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Original Article

miR-126 promotes the growth and proliferation of leukemia stem cells by targeting DNA methyltransferase 1

Qian Ding, Qing Wang, Yi Ren, Hongqian Zhu, Zhujun Huang

Department of Hematopathology, Guizhou Provincial People's Hospital, Guiyang, Guizhou Province, China

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Abstract: Previous studies have showed that the interaction between microRNAs (miRNAs) and leukemia stem cells (LSCs) may be a cause of drug resistance of acute myeloid leukemia (AML). However, whether miR-126 participates in the pathogenesis of AML remains unclear. In our study, we first examined the expression of miR-126 in CD34+ or CD34- cells isolated from blood samples and LSC cell line: KG-1a-LSCs and MOLM13-LSCs by qRT-PCR analysis. Then miR-126 inhibitor and mimics were applied to evaluate the roles of miR-126 in cell proliferation of LSC cell lines using CCK-8 assay and Ki-67 staining. Moreover, flow cytometry analysis was used to assess the apoptosis of LSC cell lines treated with miR-126 inhibitor of mimics. In addition, we analyzed the relationship between miR-126 and DNA methyltransferase 1 (DNMT1) by bioinformatics analysis and dual-luciferase reporter assay. Western blot analysis was applied to examine the protein expression level of DNMT1 in miR-126 mimics treated LSC cells. Results showed that miR-126 expression was significantly higher in CD34+ cells and KG-1a-LSCs and MOLM13-LSCs. Knockdown of miR-126 in KG-1a-LSCs and MOLM13-LSCs inhibited cell proliferation, and promoted apoptosis. miR-126 could regulate DNA methyltransferase 1 (DNMT1) expression by directly binding to it. In conclusion, these findings suggested that miR-126 may promote cell proliferation of LSCs by targeting DNMT1.

Keywords: Acute myeloid leukemia (AML), leukemia stem cells (LSCs), miR-126, DNA methyltransferase 1 (DNMT1), proliferation, apoptosis

Introduction

Acute leukemia (AL), characterized by abnormal proliferation of hematopoietic stem cells in bone marrow, is one of the most serious malignant tumors in the world [1-3]. According to lineage, it can be classified into two major subtypes: acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML) [4]. AML, accounting for more than 70% of AL, is predominantly found in adults with a median age at 67 years [5, 6]. It has been established that 19,950 new cases of AML were diagnosed and 10,430 of them were dead in the United States in 2016 according to the American Cancer Society [7]. Evidence has suggested that more than half of elderly AML patients die within the first year of disease diagnosis, and the 2-year survival rate of them is less than 20% [8, 9]. Despite the tremendous advances in treatments for AML in the past decades, it is still challenging to treat patients with serious conditions.

Leukemia stem cells (LSCs), confined to the CD34+ and CD38- subgroup, were first found by J. E. Dick and confirmed in later numerous studies [10, 11]. It was reported that LSCs may be produced by the malignant transformation of normal hematopoietic stem cells [12, 13]. Recent studies have revealed that the presence of LSCs may be the fundamental cause of drug-resistance, relapse, and obstinacy of AML [14-16]. However, it is remains unclear how LSCscontribute to the initiation and development of AML. MicroRNAs (miRNAs), known as a new gene regulator in various biological processes, are an important subtype of non-coding RNA that lack the ability of protein translation [17-19]. Evidence has indicated that miRNAs could regulate proliferation, apoptosis, and metastasis of tumor cells by interacting

with the target mRNA [20, 21]. In recent years, miRNAs were demonstrated to be involved in the pathogenesis of various types of leukemia, including AML, suggesting that miRNAs may play a role in the initiation and development of AML [22, 23].

miR-126 was demonstrated to participate in the tumorigenesis of various cancers, such as gastric cancer, colorectal cancer, and lung cancer [24-26]. It was also reported to function as an oncogene in AML, and knockdown of it would induce LSCs to enter the cell cycle so that drugs could kill LSCs [27-29]. However, the mechanisms of how miR-126 regulates LSCs in AML remain undetermined.

