, J. HU, C.-X. JUAN, S. LI, W.-X. KUAI

Department of Pediatrics, The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University, Huaian, China

Abstract. – OBJECTIVE: To elucidate the regulatory effect of microRNA-34b on the occurrence of pediatric acute myeloid leukemia and the underlying mechanism.

PATIENTS AND METHODS: The expression of microRNA-34b in the bone marrow of 72 children with newly diagnosed acute myeloid leukemia (AML) was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between microRNA-34b expression and pathological characteristics was analyzed. Kaplan-Meier curve was introduced for evaluating the prognostic value of microRNA-34b in pediatric AML. The regulatory effects of microRNA-34b on proliferation, cell cycle, and apoptosis of leukemia cells were accessed by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Bioinformatics prediction and dual-luciferase reporter gene assay were conducted to evaluate the binding between microRNA-34b and lactate dehydrogenase A (LDHA). LDHA expression after overexpression of microRNA-34b was determined by qRT-PCR and Western blot. Rescue experiments were conducted to verify whether microRNA-34b could regulate proliferative and apoptotic behaviors of leukemia cells by suppressing LDHA expression.

RESULTS: MicroRNA-34b was markedly downregulated in AML children. Low expression of microRNA-34b was correlated to FAB typing, cytogenetic abnormality, and day 7 response to the treatment of pediatric AML. By collecting the follow-up data, it was found that low expression of microRNA-34b was correlated to the poor prognosis of AML. Overexpression of microRNA-34b inhibited proliferative ability and cell cycle progression, but accelerated apoptosis of AML cells. Dual-luciferase reporter gene assay verified that microRNA-34b could bind to LDHA, thereafter inhibiting LDHA expression. Overexpression of LDHA reversed the regulatory effects of microRNA-34b on proliferation, cell cycle, and apoptosis of AML cells.

CONCLUSIONS: We found that microRNA-34b is lowly expressed in pediatric AML patients, and low expression of microRNA-34b may serve as an indicator of malignant progression and poor prognosis of pediatric AML. MicroRNA-34b may affect the proliferation and apoptosis of leukemia cells by regulating the expression of LDHA.

Key Words:
Pediatric AML, MicroRNA-34b, Proliferation, Apoptosis.

Introduction

Leukemia is a malignant tumor of the hematopoietic system. It is a heterogeneous disease characterized by abnormal proliferation of myeloid or lymphoid blood precursor cells[1]. During the pathological progression of leukemia, the infinite proliferation of leukemia cells in bone marrow and other hematopoietic tissues change the amount and quality of peripheral blood cells. Leukemia is the most frequent malignant tumor in children, with an incidence of 34/1 000 000, and is still on the rise. There are about 15,000 new pediatric leukemia cases in China each year. More than 90% of pediatric leukemia pathologically belong to acute leukemia (AL), and chronic leukemia only accounts for 3-5%[2-4]. With the clinical application of combination chemotherapy and hematopoietic stem cell transplantation technology, the survival rate of pediatric leukemia has been greatly improved[5]. Unfortunately, there are still 25-30% of pediatric leukemia cases experience bone marrow, testicular or central nervous system recurrence[6]. Searching for new therapeutic and prognostic markers are necessary to improve the clinical outcomes of pediatric leukemia. Researches on the molecular mechanism of leukemia mainly focus on chromosomal abnormalities and protein-coding genes for a long time[7]. In recent years, microRNAs have been identified to be closely related to the incidence, development, clinical manifestations, and prognosis of leukemia[8]. They serve as oncogenes or tumor suppressors in leukemia[9].

Corresponding Author: Wenxia Kuai, MD; e-mail: kwx9743@163.com

MicroRNAs are a class of endogenous, single-strand, non-coding small RNAs of approximately 22 nt in length, and are widely found in eukaryotic cells[10]. As an essential regulatory factor, microRNAs regulate gene expressions at the post-transcriptional level through specific binding to target gene mRNA and thus degrade or inhibit target mRNA expression. They are widely involved in individual development, cell differentiation, proliferation, apoptosis, aging, and stress[11-13]. Numerous studies[14,15] have shown that abnormal expression levels of microRNAs in tumor cells are closely related to the incidence, development, and prognosis of tumors. The regulatory role of microRNAs in normal hematopoietic function has been recently confirmed, which may participate in the occurrence and progression of leukemia. These microRNAs are expected to serve as prognostic markers for leukemia[16].

