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Original Research Article

MiR-26 targeting methyltetrahydrofolate reductase inhibits the proliferation of acute myeloid leukemia cells via JAK/STAT pathway

Yudi Miao[1], Qiaojiajie Zhao[2], Chengliang Li[3]*
[1]Department of Hematology, [2]Blood Research Department, Shaanxi Provincial people's Hospital, Xi'an, [3]Department of General Practice, Guangzhou First People's Hospital, Guangzhou, China

For correspondence: **Email: lichengliang79@sina.com; **Tel:** 86013991166493*

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Abstract

Purpose: *To investigate the effect of microribonucleic acid (miR)-26 targeting methyltetrahydrofolate reductase (MTHFR) on cell proliferation, cycle, and apoptosis in acute myeloid leukemia (AML).*
Methods: *The expressions of miR-26 in three types of human AML cell lines (HL-60, Kasumi-1, and KG-1a) and normal myeloid cell line (HS-5) were determined via quantitative reverse transcription-polymerase chain reaction (qRT-PCR), while the effect of the over-expression of miR-26 on the proliferation, cell cycle and apoptosis of AML cells was evaluated using cell counting kit-8 (CCK-8) assay and flow cytometry. The potential target for miR-26 was predicted using public miRNA database TargetScan, and whether miR-26 binds to the predicted target was determined using a dual-luciferase reporter assay. Western blotting was performed to determine the effect of miR-26 on Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway-associated proteins.*
Results: *Expression level of miR-26 was significantly lower in all AML cell lines than in HS-5 cells ($p < 0.05$). Overexpression of miR-26 inhibited the proliferation of KG-1a cells, reduced the percentage of cells in S phase, increased those in G0/G1 phase, and enhanced apoptosis of KG-1a cells ($p < 0.05$). After overexpression of miR-26, protein expression levels of phosphorylated (p)-JAK and p-STAT were down-regulated, while those of JAK and STAT did not change significantly.*
Conclusion: *Expression of miR-26 is down-regulated in AML, while MiR-26 targeting of MTHFR induces apoptosis and cycle arrest of AML cells through the JAK/STAT pathway, thus inhibiting AML cell proliferation in vitro.*

Keywords: *miRNA, Leukemia, Proliferation, Apoptosis*

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INTRODUCTION

Acute myeloid leukemia (AML) is a type of cytogenetic and/or molecular heterogeneous disease characterized by the uncontrolled proliferation of clonal myeloid precursors, leading to the accumulation and maturation arrest of myeloid cells in the bone marrow [1,2]. Despite great improvement in the understanding of the mechanism of AML and considerable progress in the optimized therapeutic methods, the prognosis of AML patients remains poor [3]. The 5-year survival rate is only 31 % in AML patients aged below 65 years, and 4 % in elderly patients [4].

There is therefore the need to identify more effective therapeutic targets and precise treatment of AML by revealing the potential mechanism of AML pathogenesis. Microribonucleic acids (miRNAs) are a class of small non-coding RNAs of about 18 - 22 nucleotides in length, which specifically bind to the 3'-untranslated region (UTR) of their target mRNAs, and reduce protein translation levels through the unstable or inhibitory effect of the transcript [5,6]. The miRNA dysregulation has been observed in many human solid tumors and hematological malignancies, and it promotes malignant transformation by intervening in key processes, including differentiation, proliferation, and apoptosis [7,8]. Many miRNAs have been identified as tumor promoters or tumor suppressors in AML [9,10], and there are different miRNA expression profiles in the cytogenetics of AML [11-13]. Zhu *et al* [14] clarified the pathogenesis of leukemia involving miR-9 and found the differential expressions of miR-9 and C-X-C chemokine receptor 4 (CXCR4) in myeloid leukemia, especially AML. MiR-9 inhibits the proliferation, apoptosis, migration, and invasion of AML cells *in vitro*. It has been confirmed by a dual-luciferase assay that CXCR4 is a direct target for miR-9. The inhibitory effect of miR-9 on AML cells is offset by the overexpression of CXCR4. He *et al* [15] explored the effect of miR-26 on the development of non-small cell lung cancer (NSCLC) and found the abnormal expression of miR-26 in human NSCLC tissues. The MiR-26 induces apoptosis and suppresses the autophagy of human NSCLC cells through the TGF-β1-JNK signaling pathway, demonstrating that miR-26 may be a potential new therapeutic target for NSCLC. Whether miR-26 also has similar biological functions in AML remains unknown. In the present study, therefore, the effects of miR-26 on the proliferation and apoptosis of AML cells were investigated.

