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MicroRNA-125a regulates proliferation and apoptosis of acute myeloid leukemia through targeting NF-κB pathway

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Abstract. – OBJECTIVE: To elucidate the influence of microRNA-125a on the biological behaviors of acute myeloid leukemia (AML) cells.

MATERIALS AND METHODS: MicroRNA-125a mimic and negative control (NC) were constructed and transfected into AML cell line HL60, respectively. Cell viability of HL60 cells transfected with microRNA-125a mimic or NC was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Regulatory effects of microRNA-125a on enzyme activities of B-cell lymphoma-2 (Bcl-2), Bcl-xl, caspase-3, and caspase-9 in HL60 cells were quantified by a spectrophotometry. Changes in apoptosis and invasion of HL60 cells overexpressing microRNA-125a were detected by flow cytometry and transwell assay, respectively. Protein levels of cell cycle genes (cyclin B, cdc-2, mdm-2), pro-apoptotic gene p53 and anti-apoptotic gene Bcl-2 in HL60 cells transfected with microRNA-125a mimic or NC were assessed by Western blot. Finally, the mRNA levels of Bax, caspase-8, nuclear factor-κB (NF-κB), and c-myc in HL60 cells with microRNA-125a overexpression were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: MicroRNA-125a expression remarkably increased by transfection of microRNA-125a mimic into HL60 cells, suggesting its sufficient transfection efficacy. MTT assay revealed an inhibited viability after microRNA-125a overexpression. Transfection of microRNA-125a mimic markedly enhanced enzyme activities of caspase-3 and caspase-9, but reduced activities of Bcl-2 and Bcl-xl in HL60 cells than controls ($p<0.05$). Moreover, microRNA-125a overexpression elevated apoptotic rate as FCM data indicated. Transwell assay demonstrated a decrease in the invasive rate of HL60 cells overexpressing microRNA-125a. Western blot analyses revealed that cell cycle genes all downregulated by transfection of microRNA-125a mimic in HL60 cells. The protein level of p53 upregulated and Bcl-2 downregulated in HL60 cells overexpressing microRNA-125a ($p<0.05$). Furthermore, mRNA levels of pro-apoptotic genes Bax and caspase-8 were enhanced after microRNA-125a overexpression, while mRNA levels of NF-κB and c-myc were reduced ($p<0.05$).

CONCLUSIONS: MicroRNA-125a inhibits proliferative and invasive potentials, arrests the cell cycle in the G2/M phase of AML cells by regulating the NF-κB pathway.

Key Words
MicroRNA-125a, NF-κB pathway, Proliferation, Apoptosis.

Introduction

Acute myeloid leukemia (AML) is a hematological malignancy with a high heterogeneity in cell morphology, molecular biology, cytogenetics, and immunophenotyping. AML is characterized by maturation arrest of myeloid progenitor cells at different stages, malignant proliferation and impaired apoptosis, and are accompanied by cytogenetic abnormalities and mutations[1]. Although progresses in supportive treatment and prognostic risk stratification greatly optimize the treatments, the overall long-term survival of AML still remains very low[2]. Novel therapeutic approaches for AML are urgently required. Currently, the specific pathogenesis of AML has not been comprehensively elucidated, which greatly hinders the development of novel treatments for AML[3].

MicroRNAs are a class of endogenous, small RNAs with a length of about 20-24 nucleotide residues, which are widely present in various organisms[4]. MicroRNAs could not encode proteins, but regulate gene expressions, cellular behaviors, and individual development. They are very important in the regulation of cell proliferation, differentiation, and apoptosis through inhibiting translation

