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Original Article

miR-9-5p prompts malignancies of acute myeloid leukemia cells mainly by targeting p27

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Abstract: MicroRNAs play key roles in the initiation and progression of acute myeloid leukemia (AML). The abnormal expression of miR-9-5p has been widely reported in various tumors. However, the expression pattern of miR-9-5 in AML has never been explored. The current study aims to explore the role of miR-9-5p in the progression of AML. First, real-time quantitative PCR was used to determine the level of miR-9-5p in the bone marrow and whole blood of AML patients. Then, a dual-luciferase reporter assay was performed to explore the possible target genes of miR-9-5p in HL-60 cells. According to our study, the level of miR-9-5p was significantly increased in the bone marrow and peripheral blood of AML patients compared with that of healthy controls. Furthermore, a close correlation was identified between the miR-9-5p level and clinicopathological parameters of AML, including FAB classification and cytogenetics. Additionally, the dual-luciferase reporter assay demonstrated that the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 (p27) was a target of miR-9-5p. Transfection with miR-9-5p inhibitors into HL-60 cells resulted in decreased proliferation and enhanced cell apoptosis. In summary, these results indicated that miR-9-5p functions as an oncogenic regulator mainly by targeting p27 in HL-60 cells.

Keywords: MiR-9-5p, acute myeloid leukemia, cell cycle, p27

Introduction

As a key component of hematopoiesis, myeloid development is characterized by sequential regulator expression [1]. The dysregulation of those regulators may lead to the failed differentiation of myeloid development, which induces the initiation of myeloid leukemia. Myeloid leukemia can be divided into acute myeloid leukemia (AML) and chronic myeloid leukemia (CML), both of which arise from abnormal white blood cells. Among them, acute myeloid leukemia is a malignant disorder, which is characterized by the arrest of maturation and rapid proliferation of immature precursors [2]. Due to substantial genetic abnormalities, the clinicopathological features and prognosis of AML patients are widely variable. It has been reported that the early and timely diagnosis of acute myeloid leukemia (AML) is effective in reducing morbidity and mortality [3].

The aberrant progression of the cell cycle contributes to the initiation and development of tumors [4]. The cell cycle is modulated by the sequential activation and inactivation of cyclin-dependent kinases (CDKs) [5]. The cyclin-dependent kinase (Cdk) inhibitor p27Kip1 (p27) plays a key role in the cell cycle transition from the G0 to S phase by inhibiting the activity of Cdks [6]. In human cancer, including the lung, colon, prostate and breast cancer, the expression of p27 is reduced and is tightly correlated with poor prognosis [6]. For example, p27 is extensively reported to decrease in breast cancer, and the reduction of p27 is markedly correlated with poor prognosis in multivariate analysis [6, 7]. In addition, decreased p27 was found to enhance the risk of death by 1.3- to 4-fold in 5-17 years of follow-up [8]. In addition, in AML, the suppression of p27 was found to promote cancer progression, while the overexpression of p27 was found to contribute to reduced cell cycle progression

Table 1. Correlation of peripheral blood miR-9-5p level with clinical characteristics of 200 AML patients

Variable	No. of patients	miR-9-5p expression (n)		*P* value
		Low	High	
Gender				
Male	110	56	54	ns
Female	90	47	43	
Leukocyte (/μL)				
>10,000	115	67	58	ns
≤10,000	85	38	46	
FAB classification				
M1		7	5	ns
M2		8	9	
M3		25	18	
M4		16	24	
M5		17	16	
M6		14	13	
M7	28	18	10	
Extramedullary disease				
Absent	109	56	53	ns
Present	91	42	49	
Cytogenetics				
Favorable	68	43	25	0.001
Intermediate	103	47	56	
Unfavorable	29	12	17	
Complete remission				
Y	134	65	69	0.04
N	66	35	31	

and enhanced cell apoptosis [9, 10]. Thus, it is important to explore the possible mechanism by which the expression of p27 is regulated in AML.