In our study, we first examined the expression of miR-126 in both AML blood samples and cell lines: KG-1a-LSCs, and MOLM13-LSCs. Then, we established a miR-126 overexpressed cell model and a miR-126 knockdown cell model in KG-1a-LSCs and MOLM13-LSCs by transfecting them with miR-126 mimics or inhibitor, respectively. Subsequently, we analyzed the miR-126 effects on cell proliferation and apoptosis in these two cell models. Finally, we evaluated the relationship between miR-126 and DNA methyltransferase 1 (DNMT1), and assessed the roles of DNMT1 in the miR-126 signaling pathway in AML.

Materials and methods

Patient samples and ethics statement

Blood samples of AML patients (N = 40) or normal healthy individuals (N = 40) were collected in the Guizhou Provincial People's Hospital during May 2015 to June 2016. Informed consent was offered by each AML patient, and ethics approval was agreed by the Ethics Committee of the Guizhou Provincial People's Hospital. CD34+ or CD34- cells were isolated by magnetic-activated cell sorting (MACS) and maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 15 ng/mL epidermal growth factor.

Cell culture and transfection

KG-1a-LSCs and MOLM13-LSCs were obtained from ATCC (Rockville, Md., USA), and they were all maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2.

KG-1a-LSCs and MOLM13-LSCs were incubated in 6-well plates at 37°C for 24 h at a density of 1×10^5 cells/well, and then treated with miR-126 mimics (50 nM), inhibitors (50 nM), or negative control (miR-NC) by using Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's protocol.

Reverse transcription and real-time PCR

Cultured cells were lysed in TRIzol reagent (Invitrogen, CA, USA) for the extraction of total RNAs, and RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA) was used to transformed RNAs into cDNA. Subsequently, cDNA was used as the template in real-time PCR by SYBR® Premix Ex TaqTM II (Tli RNaseH Plus; Takara Bio Inc., Shiga, Japan). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 45 s and 58°C for 45 s, 72°C, 10 mins, and 4°C infinity. The primers for miR-126 were 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGCATTA-3' (for reverse transcription); 5'-GGGGTCGTACCGTGAGT-3' (GSP: the specific primer for miR-126), R: 5'-CAGTGCGTGTCGTGGAGT-3' (real-time PCR); and the sequences of U6 primers were 5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATT TGCGT -3'. All primers were designed and purchased form Sangon, China, and U6 was used the internal control. The mRNA expression levels of miR-126 were normalized to U6.

Western blotting analysis

Total proteins of treated KG-1a-LSCs and MOLM13-LSCs were extracted by lysing cells with RIPA buffer (0.1% SDS, 1% Triton X-100, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), and then breaking cells with ultrasonication at low temperature. Subsequently, broken cells were subjected to a centrifuge at 12,000 rpm/min for 20 min at 4°C, then supernatant was collected and the concentration of proteins was determined by Pierce™ BCA Protein Assay Kit. Each protein sample was separated by 10% SDS-PAGE at room temperature, and then it was transferred onto the nitrocellulose membranes (Millipore, Billerica, MA, USA) at low temperature for 2 h. After blocking with 5% low fat dried milk at room temperature for 2 h, membranes

were incubated overnight with primary antibodies against DNMT1 (1:2000, Rabbit polyclonal to Dnmt1, ab19905, Abcam). Finally, the membranes were incubated with corresponding HRP-conjugated secondary antibodies (1:5000, Donkey anti-rabbit IgG, ab6801, Abcam) for 2 h at room temperature. GAPDH was used as an internal control, and signals were detected by enhanced chemiluminescent reagents.