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or degrading target mRNA by complementary binding to its 3' untranslated region (3'UTR)[5]. The latest studies[6-8] have demonstrated the involvement of microRNAs in the proliferation, differentiation, apoptosis, and migration of tumor cells. They are expected to be potential and promising tools for tumor diagnosis and treatment. Relative microRNAs have been identified to participate in the occurrence, development, and prognosis of AML as oncogenes or tumor suppressors. For example, miRNA-192 regulates proliferation and cell cycle progression of AML cells, thereby controlling the disease development[9,10]. MicroRNA-223 can inhibit cell proliferation and promote apoptosis of leukemia cells by targeting the FBXW7 pathway. MicroRNA-125a exerts a crucial role in the normal hematopoietic system by regulating blood cell proliferation and differentiation. Several studies have confirmed the different functions of the microRNA-125 family in the development of various tumors. MicroRNA-125a is usually considered as a tumor suppressor in solid tumors. As an oncogene, microRNA-125b is highly expressed in AML with chromosomal abnormalities and gene mutations, and its expression serves as an indicator for monitoring the disease state[11].

NF-κB (nuclear factor-κB) is a nuclear transcription factor regulating multiple cellular functions by mediating expressions of a series of related genes[12]. Under normal physiological conditions, NF-κB mainly distributes in the cytoplasm and binds to the subunit of NF-κB inhibitor IκBs in a rest state. External stimuli release NF-κB from the IκBs subunit, phosphorylate and degrade IκB-α. NF-κB subunit p65 subsequently releases a nuclear sublocalization signal and thus initiates transcription of specific genes[13]. According to previous studies[14,15], NF-κB expression markedly upregulates in some tumor tissues, including colon and pancreatic cancer. It is believed that activated NF-κB serves as a potential therapeutic target for malignant tumors[16,17]. In addition, NF-κB has been proved to be a target gene for some microRNAs in malignancies. For example, miRNA-223 promotes the progression of lung cancer by activating NF-κB pathway[18]. MiR-429 prevents cervical cancer progression through negatively regulating NF-κB activation[19].

In this work, the expression level of microRNA-125a in leukemia cells was altered by cell transfection technology. We investigated the potential role of microRNA-125a in AML through a series of functional experiments. Our study aims to provide novel therapeutic approaches for AML.

Materials and Methods

Cell Culture

The human AML cell line HL60 was routinely cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL streptomycin and 100 U/mL penicillin. Cells were maintained in a 37°C, 5% CO_2 incubator and passaged every 2-3 days depending on the cell growth. Cells in the logarithmic growth phase were harvested and adjusted to an appropriate density for use.

Transfection

Cell transfection was performed at 70-80% of confluence. HL60 cells were transfected with microRNA-125a mimic or NC using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), respectively. In brief, transfection plasmid and Lipofectamine 3000 were respectively diluted in an appropriate amount of Opti MEM. After the mixture of these two solutions and maintained for 5 min, it was added in each well for 48 h transfection.

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

Total RNA was extracted from transfected HL60 cells for 48 h and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the relative kit (TaKaRa, Otsu, Shiga, Japan). Primers of microRNA-125a and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were constructed using Primer 5.0 Software according to their mRNA sequences searched from the Gen Bank. Relative expressions of target genes were analyzed by qRT-PCR and calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: Bax, F: 5'-C ACGATGCACCTGTACGATCAC-3', R: 5'-CCTTTCAACACGCAGGACGA-3'; microRNA-125a, F: 5'-CATAAAGACATACTCCAAACTG-3', R: 5'-CTTCTCCACAACCCTCTGG-3'; caspase-8, F: 5'-CAGAGGGAAGAGTTCCCCAG -3', R: 5'-TCCTTGGTCTGGTAGGAGACGT-3'; NF-κB, F: 5'-AGCAGCGGCATTTGGACAA-3', R: 5'-CGTGCGAATAGCGACAGTTCT-3'; c-myc, F: 5'-CGTGAATGATAGTGAGGAAC-3', R: 5'-GTGAACGATTTGCCACACACAT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

200 μL of a suspension containing 1×10^4 transfected cells was added in each well of 96-well plates, with 6 replicates in each group. MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added, gently mixed and incubated for 4 h. Cells in each well were collected into a 1.5 mL Eppendorf (EP) tube, centrifuged, and the precipitate was further incubated with 150 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). EP tubes were placed on a plate shaker for 10 min. Absorption value A at the wavelength of 490 nm of each well was recorded by a MTT enzyme-linked immunometric meter. Inhibitory rate of proliferation (%) = (1 – A value of experimental group / A value of blank control group) × 100%.