MicroRNA-34b is a member of the microRNA-34 family and locates on chromosome 1p36[17]. Some researches[18,19] have shown that microRNA-34b is abnormally expressed in many malignant tumors. MicroRNA-34b is lowly expressed in colorectal cancer and gastric cancer tissues due to the high methylation of DNA in CpG island of the promoter region, thereafter alleviating tumor development. MicroRNA-34b inhibits the development of pancreatic cancer by targeting the proto-oncogene Smad3. Its low expression is positively correlated with TNM stage, lymph node metastasis, and overall survival of pancreatic[20]. However, the potential function of microRNA-34b in hematological malignancies is rarely reported.

This study determined the expression of microRNA-34b in pediatric AML at first, and explored its regulatory effects on proliferation and apoptosis of leukemia cells. We investigated whether microRNA-34b could exert its biological functions in pediatric AML by targeting lactate dehydrogenase A (LDHA).

Patients and Methods

Patients

Bone marrow samples were taken from 72 newly diagnosed pediatric AML patients. Meanwhile, bone marrow samples from 20 children with non-malignant tumors were collected as normal controls. Pediatric AML patients were diagnosed by bone marrow smear, immunophenotyping, fusion gene, and chromosome examination, and classified into M0-M7 according to FAB (French-American-British) criteria. The clinical information of enrolled pediatric AML patients was shown in Table I. This investigation got the approval of the Ethics Committee of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University and obtained the informed consent of their guardians.

Cell Lines

Leukemia cell lines HL60 and K562 were purchased from ATCC. $CD14^+$ bone marrow cells were isolated from peripheral blood of healthy subjects as normal controls. Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and 1% penicillin-streptomycin. They were maintained at 37°C, 90% of relative humidity, and 5% CO_2.

Cell Transfection

Cells with good viability were selected and transfected according to instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were transfected with microRNA-34b mimics, pcDNA-LDHA or corresponding negative controls. MicroRNA-34b mimics and pcDNA-LDHA were generated by GenePharma (Shanghai, China). Transfected cells were harvested at 48 h for experiments.

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

Tissue and cellular RNA were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The upper aqueous phase after centrifugation was incubated with isopropanol. After another centrifugation, the precipitate was washed, air dried, quantified and finally diluted in 20-30 μL of diethyl pyrocarbonate (DEPC). RNA samples were preserved in a -80°C refrigerator. Reverse transcription was performed according to the instructions of the TaKaRa OneStep PrimeScript® miRNA complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). PCR was performed using the SYBR Green I fluorescent dye method. The primer sequences were as follows: MicroRNA-34b, F: 5'-TTTAGTTACGCGTGTTGTGC-3', R: 5'-ACTACAACTCCCGAACGATC-3'; LDHA, F: 5'-AGGAGAAACACGCCTTGATTTAG-3', R: 5'-ACGAGCAGAGTCCAGATTACAA-3'; GAPDH, F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'; U6, F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'.

Table I. Correlations between microRNA-34b expression and the clinicopathological features of pediatric AML (n=72).

Parameters	Number of cases	MiR-34b expression		*p*-value
		Low	High	
Age (years)				0.313
≤6	29	17	12	
>6	43	20	23	
Sex				0.780
Male	42	21	21	
Female	30	16	14	
FAB classification				0.016
M1-M6	63	29	34	
M7	9	8	1	
Leukocyte counts (/uL)				0.436
>10,000	44	21	23	
≤10,000	28	16	12	
Cytogenetics				<0.001
Favorable	18	2	16	
Intermediate	40	22	18	
Unfavorable	14	13	1	
Day 7 response to treatment				0.006
Favorable	46	18	28	
Unfavorable	26	19	7	

Western Blot

Cells were lysed in phenylmethylsulfonyl fluoride (PMSF) containing a protease inhibitor and were collected on ice. Lysis was centrifuged at 12 000 r/min for 20 min. The supernatant was taken for determining total protein concentration using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). 50 μg of total protein of each sample was harvested for electrophoresis and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking the non-specific sites, membranes were incubated with the primary and corresponding secondary antibodies, followed by band exposure using chemiluminescence.