Plasmid construction and dual luciferase activity assay

The fragment of DNMT1 containing miR-126 binding sites was amplified by PCR with specific primers. Then it was cloned into psi-CHEK (Promega, Madison, USA) to produce DNMT1 wild type (WT) plasmid. The fragment of mutant DNMT1 containing mutant miR-126 binding sites was also amplified and inserted into psi-CHEK to establish DNMT1 Mutant plasmid (MUT). KG-1a-LSCs and MOLM13-LSCs were incubated overnight in 24-well plates and then cells were co-transfected with DNMT1 WT or MUT plasmids and miR-126 mimics or miR-126 negative control (miR-NC) by Lipofectamine 2000 (Invitrogen). Finally, luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega) following the instructions of the manufacturer, and renilla luciferase was normalized to firefly luciferase activity.

Cell apoptosis analysis

Cell apoptosis rate of miR-126 overexpressed or knockdown KG-1a-LSCs and MOLM13-LSCs was determined by flow cytometry with Annexin-V/7-AAD double staining. After cultured overnight at 37°C in RPMI 1640 medium, treated KG-1a-LSCs and MOLM13-LSCs were collected and re-suspended with 0.5 mL binding buffer containing 5 g/mL of annexin V-FITC (BD Biosciences) and 7-aminoactinomycin D (7-AAD, Sigma), and incubated at room temperature for 20 min. Finally, cell apoptosis rates of treated KG-1a-LSCs and MOLM13-LSCs were analyzed by flow cytometry.

Cell proliferation analysis

Cell Counting Kit-8 (CCK-8) analysis was performed to determine the cell proliferation of KG-1a-LSCs and MOLM13-LSCs transfected with miR-126 mimics or miR-126 inhibitors. Briefly, treated AML cell lines were seeded in 96-well plates at a density of 3,000 cells/per well, and incubated at 37°C for 24 h. Cell proliferation was determined by CCK-8 detection kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at every 24 h for six consecutive days, according to the protocols of manufacturer. The absorbance of each group was assessed at 450 nm using an ELISA reader (Elx800; Bio-Rad Laboratories, Inc.).

Immunofluorescence

After transfection with miR-126 mimics or inhibitors, KG-1a-LSCs and MOLM13-LSCs were fixed with 4% paraformaldehyde for 20 mins, and washed by phosphate buffered saline (PBS) three times. Treated cells were then blocked with 1% bovine serum albumin (BSA, Sigma) at room temperature for 2 h. Next, treated AML cells were stained overnight with primary antibody against Ki-67 (1:500, Rabbit polyclonal to Ki67, ab15580, Abcam) at 4°C. After washing with PBS three times, cells were then stained with corresponding secondary antibody conjugated to Alexa Fluor-488 (1:200, ab150073, Donkey Anti-Rabbit IgG, Abcam) at room temperature for 2 h. Finally, images were taken with a confocal laser-scanning microscope.

Statistical analysis

All data from this study were presented as means ± SEM. The statistical significance of differences was determined by Graphpad (Ver. Prim 7.0, USA) with one-way ANOVA analysis. Differences between each group were considered significant if $P<0.05$.

Results

miR-126 expression was upregulated in blood samples of AML patients and LSCs

To investigate whether miR-126 plays a role in the pathogenesis of leukemia, CD34+ or CD34- cells were isolated from blood samples of AML patients, and normal blood samples were collected from healthy subjects. Then, qRT-PCR was performed to measure the miR-126 expression, and results indicated that miR-126 expression was significantly upregulated in CD34+