Enzyme Activity Determination of Bcl-2, Bcl-xl, Caspase-3, and Caspase-9

Cells were collected by centrifugation at 600 g/min, 4°C for 5 min. After phosphate-buffered saline (PBS) wash, cells were lysed in 100 μL of lysate on ice for 15 min. After centrifugation for 12 min, the supernatant was transferred on an ice-cold 96-well plate for determining absorbance at 405 nm and plotting the standard pNA curve.

Apoptosis Determination

HL60 cells transfected with microRNA-125a mimic or NC for 48 h were collected, washed with PBS and incubated with 300 μL of loading buffer. Subsequently, 3 μL of Propidium Iodide (PI) and Annexin V were added, and the mixture was incubated for 10 min in the dark. Cell apoptosis was determined using flow cytometry (FCM) (Partec AG, Arlesheim, Switzerland).

Cell Invasion Assay

Cells were collected, washed with PBS and serum-free RPMI-1640, and centrifuged. Cells were resuspended in serum-free RPMI-1640 with the adjusted cell density at 2×10^5/mL. In the basolateral chamber (i.e., at the bottom of the 24-well plate), 600 μL of RPMI-1640 containing 10% FBS was added. 100 μL of cell suspension (about 2×10^4 cells) was added to the apical chamber. Three replicate wells were set in each group. After cell culture for 24 h, the number of cells invading into the basolateral chamber was counted.

Western Blot

Total protein was extracted from cell lysis, quantified and electrophoresed. After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were incubated with primary antibodies of cyclin B, cdc-2, mdm-2, p53, and B-cell lymphoma-2 (Bcl-2) at 4°C. At the other day, membranes were incubated with the corresponding secondary antibody for 2 h. Bands were exposed with the enhanced chemiluminescence (ECL), and integrated optical density was analyzed by gel imaging analysis system with the internal reference of GAPDH.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 17.0 statistical software (IBM, Armonk, NY, USA). Quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). Data were analyzed by the Chi-square test and *t*-test, respectively. $p<0.05$ was considered as statistically significant.

Results

Transfection Efficacy of MicroRNA-125a Mimic

Relative expression of microRNA-125a was remarkably higher in HL60 cells transfected with microRNA-125a mimic compared to that in NC group. The difference in microRNA-125a expression was statistically significant, confirming the effective transfection ($p<0.001$, Figure 1A).

Overexpression of MicroRNA-125a Inhibited Viability of HL60 Cells

After transfection of microRNA-125a mimic or NC into HL60 cells for 48 h, MTT assay was conducted to determine the cell viability. As the data indicated, microRNA-125a overexpression reduced cell viability significantly ($p<0.05$, Figure 1B).

Overexpression of MicroRNA-125a Accelerated Apoptosis of HL60 Cells

At 48 h of transfection, HL60 cells overexpressing microRNA-125a had higher apoptotic rate than those transfected with NC ($p<0.001$, Figure 1C). It is indicated that microRNA-125a markedly accelerated apoptosis of AML cells.

Overexpression of MicroRNA-125a Inhibited Invasion of HL60 Cells

HL60 cells overexpressing microRNA-125a presented a lower invasive rate than controls, suggesting an inhibitory effect of microRNA-125a on the invasion of AML cells ($p<0.05$, Figure 1D).