microRNAs (miRNAs) are a class of small, non-coding RNAs with up to 22 nucleotides, which are stable in the blood and exhibit tissue-specific expression patterns [11]. Therefore, miRNAs may be utilized as non-invasive biomarkers of specific cancers. In the current study, we mainly focused on miR-9-5p, which has been widely explored in various tumors. For example, in cervical cancer cells, miR-9-5p was reported to act as a tumor suppressor mainly by targeting astrocyte-elevated gene-1 [12]. Furthermore, miR-9-5p was also found to be upregulated in the bone marrow of AML patients carrying *NPM1* mutations, which are present in approximately 35% of patients with AML [13, 14]. However, few studies have focused on the expression of miR-9-5p in pediatric AML patients. Here, we evaluated the role of miR-9-5p in AML, which has never been explored. Therefore, the present study aims to evaluate the expression of miR-9-5p in the peripheral whole blood of AML patients and healthy controls, thereby investigating the potential of blood miR-9-5p level as a non-invasive AML diagnostic biomarker.

Materials and methods

Patient samples

Bone marrow (BM) specimens were isolated from 200 pediatric AML patients and 30 healthy volunteers before any interventional measures between February 2014 and October 2015 in Hongqi Hospital. All peripheral whole blood samples of patients and specimens were handled anonymously according to ethical and legal standards. The study was approved by the hospital's Ethical Committee with informed consent from the patients.

The diagnosis of AML was performed according to a morphologic assessment of Wright-Giemsa stained smears of bone marrow aspirates along with special stains and immunophenotyping by flow cytometry. Laboratory investigation included conventional and molecular cytogenetic analyses. The median leukocyte count at diagnosis was 20,606/μL (range 420-352, 906/μL). The clinical characteristics of 200 pediatric AML patients are summarized in **Table 1**. The healthy control group consisted of 30 healthy volunteers with no clinical symptoms of cancer or other diseases.

Leukemic blasts from AML samples and mononucleated fractions from normal BM specimens were isolated by Ficoll-Hypaque (Nygaard) centrifugation and then cryopreserved. After thawing, cells were washed with HBSS and processed for RNA isolation.

RNA extraction

Total RNA from the bone marrow (2 mg) and whole blood samples (5 mL) collected in tubes containing EDTA was extracted with RNAVzol

LS (Vigorous Biotechnology, Beijing) in strict accordance with the manufacturer's instructions. The concentration and purity of the RNA samples was determined by OD_{260}/OD_{280}.

Cell culture

The human AML cell line HL-60 was derived at the Institute of Haematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China). HL-60 was cultured in IMDM (HyClone, Logan, UT, USA) supplemented with 20% fetal bovine serum (Gibco, Life Technologies, USA).

Transient transfection

Cells were seeded at 10^6 cells/well in 6-well plates. Meanwhile, miR-9-5p mimic, inhibitor, or miR negative control (GenePharma) was mixed with HiPerFect transfection reagent (Qiagen) and incubated at room temperature for 10 min. Then, the complex was added to the culture medium for 48 h.

Reverse transcription (RT) and quantitative (q) polymerase chain reaction (PCR)

For the synthesis of the cDNA of the specific miR, a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used. To quantify miR-9-5p, a quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) on a Bio-Rad iCycler iQ real-time PCR detection system according to the manufacturer's instructions. The thermal cycling conditions included a hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The 2-delta delta Ct analysis method was used to determine the relative quantity of miR-9-5p. U6 was used as an internal control. The primers were listed as follows: RT-miR-9-5p: CGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCAAT; RT-U6: TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATATG; miR-9-5p-F: GCTCTTAGCAGCACAGAAAT; U6-F: GCGTCGTGAAGCGTTC; Universe reverse primer: GTGCAGGGTCCGAGGT.

Protein extraction and Western blot analysis

Protein samples were extracted in RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China)) supplemented with a protease and phosphatase inhibitor cocktail (Sigma) and then separated by 10% SDS-PAGE, followed by electrophoretic transfer to PVDF membranes. After incubation with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-p27, anti-p-AKT, anti-AKT, anti-Bcl-2, anti-Bax and anti-β-actin (Cell Signalling). Immunodetection was performed by an enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. The housekeeping gene GAPDH was used as the internal control.

Luciferase target assay

The TargetScan program (http://www.targetscan.org/vert_71/) was used to identify the possible target genes of miR-9-5p. The 3'untranslated region (UTR) of p27 containing the predicted binding site was cloned into the pmirGLO (Promega) luciferase reporter vector. The PCR procedures were as follows: a hot start step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 55°C for 45 s, and 72°C for 30 s. To construct the mutant vector, the Fast Mutagenesis System was used (TransGen Biotech, Beijing, China).