Cell Proliferation Assay

After transfection for 48 h, cells were cultured in 96-well plates at a cell density of 1×10^6/mL with 100 μL per well. Each group set 5 replicates. 10 μL of cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added at 6 h, 24 h, 48 h, and 72 h, respectively. Two hours later, the absorbance at 450 nm was recorded using a microplate reader. The growth curve was introduced with the culture time as the horizontal axis and absorbance as the vertical axis.

FCM

Transfected cells were inoculated into 6-well plates with 1×10^5/well. After 24 hours of culture, cells were collected by trypsin, washed three times with pre-cooled PBS and fixed with ethanol at 4°C overnight. The propidium iodide (PI) was added and incubated in the dark for 25 min, followed by cell cycle determination using FCM.

For cell apoptosis determination, cells were digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin. A total of 1×10^5 cells were suspended in 100 μL of 1×Annexin buffer, stained with 5 μL of Annexin V and 1 μL of PI. After a 15-min incubation, 400 μL of 1×loading buffer was supplied and apoptosis was determined using FCM.

Dual-Luciferase Reporter Gene Assay

The binding site of microRNA-34b and LDHA was predicted by bioinformatics. The LDHA 3'UTR containing the wild-type or mutant-type sequences of the binding sites was cloned into the psi-CHECK2 luciferase reporter vector, respectively. Cells were co-transfected with microRNA-34b mimics and wild-type or mutant-type sequences for determining the luciferase activity.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad statistical software (La Jolla, CA, USA). The chi-square test was used for comparing classification data and the Student's *t*-test was used for comparing measurement data. All measurement data were expressed as mean±SD. Survival analysis was conducted to evaluate the prognosis of pediatric AML based on the expression of microRNA-34b. The difference was statistically significant at $p<0.05$.

Results

MicroRNA-34b Expression Downregulates in Pediatric AML

We detected the expression of microRNA-34b in the bone marrow of 72 pediatric AML patients and 20 healthy controls by qRT-PCR. MicroRNA-34b was lowly expressed in pediatric AML patients than controls, suggesting that microRNA- 34b may be a tumor suppressor in AML (Figure 1A). Identically, the expression level of microRNA-34b markedly decreased in AML cell lines HL60 and K562 (Figure 1C).

Low Expression of MicroRNA-34b Correlates with Advanced Clinicopathological Features and Poor Prognosis of Pediatric AML Patients

We thereafter analyzed the relationship between microRNA-34b expression with age, sex, FAB, leukocyte counts, cytogenetic abnormality, and day 7 response to the treatment of pediatric AML patients. Pediatric AML patients with low expression of microRNA-34b were more prone to FAB classification of subtype M7, unfavorable cytogenetic abnormality, and day 7 response to the treatment than those with low expression. We did not observe a correlation between microRNA-34b expression with age, sex and leukocyte counts of pediatric AML patients (Table I). Besides, Kaplan-Meier curves showed that pediatric AML patients with high expression of microRNA-34b were expected to have a better prognosis than those with low expression (Figure 1B).