EXPERIMENTAL

Cell culture

Three kinds of human AML cell lines (HL-60, Kasumi-1, and KG-1a) and a normal myeloid cell line (HS-5) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in the Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10 % heat-inactivated fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified incubator with 5 % CO_2 at 37 °C.

MiRNA transfection and plasmid construction

The miR-26 mimic and negative control miR-NC were designed and chemically synthesized by GenePharma (Shanghai, China), while the methyltetrahydrofolate reductase (MTHFR) expression plasmid pcDNA3.1-MTHFR and the empty plasmid pcDNA3.1 were designed and constructed by Guangzhou FITGENE Biotechnology Co., Ltd. (Guangzhou, China). The cells were inoculated into a 6-well plate (5×10^5 cells/well), transfected with miR-26 mimic, miR-NC, or plasmid according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and incubated under 5 % CO_2 at 37 °C. The transfected cells were collected at different time points and used for subsequent experiments.

Extraction of RNA and qRT-PCR

The total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribonucleic acid (cDNA) using the TaqMan MicroRNA RT kit. The expression of miR-26 was determined using 1 μL of the cDNA template and TaqMan MicroRNA Assay kit. The primers of miR-26 and endogenous control U6 were synthesized by BGI, and the primer sequences are shown in Table 1. The PCR conditions are as follows: 95 °C for 30 s, 95 °C for 15 s, and 60.5 °C for 30 s, for a total of 40 cycles, and the Ct value was normalized using the $2^{-\Delta\Delta Cq}$ method.

Cell proliferation analysis

The effect of miR-26 on the proliferation of AML cells was evaluated via cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). The transfected cells in the medium were inoculated into a 96-well plate at a density of 3×10^3 cells/well, and the cell proliferation was detected every 24 h for 3 days. A total of 10 μL of CCK-8 solution was added into each well at each time point. After incubation under 5 % CO_2 at 37 °C for 2 h, the optical density was measured at a wavelength of 450 nm using a microplate spectrophotometer.

Cell cycle analysis

At 72 h after transfection, the cells were collected, washed with phosphate-buffered saline (PBS), and fixed with 75 % ethanol for 1 h. After 75 % ethanol was removed, the cells were incubated in the cell cycle staining solution (50 μg/mL propidium iodide) for 30 min,

Table 1: Primer sequences used in the study

Gene		Primer sequences (5'-3')
miR-26	Forward	AGGCGATGCGTAGCGATGGCG
	Reverse	CGTAGGGGCGAGGGCTGAGC
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT

and then the cell cycle distribution was detected using a flow cytometer (Partec AG, Arlesheim, Switzerland).

Analysis of apoptosis rate

Apoptosis was detected using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit. At 24 h after transfection, the cells were collected, centrifuged, and resuspended in 100 µL of FITC binding buffer. About 5 µL of Annexin V-FITC and 5 µL of propidium iodide were added into the mixture for incubation in the dark for 30 min. Annexin V-FITC and propidium iodide fluorescence were determined using the BD FACSCalibur flow cytometer and analyzed using the CellQuest software.

Luciferase activity assay

The luciferase reporter plasmids containing the wild-type (WT) and mutant-type (MUT) MTHFR 3'-UTR (pmirGLO-MTHFR-WT & pmirGLO-MTHFR-MUT) were designed and obtained by Shanghai GenePharma (Shanghai, China). The cells were inoculated into a 24-well plate (1×10^5 cells/well), and co-transfected with miR-26 mimic or miR-NC and pmirGLO-MTHFR-WT or pmirGLO-MTHFR-MUT using Lipofectamine 2000. After 48 h, the transfected cells were collected to determine the firefly and Renilla luciferase activity using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical analysis

The SPSS analysis software (version 26.0) was used for statistical analysis. Data are expressed as mean ± standard deviation. The paired-sample t-test was performed for the comparison between the two groups. $P < 0.05$ suggested the statistically significant difference.