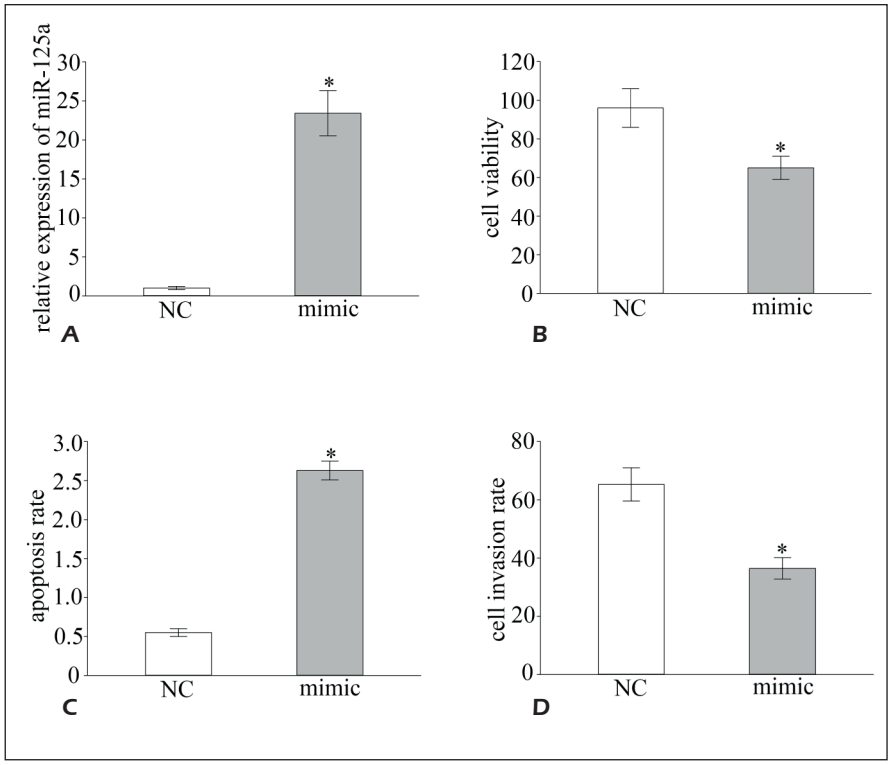

Figure 1. Effects of microRNA-125a on viability, apoptotic rate and invasive rate of HL60 cells. **A**, MicroRNA-125a expression in HL60 cells transfected with NC or microRNA-125a mimic. **B**, Cell viability of HL60 cells transfected with NC or microRNA-125a mimic. **C**, Apoptotic rate of HL60 cells transfected with NC or microRNA-125a mimic. **D**, Invasive rate of HL60 cells transfected with NC or microRNA-125a mimic. *p<0.05 *vs*. NC group.

Enzyme Activities of Bcl-2, Bcl-xl, Caspase-3, and Caspase-9

Enzyme activities of apoptosis-related genes were detected by spectrophotometry. Enzyme activities of Bcl-2 and Bcl-xl in cells with microRNA-125a overexpression were significantly lower than those of controls (p<0.05, Figure 2A and 2B). The activities of caspase-3 and caspase-9 were enhanced by microRNA-125a overexpression (p<0.05, Figure 2C and 2D).

Effect of MicroRNA-125a on NF-κ Pathway

After transfection of HL60 cells with microRNA-125a mimic or NC for 48 hours, protein expressions of cyclin B, cdc-2, mdm-2, and Bcl-2 in microRNA-125a overexpression group were reduced (p<0.05, Figure 3A and 3B). On the contrary, the microRNA-125a overexpression upregulated the protein expressions of p53 (p<0.05, Figure 3C and 3D).

Effect of MicroRNA-125a on Apoptosis

QRT-PCR was carried out to determine changes in apoptotic genes influenced by microRNA-125a in AML cells. By comparison with NC group, the mRNA levels of pro-apoptotic genes Bax and caspase-8 increased, whereas anti-apoptotic genes NF-κB and c-myc decreased (p<0.05, Figure 4).

Discussion

NF-κB belongs to a transcription factor family, which is widely present in cells and involved in various biological responses. It is a general term for a complex composed of multi-gene NF-κB-REL clusters. NF-κB is capable of regulating multiple gene expressions, thereby affecting a variety of physiological functions[19, 20]. In recent years, the biological effects of nuclear transcription factors have been gradually clarified. Immune response and inflammation regulation are the most significant functions of NF-κB[21,22]. In addition, it is also involved in regulating cell proliferation, differentiation, autophagy, aging, apoptosis, and adhesion[23,24]. Under pathological conditions, dysregulated NF-κB is observed in cancer, diabetes, autoimmune diseases, arthritis, chronic inflammation, cardiovascular disease, asthma, and neurodegenerative diseases[25-28].