For luciferase reporter assays, cells were seeded at 5×10^4 cells/well in 24-well plates in a 500-μL volume for 18 h. Then, the modified firefly luciferase vector (500 ng/μL) was mixed with the VigoFect transfection reagent according to the manufacturer's instructions. After transfection for 48 h, the dual-luciferase reporter assay system (Promega) was used to determine the changes in relative luciferase units (RLU). Renilla luciferase activity was used as the internal control.

Apoptosis assay

First, the cells were washed three times with PBS. To determine apoptosis rates, the Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA) was used. Briefly, the cells were washed with 1× Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of $2\text{-}3\times10^6$ cells/mL. Then, the Annexin-V FITC and propidium iodide buffer was added, followed by incubation at room temperature for 15 min. After treatment, the cells were filtered with a 300-mesh filter and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

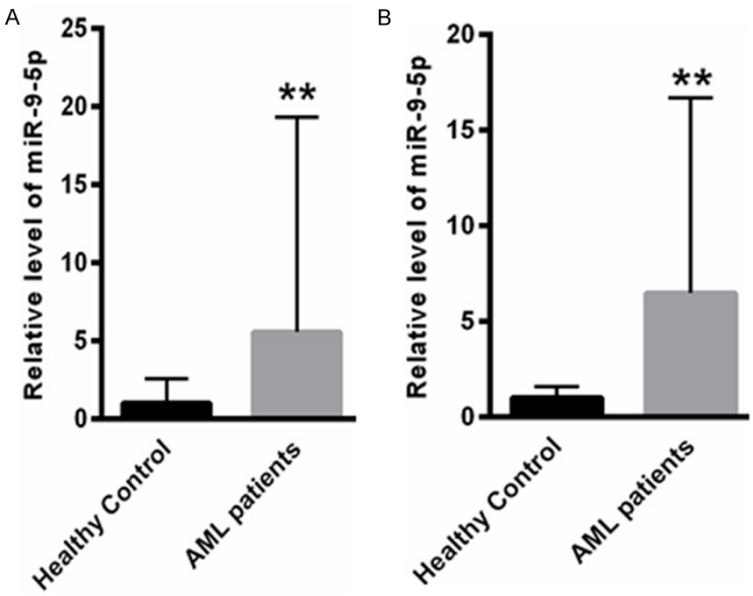

Figure 1. Increased miR-9-5p level in the bone marrow and peripheral blood of AML patients. Real-time PCR analysis of miR-9-5p expression in the bone marrow (A) or peripheral blood (B) of AML patients compared with that of healthy controls. **P < 0.01, ***P < 0.001 *vs.* control.

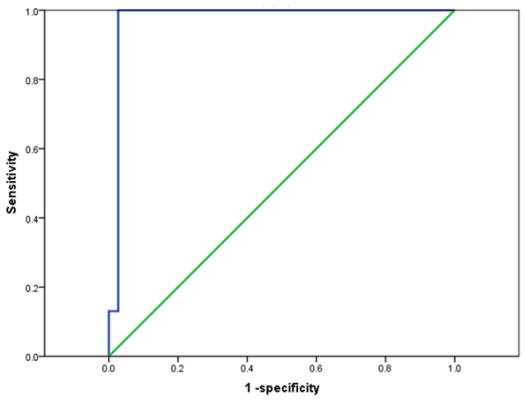

Figure 2. ROC analysis showing that miR-9-5p could be used to differentiate AML patients from healthy controls.

TUNEL

Nuclear fragmentation was detected by TUNEL staining with an apoptosis detection kit (R & D Systems) according to the manufacturer's protocol.

Statistical analysis

The data are presented as the mean ± SD. Differences were analyzed with Student's t test. ROC curves were used to assess miR-9-5p as a biomarker, and the AUC was reported. P < 0.05 was used to define a statistically significant difference.

Results

Increased miR-9-5p in the bone marrow and peripheral blood of AML patients

First, the expression level of miR-9-5p was explored in leukemia cells isolated from the bone marrow and peripheral blood of AML patients and healthy controls. As shown in **Figure 1A**, the level of miR-9-5p was markedly enhanced in the leukemic cells of bone marrow from AML patients (5.55 ± 13.75) compared with that of healthy controls (1.0 ± 1.56). We also found that miR-9-5p was increased in the leukemic cells isolated from the peripheral blood of AML patients (6.47 ± 10.22) compared with that of healthy controls (1.0 ± 0.58).