RESULTS

Expression of miR-26 was down-regulated in AML

The result of the biological effect of miR-26 in AML showed that the expression level of miR-26 was significantly lower in all AML cell lines than that in HS-5 cells ($P < 0.05$, Figure 1), indicating that the down-regulation of miR-26 may be related to the development of AML.

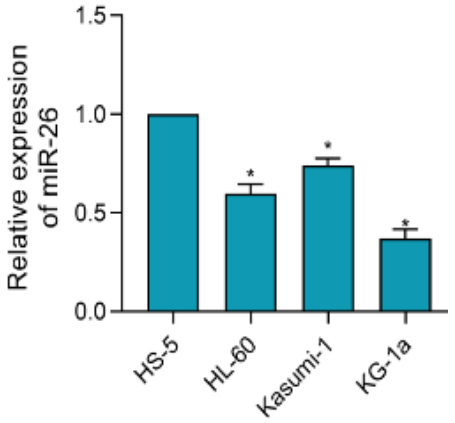

Figure 1: Expression of miR-26 in AML; *$p < 0.05$

MiR-26 overexpression inhibited proliferation of KG-1a cells

MiR-26 mimic was transfected into KG-1a cells for exogenous expression of miR-26, and its overexpression was confirmed via qRT-PCR (Figure 2 A, $p < 0.05$). Besides, the effect of miR-26 overexpression on AML cell proliferation was evaluated using CCK-8 assay. It was found that the overexpression of miR-26 inhibited the proliferation of KG-1a cells compared with cells transfected with miR-NC (Figure 2 B; $p < 0.05$).

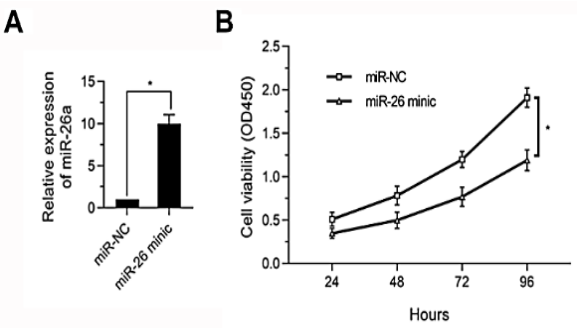

Figure 2: Effect of miR-26 expression on KG-1a cell proliferation. (A) Overexpression of miR-26. (B) The effect of miR-26 overexpression on KG-1a cell proliferation detected using the CCK-8 assay (*$p < 0.05$)

MiR-26 overexpression caused cell cycle arrest

The results revealed that the overexpression of miR-26 reduced the percentage of KG-1a cells in the S phase and increased that in G0/G1 phase ($p < 0.05$, Figure 3 A).

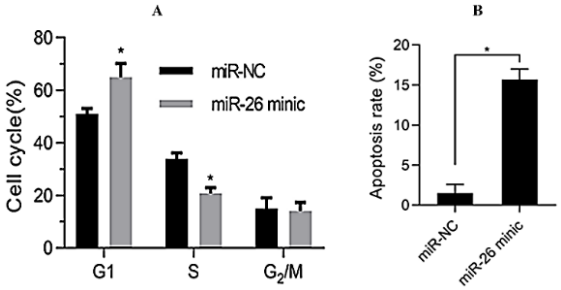

Figure 3: Effect of miR-26 expression on (A) KG-1a cell cycle. (B) KG-1a cell apoptosis detected using flow cytometry. *$P < 0.05$

MiR-26 overexpression enhanced apoptosis of KG-1a cells

It was observed that the percentage apoptosis of KG-1a cells rose after transfection with miR-26 mimic ($p < 0.05$, Figure 3 B). These results suggest that miR-26 inhibited AML cell proliferation *in vitro* by inducing apoptosis and cell cycle arrest.