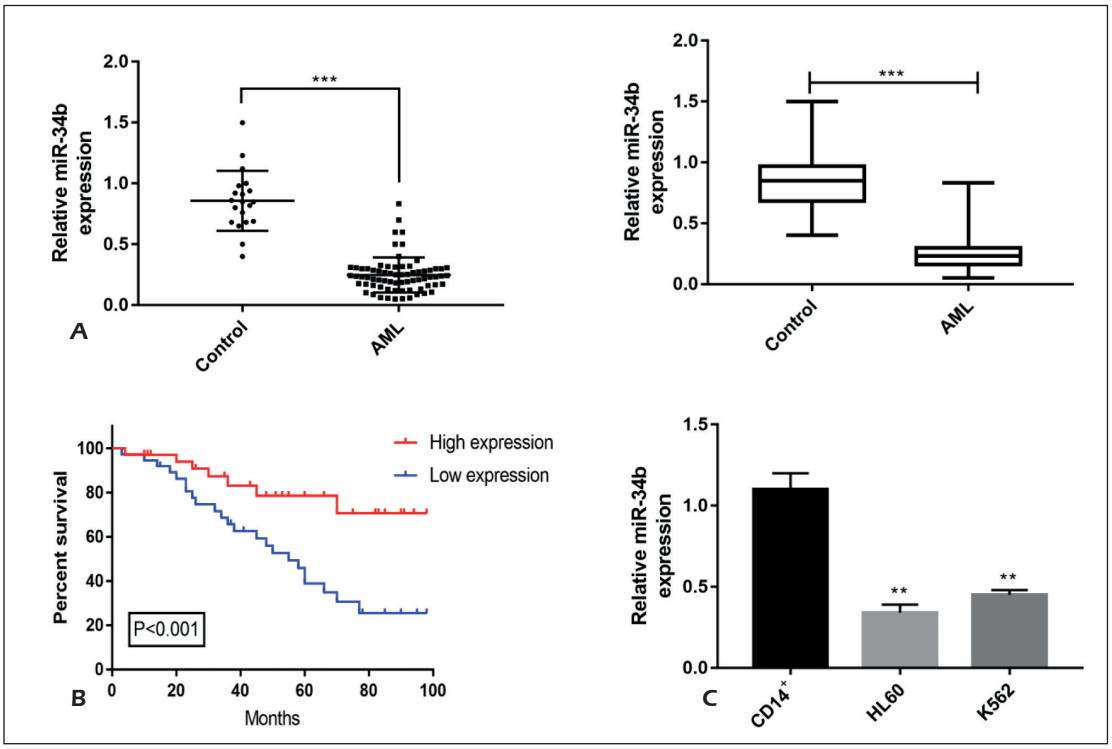

Figure 1. MicroRNA-34b expression downregulates in pediatric AML. **A**, MicroRNA-34b was lowly expressed in pediatric AML patients than controls. **B**, Kaplan-Meier curves showed that pediatric AML patients with high expression of microRNA-34b were expected to have a better prognosis than those with low expression. **C**, MicroRNA-34b was lowly expressed in AML cell lines. Data were expressed as mean±SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

MicroRNA-34b Overexpression Inhibits Proliferative Potential of Leukemia Cells

We detected the expression of microRNA-34b in HL60 and K562 cells transfected with microRNA-34b mimic or NC, respectively by qRT-PCR. The results showed that microRNA-34b mimic transfection markedly increased the expression of microRNA-34b in HL60 and K562 cells (Figure 2A). Subsequently, proliferative potential of leukemia cells was reduced by microRNA-34b overexpression as CCK-8 assay indicated (Figure 2B).

MicroRNA-34b Overexpression Inhibits Cell Cycle and Accelerates Apoptosis of Leukemia Cells

We observed the cell cycle in HL60 and K562 cells following microRNA-34b mimic transfection to determine the involvement of microRNA-34b in cell cycle progression. As shown in Figure 2C, the ratios of cells in G0/G1 phase increased in microRNA-34b mimics group compared with NC group (Figure 2C). FCM data revealed that microRNA-34b overexpression remarkably induced apoptosis of leukemia cells (Figure 2D).

MicroRNA-34b Targets and Degrades LDHA

We predicted the target gene of microRNA-34b by bioinformatics. Wild-type and mutant-type LDHA plasmids were first constructed (Figure 3A). Dual-luciferase reporter gene assay demonstrated a decreased luciferase activity in cells co-transfected with microRNA-34b mimics and pGL3-LDHA WT, whereas no significant difference was observed in LDHA-MUT group (Figure 3B). The above data confirmed the binding between microRNA-34b and LDHA. Subsequently, mRNA and protein levels of LDHA in leukemia cells overexpressing microRNA-34b were determined. LDHA expression was negatively regulated by microRNA-34b at both mRNA and protein levels (Figure 3C and 3D). These results indicated that microRNA-34b can target inhibit LDHA expression.

LDHA Reverses MicroRNA-34b Activity

After co-overexpression of microRNA-34b and LDHA in HL60 and K562 cells, the proliferative capacity was detected by the CCK-8 assay. The results showed decreased proliferative capacity after overexpression of microRNA-34b, which was reversed by overexpression of LDHA (Figure 4A). Similarly, we found that overexpression of LDHA partially reversed the increase in apoptotic rate caused by overexpression of microRNA-34b (Figure 4B). These results elucidated that microRNA-34b could inhibit cell proliferation and accelerate apoptosis by inhibiting the expression of LDHA.