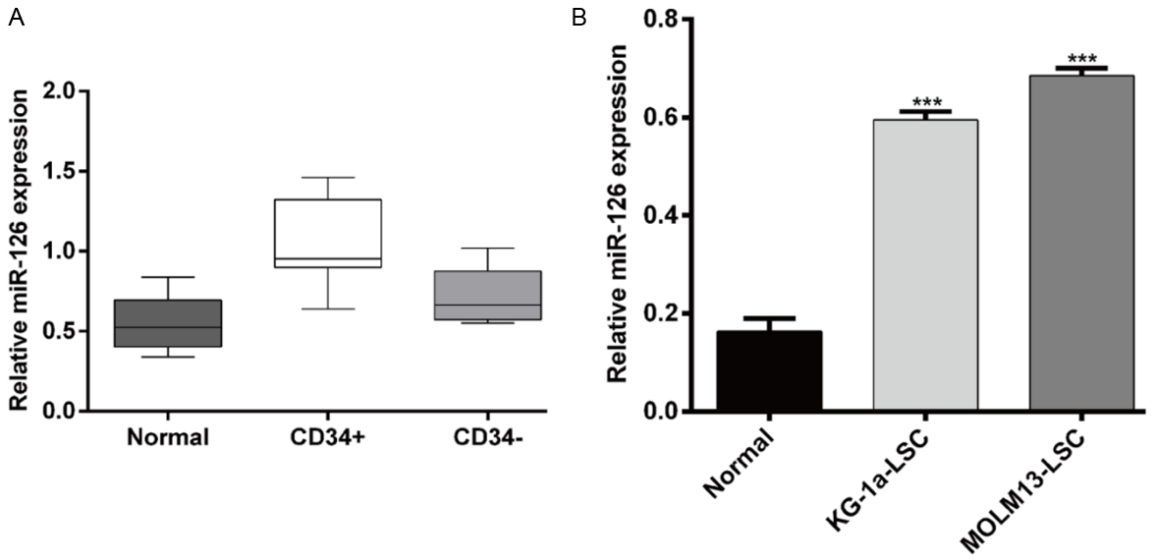

Figure 1. miR-126 expression was upregulated in blood samples of AML patients and LSCs. A. The relative miR-126 expression was detected in blood samples from normal volunteers and AML patients who were CD34 positive or negative. B. qRT-PCR analysis of miR-126 expression was performed in leukemia cell lines: KG-1a and MOLM13 (****P*<0.001).

cells compared with CD34- cells and normal specimens (**Figure 1A**). In addition, miR-126 expression was also found to be increased in LSC cell lines KG-1a-LSC and MOLM13-LSC, compared to normal cells (**Figure 1B**).

miR-126 knockdown inhibited cell proliferation in LSC cell lines

The upregulated miR-126 expression in LSCs suggested that it may participate in the initiation and development of leukemia. To further understand the underlying functions of miR-126 in AML, we established a miR-126 overexpressed cell model and a miR-126 silenced cell model in KG-1a-LSCs and MOLM13-LSCs by transfecting them with miR-126 mimics or inhibitors. Subsequently, CCK-8 assay and Ki-67 staining were used to measure cell proliferation in the miR-126 overexpressed or silenced cell models. Results showed that cell proliferation was significantly inhibited in both KG-1a-LSCs and MOLM13-LSCs transfected with miR-126 inhibitors compared with those transfected with miR-126 mimics or miR-NC (**Figure 2A** and **2B**).

miR-126 knockdown promoted cell apoptosis in LSC cell lines

In order to assess the effects of miR-126 on cell apoptosis, Annexin V/7-AAD staining and flow cytometry analysis were carried out in KG-1a-LSCs and MOLM13-LSCs transfected with miR-inhibitors or mimics. Results indicated that knockdown of miR-126 in KG-1a-LSCs remarkably increased the proportion of dead cells (Annexin V^+/7-ADD+). In addition, both the proportion of apoptotic cells (Annexin V^+/7-ADD) and dead cells (Annexin V^+/7-ADD+) were significantly upregulated in miR-126 silenced MOLM13-LSCs (**Figure 3**).

miR-126 directly regulated DNMT1 expression in LSCs

DNMT1 was reported to be involved in the pathogenesis of various diseases by interacting with miRNAs. In our study, we found that there was a complementary site in DNMT1 for miR-126 by bioinformatics analysis (**Figure 4A**). To further evaluate the correlation between miR-126 and DNMT1, luciferase activity analysis was performed. Firefly luciferase plasmid containing DNMT1 wild type (WT) or mutant (MUT) 3'-UTR was co-transfected with miR-NC or miR-126 mimics to KG-1a-LSCs and MOLM13-LSCs. The firefly luciferase activity was significantly reduced in KG-1a-LSCs and MOLM13-LSCs transfected with DNMT1 WT plasmid and miR-126 mimics, but not those cells treated with DNMT1 MUT plasmid and miR-126, indicating that miR-126 could directly bi-