Activated NF-κB is proved to enhance the anti-apoptotic ability of different types of tumor cells[29,30]. NF-κB activation is closely related to tumorigenesis, development, invasion, metastasis, and poor differentiation of tumor cells.

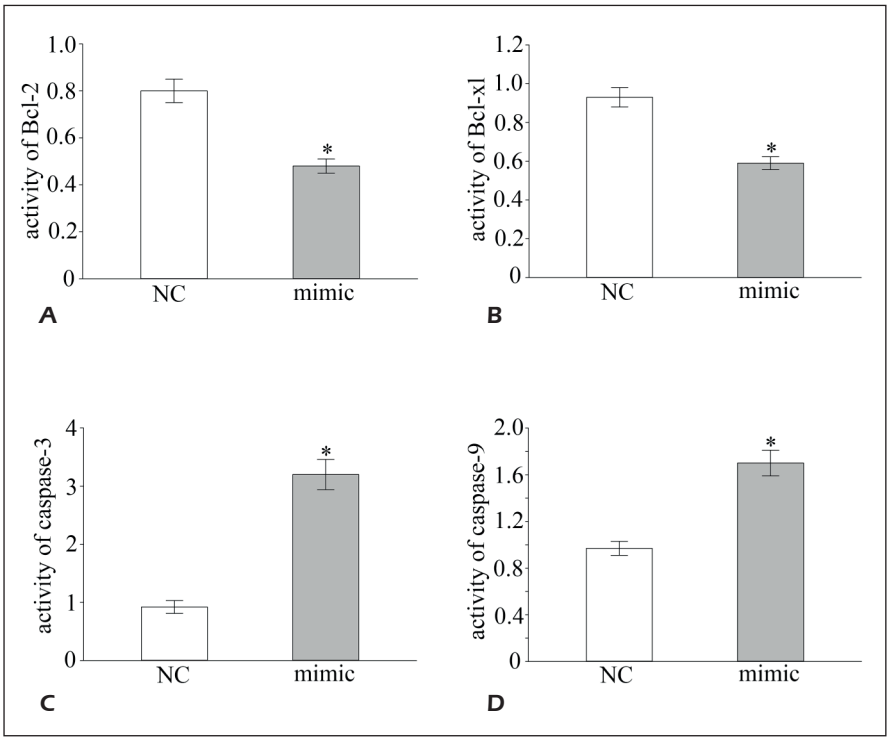

Figure 2. Enzyme activities of Bcl-2, Bcl-xl, caspase-3, and caspase-9. **A**, Bcl-2 activity in HL60 cells transfected with NC or microRNA-125a mimic. **B**, Bcl-xl activity in HL60 cells transfected with NC or microRNA-125a mimic. **C**, Caspase-3 activity in HL60 cells transfected with NC or microRNA-125a mimic. **D**, Caspase-9 activity in HL60 cells transfected with NC or microRNA-125a mimic. ***p*<0.05 *vs.* NC group.