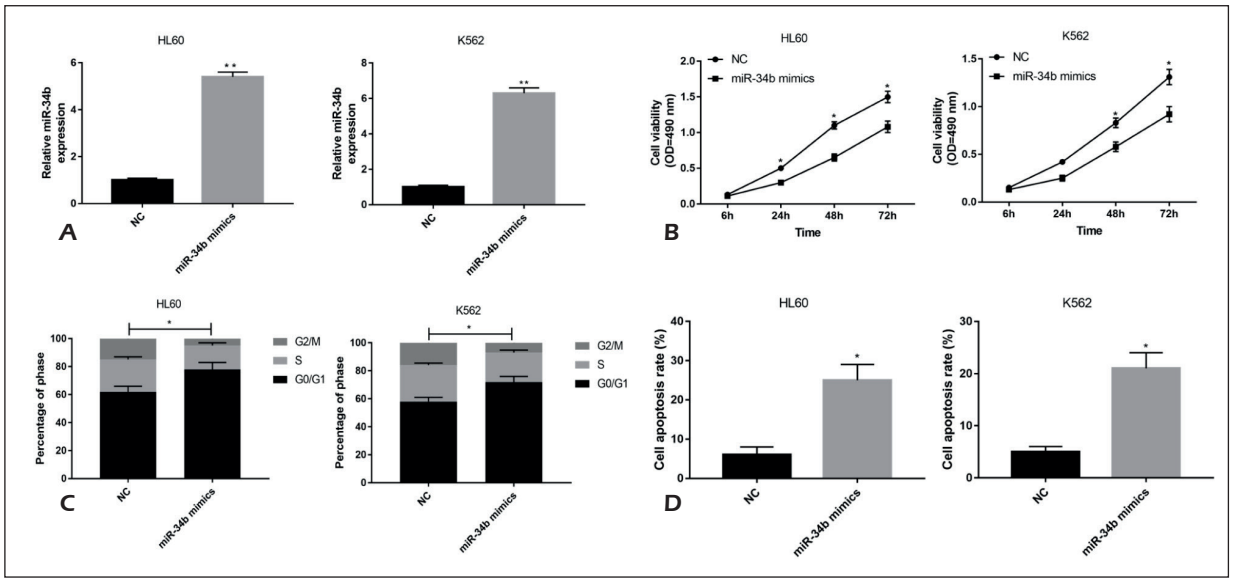

Figure 2. MicroRNA-34b overexpression inhibited proliferative potential but accelerated apoptosis of leukemia cells. **A**, Transfection of microRNA-34b mimic in HL60 and K562 cells upregulated microRNA-34b expression. **B**, CCK-8 assay showed that transfection of microRNA-34b mimic in HL60 and K562 cells decreased proliferation. **C**, Transfection of microRNA-34b mimic in HL60 and K562 cells arrested cell cycle in G0/G1 phase. **D**, Transfection of microRNA-34b mimic in HL60 and K562 cells accelerated apoptosis. Data were expressed as mean±SD (*p<0.05, **p<0.01).