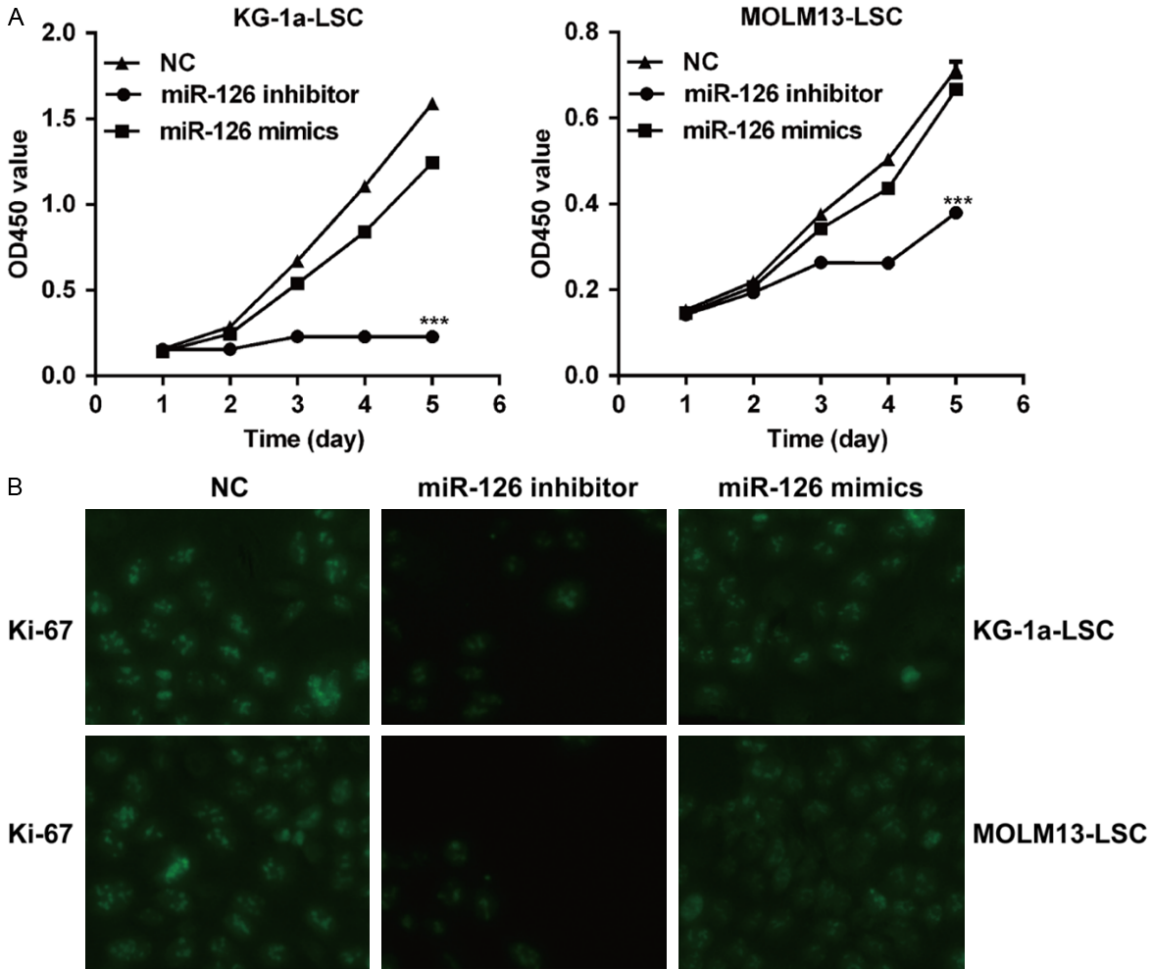

Figure 2. miR-126 knockdown inhibited cell proliferation in LSC cell lines. (A) CCK-8 assay and (B) Ki-67 (proliferation marker) staining were performed to examine cell proliferation in KG-1a-LSC and MOLM13-LSC cells transfected with miR-NC, miR-126 inhibitor, or miR-126 mimics (***P<0.001).