Correlation between miR-9-5p expression in the peripheral blood with clinical parameters of AML

Next, the correlation between miR-9-5p expression in the peripheral blood and clinical parameters of AML was analyzed. According to **Table 1**, no correlation was identified between miR-9-5p and gender, age, leukocytes, extramedullary disease and complete remission. Strikingly, a significant correlation between the peripheral blood miR-9-5p level and FAB classification (p=0.0023) and cytogenetics (p= 0.001) was found in AML patients (**Table 1**). In addition, the ROC curve was applied to evaluate whether *miR-9-5p* expression can be used as a potential diagnostic marker for AML. It was shown that the level of *miR-9-5p* expression could be available as a potential diagnostic biomarker for screening AML from controls with an AUC of 0.976 (95% CI=0.931-1.000; *P* < 0.0001; **Figure 2**).

Decreased miR-9-5p induced cell cycle arrest and enhanced apoptosis

Next, we explored the specific role of miR-9-5p in AML cells. As shown in **Figure 3A**, the inhibi-

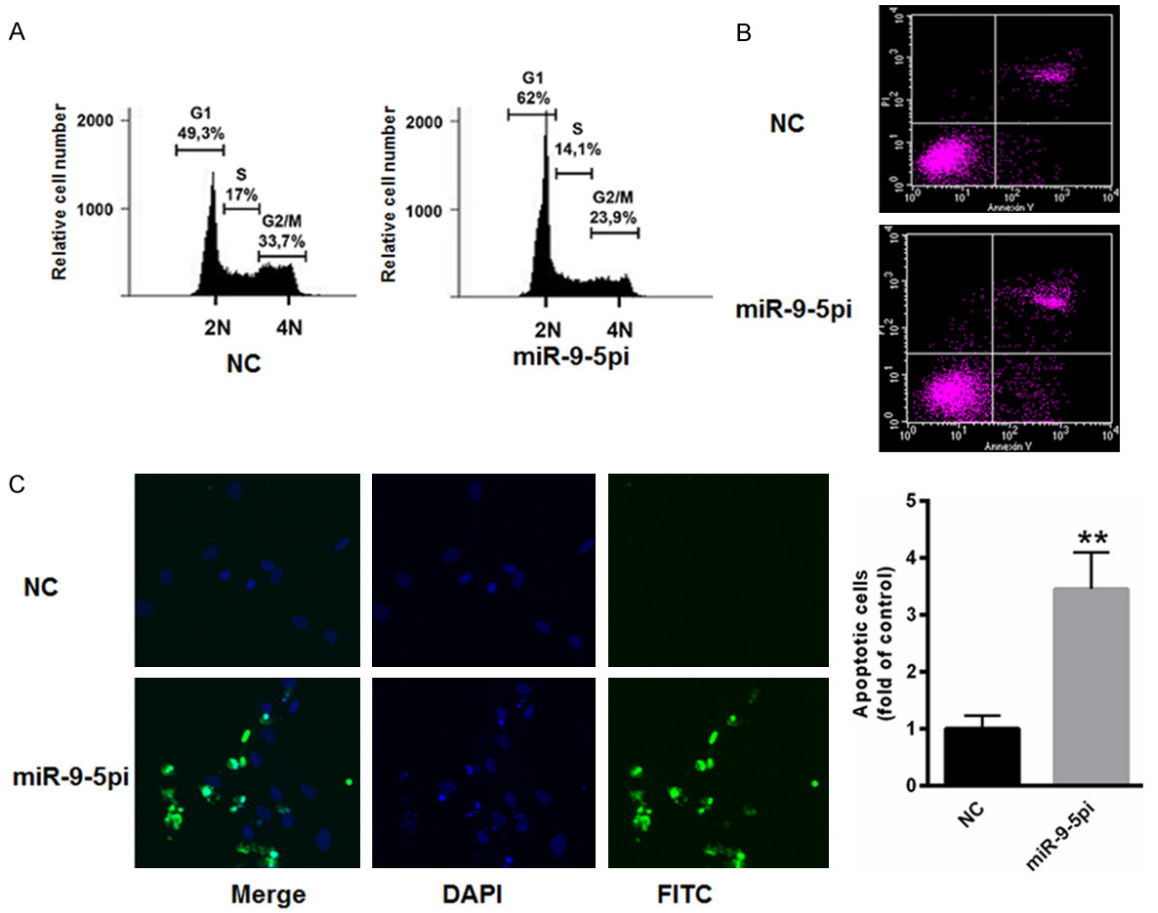

Figure 3. The decreased miR-9-5p level induced cell cycle arrest and enhanced apoptosis. A. Inhibition of miR-9-5p in HL-60 cells markedly induced cell cycle arrest. B. TUNEL staining demonstrated that the suppression of miR-9-5p significantly enhanced the apoptosis of HL-60 cells. C. Apoptosis was increased by ~3.45-fold in HL-60 cells transfected with miR-9-5p inhibitors compared with those transfected with the negative control. *P < 0.05, **P < 0.01 *vs.* control.