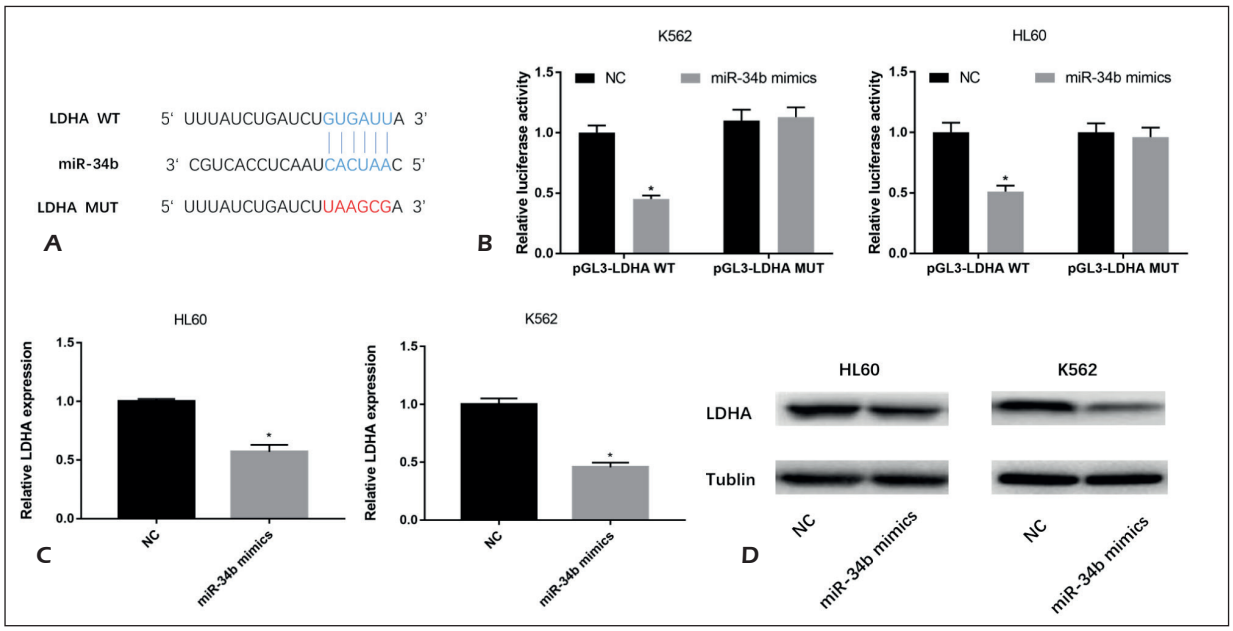

Figure 3. MicroRNA-34b targets and degrades LDHA. **A**, Construction of wild-type and mutant-type LDHA plasmids. **B**, Dual-luciferase reporter gene assay demonstrated a decreased luciferase activity in cells co-transfected with microRNA-34b mimics and pGL3-LDHA WT, whereas no significant difference was observed in LDHA-MUT group. **C**, The mRNA level of LDHA in HL60 and K562 cells overexpressing microRNA-34b decreased. **D**, The protein level of LDHA in HL60 and K562 cells overexpressing microRNA-34b decreased. Data were expressed as mean±SD (*p<0.05).