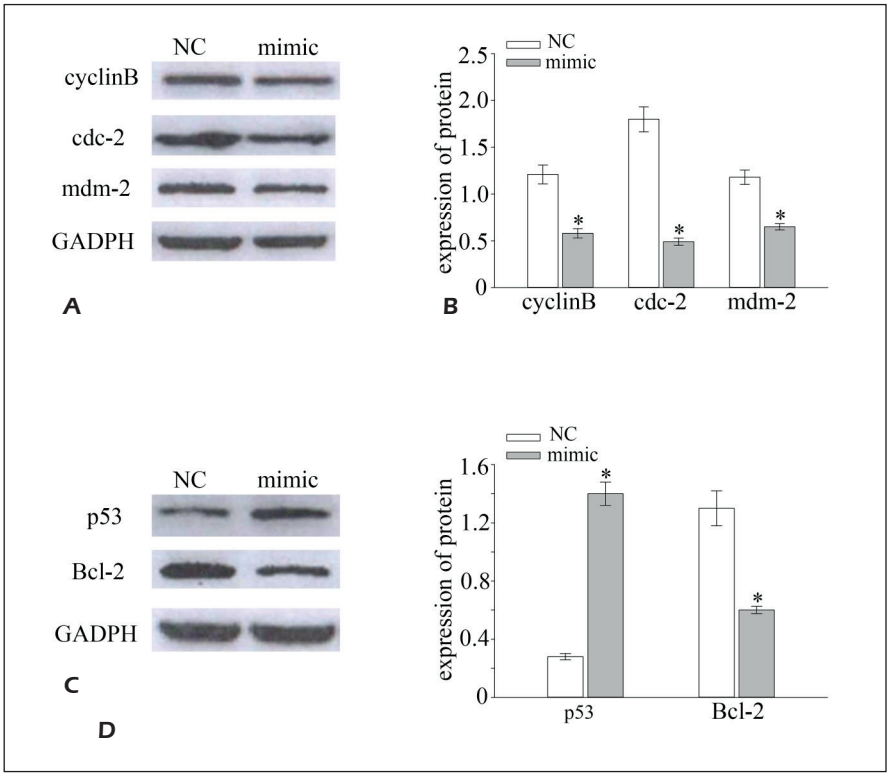

Figure 3. Effects of microRNA-125a on NF-κB pathway. **A**, Western blot analyses of cyclin B, cdc-2, and mdm-2 in HL60 cells transfected with NC or microRNA-125a mimic. **B**, Protein expressions of cyclin B, cdc-2, and mdm-2 in HL60 cells transfected with NC or microRNA-125a mimic. **C**, Western blot analyses of p53 and Bcl-2 in HL60 cells transfected with NC or microRNA-125a mimic. **D**, Protein expressions of p53 and Bcl-2 in HL60 cells transfected with NC or microRNA-125a mimic. **p*<0.05 *vs.* NC group.