nd to DNMT1 in LSCs (**Figure 4B**). In addition, we measured the protein expression levels of DNMT1 in miR-126 treated KG-1a-LSCs and MOLM13-LSCs, and found that DNMT1 protein expression was significantly decreased in miR-126 overexpressed LSCs (**Figure 4C**).

Discussion

Despite the extraordinary advancements in medicine, the prognosis of AML has not improved obviously during the past decades, with long-term survival rates of less than 50% [30]. Cancer stem cells (CSC), characterized by self-renewal and production of other tumor cells, are a kind of cancer cell found within tumors with characteristics associated with normal stem cells [31, 32]. Recently, CSC was considered to be the primary cause of tumor re-initiation and propagation in many human cancers, including ovarian, breast, and bladder cancers [33-35]. Leukemia stem cells (LSCs) are defined as cells that can re-initiate leukemia, and originate all the cells of leukemia, when transplanted them into immune-deficient animals [10, 36]. Although the underlying pathogenic mechanisms of AML remain unclear, cumulative evidence suggests that LSC may play a vital role in the progression of this disease.

As a focal point of epigenetics, miRNAs have been demonstrated to participate in cell proliferation, apoptosis, and differentiation by regulating the expression of target genes [37]. Previous studies have suggested that various miRNAs were associated with the initiation and development of leukemia, such as miR-193b, miR-2909, and miR-107 [38-40]. miR-126 was