tion of miR-9-5p in HL-60 cells markedly induced cell cycle arrest. Furthermore, TUNEL staining demonstrated that suppressed miR-9-5p significantly enhanced apoptosis in HL-60 cells (**Figure 3B**). In addition, apoptosis was increased by up to 3.45-fold in HL-60 cells transfected with miR-9-5p inhibitors compared with those transfected with the negative control (**Figure 3C**). These data indicated an oncogenic role of miR-9-5p in AML cells.

p27 is the target gene of miR-9-5p

We further analyzed the target gene of miR-9-5p, and TargetScan prediction demonstrated that there was a conserved binding site of miR-9-5p in the 3'UTR of p27 (**Figure 4A**). Then, the 3'UTR of p27 was cloned into the pmirGLO plasmid, and further study demonstrated that miR-9-5p suppressed the relative luciferase reporter activity of pmirGLO-p27-3'UTR compared with that of the blank vector (**Figure 4B**). Next, the overexpression of miR-9-5p significantly suppressed the protein levels of p27 and bcl-2, while it markedly enhanced the expression of bax (**Figure 4C**). In contrast, the inhibition of miR-9-5p markedly increased the expression of p27 and bcl-2, while it inhibited the level of bax in HL-60 cells (**Figure 4D**). Together, these data showed that miR-9-5p inhibited the apoptosis of HL-60 cells by targeting p27.

Discussion

Among older patients, AML is a different disease due to the aging of hematopoietic stem cells (HSCs) caused by DNA damage, telomere