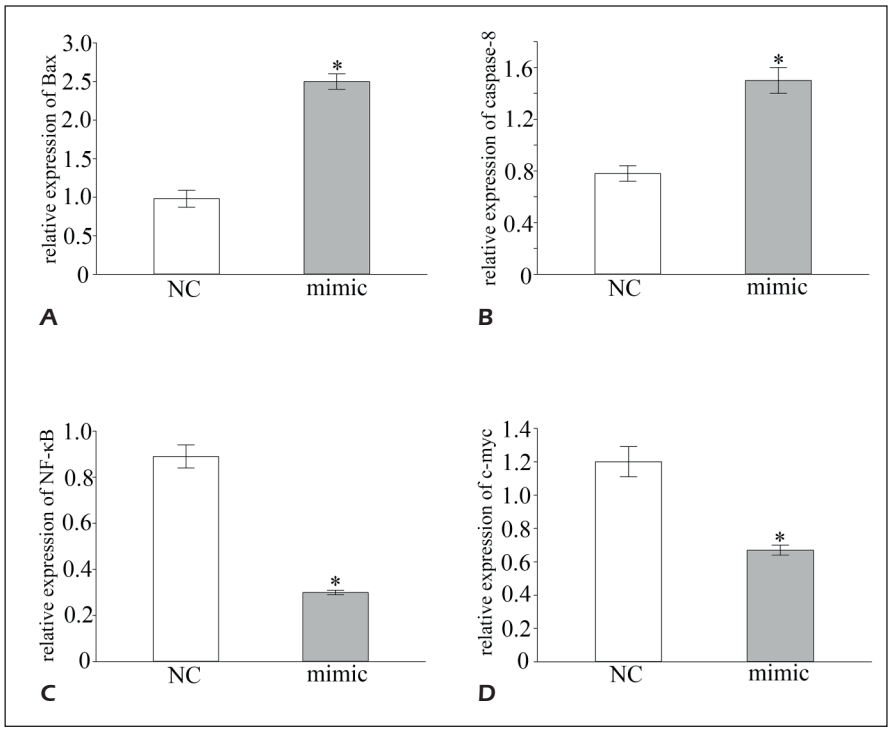

Figure 4. Effects of microRNA-125a on apoptosis. **A**, Relative expression of Bax in HL60 cells transfected with NC or microRNA-125a mimic. **B**, Relative expression of caspase-8 in HL60 cells transfected with NC or microRNA-125a mimic. **C**, Relative expression of NF-κB in HL60 cells transfected with NC or microRNA-125a mimic. **D**, Relative expression of c-myc in HL60 cells transfected with NC or microRNA-125a mimic. *$p<0.05$ *vs.* NC group.

Activated NF-κB can inhibit apoptosis and regulate proliferation[31]. NF-κB is a complex regulatory pathway. It regulates the apoptosis pathway through targeting anti-apoptotic or pro-apoptotic genes. Anti-apoptotic genes inhibit the death receptor pathway or the mitochondrial pathway by binding to NF-κB[32]. Bcl-2 family is the main apoptotic genes regulated by NF-κB. Anti-apoptosis is one of the basic characteristics of tumor cells, and high expressions of anti-apoptotic genes are frequently observed in the tumorigenesis[33]. Furthermore, NF-κB controls cell cycle progression by regulating cyclin B/cdc-2 and cyclin D1/CKD2. We believed that activated NF-κB could be utilized as a potential target for the treatment of malignant tumors[34].

The microRNA-125 family is involved in the development of various tumors as a promoter or repressor. MicroRNA-125a is commonly served as a tumor-suppressor gene to participate in the development of solid tumors. Rigolin et al[35] found that microRNA-125a expression is markedly reduced in chronic lymphocytic leukemia (CLL). Low expression of microRNA-125a is expected to be a marker for classifying subtypes of CLL. Moreover, short overall survival and poor prognosis are observed in CLL subtypes with lowly expressed microRNA-125a. This study elucidated that microRNA-125a overexpression accelerated apoptosis and inhibited invasion of AML cells, indicating a tumor inhibitory role of microRNA-125a in AML.

Apoptosis, also known as programmed cell death, is a cell-suicide program that stabilizes the homeostasis[36]. The ability to evade apoptosis of tumor cells provides us with a new tumor treatment strategy[37]. Here, we explored the influence of microRNA-125a overexpression on apoptosis of HL60 cells. Our results revealed downregulation of cell cycle genes cyclin B, cdc-2, and mdm-2 after microRNA-125a overexpression. Downregulation of genes related to G2/M phase indicated the arrested G2/M phase in HL60 cells after microRNA-125a overexpression. Expressions of pro-apoptotic proteins p53, Bax, caspase-3, caspase-8, and caspase-9 increased, whereas expressions of anti-apoptotic proteins Bcl-2 and Bcl-xl decreased in HL60 cells overexpressing microRNA-125a. Therefore, we proved the accelerated apoptosis of HL60 cells overexpressed microRNA-125a. Previous study confirmed the regulatory effect of the NF-κB pathway on cell cycle and apoptosis. Here, we found that the downstream genes of the NF-κB pathway were regulated by microRNA-125a in AML cells, showing that microRNA-125a may inhibit the carcinogenicity of NF-κB pathway activation in AML. It is concluded from this study that microRNA-125a

overexpression inhibited proliferation and accelerated apoptosis of HL60 cells through inhibiting NF-κB pathway, thus exerting its anti-tumor effect on AML.

In summary, microRNA-125a may be involved in the development of AML as a tumor suppressor. MicroRNA-125a could be utilized as a new target for diagnosis, treatment, and prognosis of AML.

Conclusions

We found that microRNA-125a inhibits proliferative and invasive potentials, as well as arrests cell cycle in the G2/M phase of AML cells. MicroRNA-125a promotes apoptosis of AML cells through regulating the NF-κB pathway.

Acknowledgements

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Conflict of Interests

The authors declare that they have no conflict of interest.

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