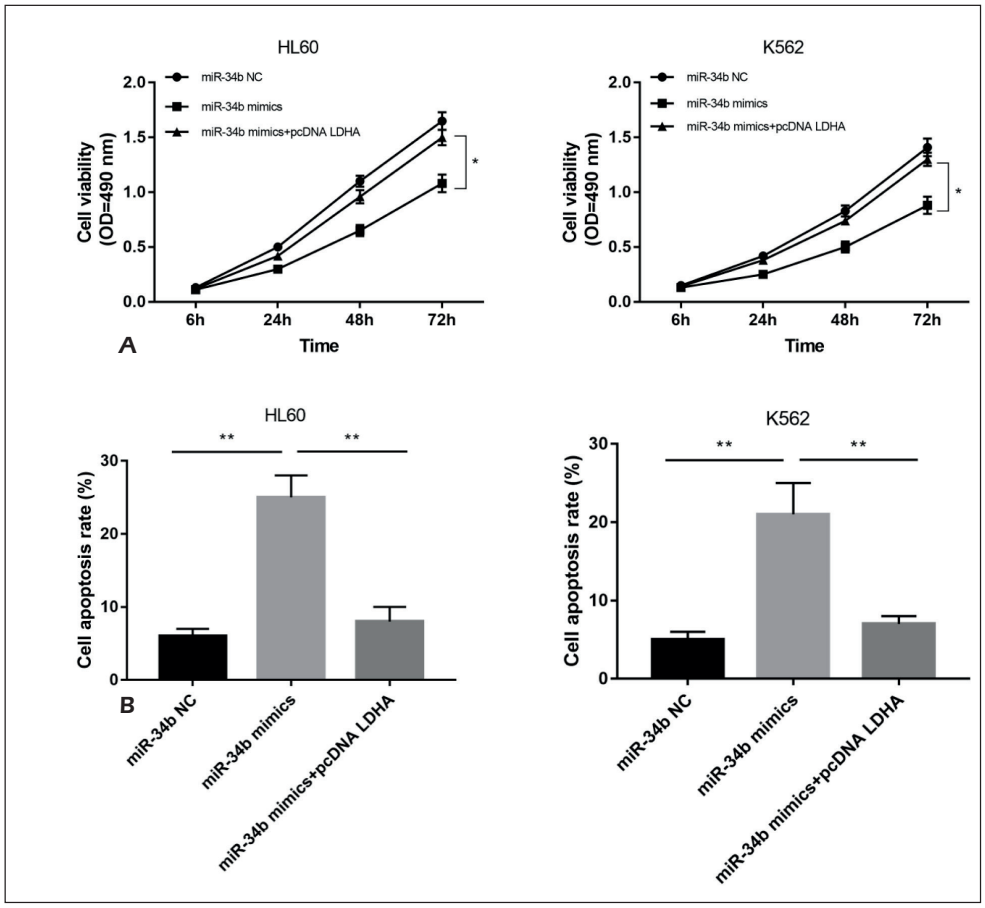

Figure 4. LDHA reverses microRNA-34b activity. **A**, CCK-8 assay showed decreased proliferation of HL60 and K562 cells overexpressing microRNA-34b, which were reversed by LDHA overexpression. **B**, Increased apoptotic rate of HL60 and K562 cells overexpressing microRNA-34b were reduced by LDHA overexpression. Data were expressed as mean±SD (*p<0.05, **p<0.01).

Discussion

AML is a frequent malignancy in children, seriously threatening children's health and life[21]. Some studies[22,23] have found the regulatory role of microRNAs in the incidence, development, invasion, metastasis, and apoptosis of tumors as oncogenes or tumor suppressors. They degrade mRNAs or inhibit post-transcriptional translation through completely or incompletely pairing with the 3'UTR of target gene mRNA, thus silencing target genes to participate in multiple biological processes[24]. In hematological diseases, abnormally expressed microRNAs can lead to the development and progression of hematological malignancies[8]. They can affect the directed differentiation of hematopoietic cells and is associated with the pathogenesis of leukemia and lymphoma.

MicroRNA-34b belongs to a member of the human microRNA-34 family alongside with microRNA-34a and microRNA-34c. It locates on chromosome 11q23 and is transcribed together with microRNA-34c as a gene cluster[25]. Some studies have shown that microRNA-34b is involved in the regulation of tumor cell proliferation. As a tumor-suppressor gene, microRNA-34b regulates the cell cycle of prostate cancer cells by synergistically acting with the p53 pathway[26]. MicroRNA-34b reduces the malignancy of bladder cancer T24 cells by downregulating the expression of c-myc[27].

This investigation focused on the function of microRNA-34b in pediatric AML and explored its possible mechanism of affecting the abnormal biological behaviors of leukemia cells. MicroRNA-34b expression remained at a low level in the bone marrow of pediatric AML patients, as well as in leukemia cell lines. Moreover, the low expression of microRNA-34b was associated with FAB typing, unfavorable cytogenetic abnormality, and day 7 response to the treatment in pediatric AML patients. Follow-up data analysis showed that low expression of microRNA-34b was correlated with poor prognosis of pediatric AML. The higher the microRNA-34b expression level, the worse the prognosis of pediatric AML. Functional studies suggested that overexpression of microRNA-34b inhibited proliferative capacity and cell cycle progression, but accelerated apoptosis of leukemia cells, indicating the crucial role of microRNA-34b in pediatric AML.

Lactate dehydrogenase A (LDHA) is an important enzyme in glycometabolism, participating in the conversion process of pyruvate into lactic acid, that is, glycolysis[28]. LDHA is able to regulate cell proliferation and apoptosis of tumor cells. It is reported that LDHA overexpression inhibits apoptosis and accelerates the proliferation of lung cancer cells and cholangiocarcinoma cells due to the insufficient energy[29]. LDHA overexpression allows the adaption of tumor cells to energy metabolism by providing a self-defense mechanism. In malignancies such as liver cancer and pancreatic cancer, LDHA deficiency induces changes of cellular energy metabolism and oxidative stress, eventually leading to cell death[30,31]. Reduction of LDHA can inhibit mitochondria-mediated apoptosis of breast cancer cells induced by oxidative stress.

LDHA was predicted to be a downstream target gene of microRNA-34b by bioinformatics, and the binding condition between them was further verified through the dual-luciferase reporter gene assay. Overexpression of microRNA-34b decreased the expression of LDHA at mRNA and protein levels. Furthermore, LDHA overexpression could reverse the regulatory effects of microRNA-34b on proliferative capacity and apoptotic rate of leukemia cells. Our study proved that microRNA-34b regulated proliferation and apoptosis of pediatric AML by targeting LDHA.

Conclusions

We found that microRNA-34b is lowly expressed in pediatric AML patients, and low expression of microRNA-34b may serve as an indicator of malignant progression and poor prognosis of pediatric AML. MicroRNA-34b may affect the proliferation and apoptosis of leukemia cells by regulating the expression of LDHA.

Conflict of Interests

The authors declare that they have no conflict of interest.

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