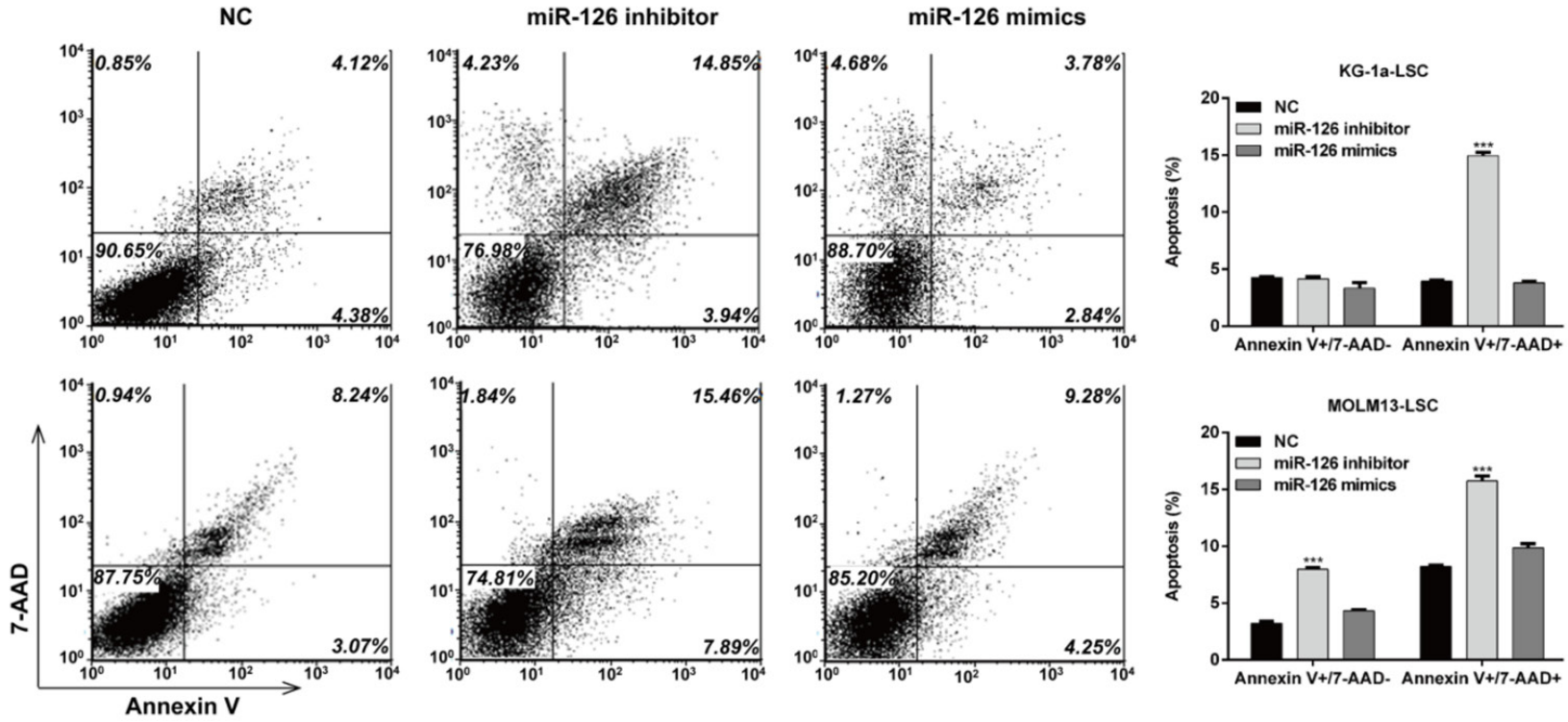

Figure 3. miR-126 knockdown promoted apoptosis in LSC cell lines. After transfection with miR-NC, miR-126 inhibitor or miR-126 mimics, apoptotic (Annexin V^+/7-ADD^-) or dead (Annexin V^+/7-ADD^+) cells of LSCs (KG-1a-LSC and MOLM13-LSC cells) were analyzed by flow cytometry (***P<0.001).