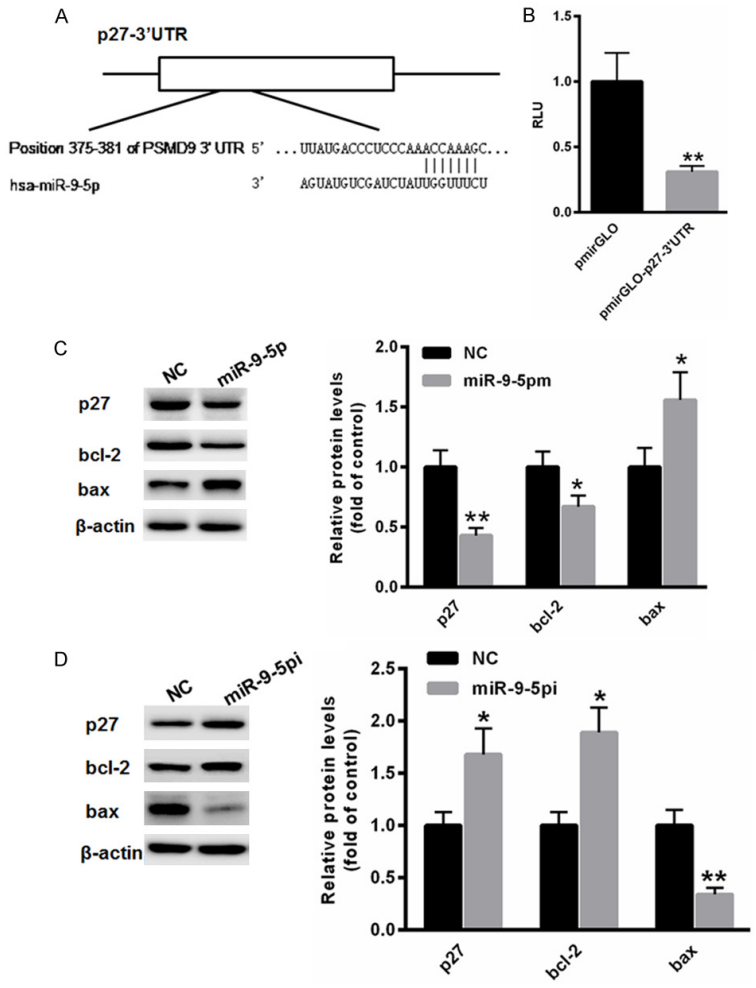

Figure 4. p27 was the target gene of miR-9-5p. A. Schematic analysis of the binding site for miR-9-5p in the 3'UTR of p27. B. Dual-luciferase reporter assay demonstrated that miR-9-5p suppressed the relative luciferase reporter activity of pmirGLO-p27-3'UTR compared with that of the blank vector. C. The overexpression of miR-9-5p significantly suppressed the protein levels of p27 and bcl-2, while it markedly enhanced the expression of bax. D. The inhibition of miR-9-5p markedly increased the expression of p27 and bax, while it inhibited the level of bax in HL-60 cells. *P < 0.05, **P < 0.01 *vs.* control.

shortening, and oxidative stress [15]. It has been reported that the 5-year survival rate is approximately 50% among pediatric AML patients, while the mortality is even higher among older AML patients [16, 17]. miRNAs have been widely reported to be dysregulated in different tumors and any disruption of miRNAs may result in oncogenesis and metastasis [18]. For example, the upregulation of miR-155 is correlated with initial progression and poor outcomes in Chinese pediatric AML patients [19]. In addition, miR-22-mediated MECOM degradation has been shown to play a key role in normal hematopoiesis and AML development, indicating its potential value in AML diagnosis and therapy [20]. Therefore, investigations on miRNAs may shed light on the understanding of AML progression.

Previous studies have shown the enhanced expression of miR-9-5p in the bone marrow of AML patients carrying NPM1 mutations [13, 14]. In addition, miR-9-5pb was also found to be overexpressed in mixed lineage leukemia *(MLL)*-associated AML in the bead-based miRNA profiling of 57 samples [21]. Here, we first explored the expression of miR-9-5p in the bone marrow and peripheral blood of AML patients and healthy controls. We first demonstrated that miR-9-5p was markedly increased in the bone marrow and peripheral blood of AML patients compared with that of healthy controls. Further studies demonstrated the significant correlation of peripheral blood miR-9-5p levels with FAB classification (p=0.0023) and cytogenetics (p=0.001). ROC analysis indicated that peripheral blood miR-9-5p could be used to distinguish AML patients from healthy controls. Together, these data indicated miR-9-5p as a potential biomarker for AML diagnosis.

Next, we explored the possible function of miR-9-5p in the progression of AML. Flow cytometric analysis showed that the decreased miR-9-5p level markedly induced cell cycle arrest. We also explored the role of miR-9-5p in cell apoptosis. Flow cytometric analysis and TUNEL staining indicated that the reduction of miR-9-5p significantly induced apoptosis in HL-60 cells. These data indicated an oncogenic role of miR-9-5p in AML cells. To gain insights into the mechanisms leading to cell cycle arrest, we analyzed the possible target genes of miR-9-5p and found a conserved binding site of miR-9-5p in the 3'UTR of p27.

p27Kip1 is an inhibitor of cell cycle progression, which is encoded by the CDKN1B gene and suppresses the activation of cyclin E-CDK2 and cyclin D-CDK4 complexes, thereby blocking the cell cycle at G1 phase [22]. Studies have indicated that p27 plays a key role in carcinogenesis and may also be utilized as a prognostic factor in different tumors [23, 24]. In the present study, we found that miR-9-5p markedly suppressed the relative luciferase reporter activity of p27, and Western blot showed that the protein level of p27 was markedly inhibited by p27, suggesting that p27 is a target gene of miR-9-5p. These findings demonstrate an oncogenic role of miR-9-5p mediated by p27 in the progression of AML.

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Disclosure of conflict of interest

None.

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