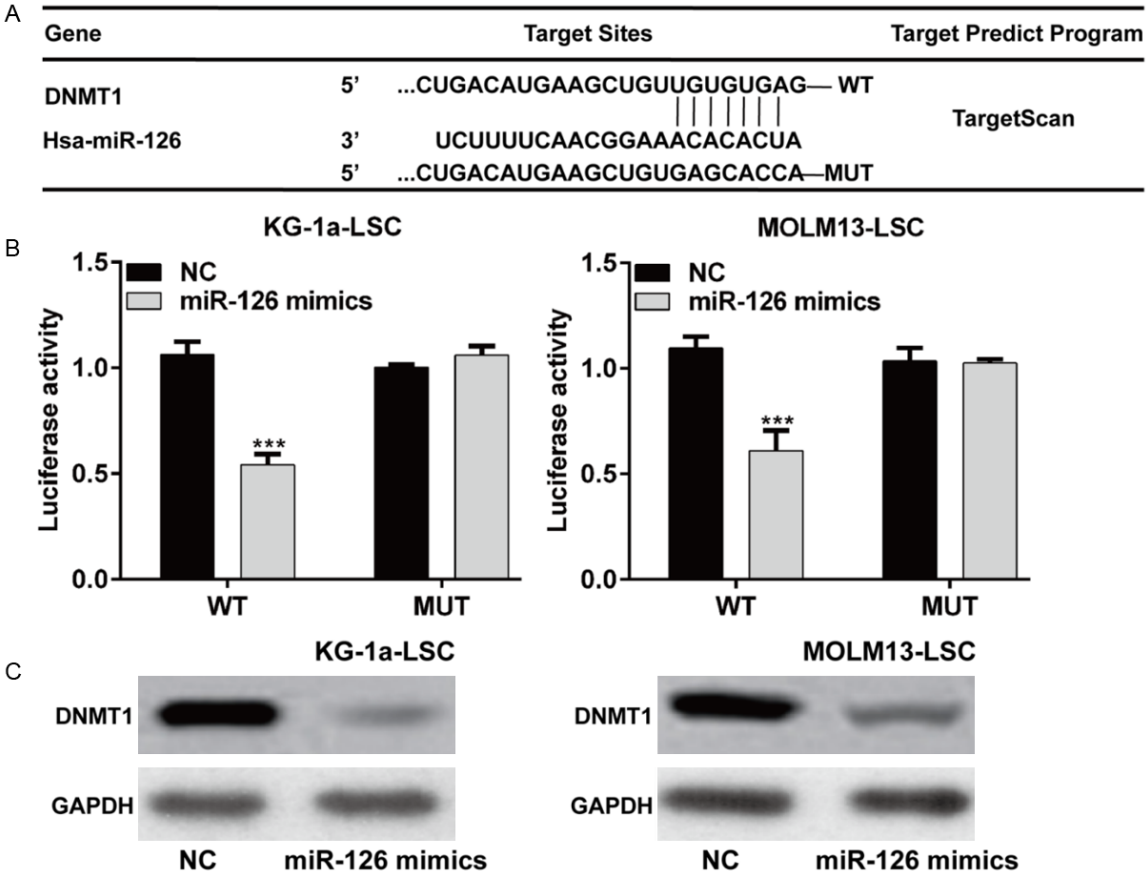

Figure 4. miR-126 directly regulated DNMT1 expression in LSC cell lines. A. Bioinformatic analysis was used to explore the underlying complementary sites between miR-126 and DNMT1. B. Luciferase activity assay was assayed to detect the correlation between miR-126 and DNMT1 in KG-1a-LSC and MOLM13-LSC cells. C. Western blot analysis was used to measure DNMT1 protein expression in KG-1a-LSC and MOLM13-LSC cells transfected with miR-126 mimics.

reported to have increased biologic activity in the LSCs, and knockdown of miR-126 induced LSCs to enter the cell cycle and sensitized LSCs to anti-carcinogens [29], suggesting that overexpression of miR-126 in LSCs may be the theoretical basis for the maintenance of the LSCs. In our study, we also found that miR-126 expression was significantly higher in leukemia tissues and LSCs. In order to further evaluate the roles of miR-126 in leukemia, we established a miR-126 overexpressed and knockdown cell model by transfecting LSCs with miR-126 mimics or inhibitors. Results from the functional assays showed that miR-126 may be an oncogenic agent in leukemia.

Evidence has shown that abnormal DNA methylation may result in the aberrant expression of miRNAs, which play a critical role in the progression of human cancer [41]. DNA methylation and demethylation are regulated by DNA methyltransferase (DNMT) [42]. Agirre et al. have demonstrated that miR-124a expression was significantly downregulated in acute lymphoblastic leukemia, due to promoter methylation modification, and decreased miR-124a expression promoted leukemia cell proliferation via CDK6 [43]. Chu et al. have revealed that DNMT1 and miR-126 formed a regulatory "loop" in esophageal squamous cell carcinoma. Overexpression of DNMT1 resulted in hypermethylation and downregulation of miR-126; however, overexpression of miR-126 inhibited DNMT1 expression [44]. In our study, we found that there is a complementary site in the 3'-UTR of DNMT1 mRNA for miR-126, according to the biologic informatics analysis and dual-luciferase reporter assay. Overexpression of miR-126

significantly inhibited DNMT1 protein expression in both KG-1a-LSCs and MOLM13-LSCs.

In conclusion, upregulated miR-126 expression functions as an oncogenic agent by decreasing the expression of DNMT1 in the progression of AML. However, additional work is needed to verify these results in AML in vivo.

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Disclosure of conflict of interest

None.

Address correspondence to: Qian Ding, Department of Hematopathology, Guizhou Provincial People's Hospital, 83 East Zhongshan Road, Guiyang 550002, Guizhou Province, China. Tel: +86-18985187674; Fax: +86-851-85924943; E-mail: dqishere@163.com

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