MTHFR was a target gene for miR-26 in AML cells

As shown in Figure 4 A, the MTHFR 3'-UTR contained highly conserved binding sites for miR-26. This hypothesis was confirmed by luciferase reporter assay. The luciferase reporter plasmids containing the WT, and MUT MTHFR 3'-UTR (pmirGLO-MTHFR-WT and pmirGLO-MTHFR-MUT) were chemically synthesized and co-transfected into KG-1a cells using miR-26 mimic or miR-NC. The results showed that restoring the expression of miR-26 reduced the luciferase activity of KG-1a cells transfected with pmirGLO-MTHFR-WT (Figure 4 B, $p < 0.05$). However, it did not influence that of KG-1a cells transfected with pmirGLO-MTHFR-MUT (Figure 4 B), demonstrating that miR-26 recognized and bound to the MTHFR 3'-UTR. Moreover, whether miR-26 affects the endogenous MTHFR expression in AML cells was explored. According to the results of qRT-PCR and Western blotting, the overexpression of miR-26 down-regulated the mRNA and protein expression levels of MTHFR in KG-1a cells ($p < 0.05$, Figure 4 C). The above results indicate that MTHFR is a direct target for miR-26 in AML cells. The protein expression levels of p-JAK and p-STAT were down-regulated, while those of JAK and STAT showed no significant changes after transfection with miR-26 mimic, thereby demonstrating that changes in the proliferation ability of AML cells after overexpression of miR-26 may be regulated through the JAK/STAT signaling pathway.

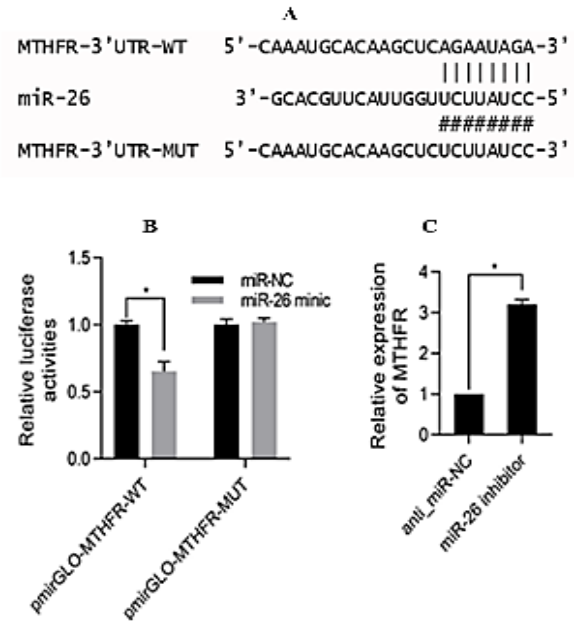

Figure 4: (A) Predicted binding sites for miR-26 and MTHFR. (B) Interaction between miR-26 and MTHFR determined using a luciferase activity assay. (C) Regulatory relation between miR-26 and MTHFR detected via qRT-PCR (*$p < 0.05$ vs. miR-NC)

DISCUSSION

Acute myeloid leukemia (AML) is an extremely invasive subtype of leukemia characterized by abnormal growth of bone marrow stromal cells [16], which accounts for about 15 - 20 % of childhood acute leukemia [17]. Genetic abnormality interferes with the growth, metastasis, and differentiation of normal hematopoietic progenitor cells. Currently, the treatment strategies for AML patients include chemotherapy, targeted therapy, and hematopoietic stem cell transplantation, and their development has greatly improved the therapeutic effect on AML patients in recent decades. Unfortunately, a large number of AML patients still have a recurrence and die of the disease. Therefore, clarifying the molecular process and mechanism of the development and progression of AML is of important significance in developing reliable therapeutic interventions for such patients. It is known that a variety of miRNAs have an abnormal expression in AML, and these dysregulated miRNAs play crucial roles in the occurrence and development of AML and may regulate major cancer-related biological

properties [9]. Therefore, a comprehensive study of the regulatory mechanism of miRNAs in the occurrence and development of AML has important significance for developing therapeutic strategies for AML patients. It has been reported that miRNAs regulate various biological processes in cancer cells, including cell differentiation, proliferation, metastasis, and apoptosis. Various miRNAs are abnormally expressed in different types of cancers, including AML [10]. Ding *et al* [18] explored the ectopic expression of miR-130a in chronic leukemia and discovered that miR-130a is notably overexpressed in AML. The expression of miR-130a will remarkably decline once the leukemia is completely relieved in patients, but will dramatically rise again in the case of recurrence. At the same time, it was also revealed that miR-130a is directly activated by AML1/ETO, and it may serve as an AML marker and chemosensitivity-related factor. In addition, the specific role of miR-26 in the progression of AML and its molecular mechanism were explored. MiR-26 is one of the most important miRNAs involved in human malignant tumors, and its abnormal expression was found in various types of cancers, including esophageal squamous cell carcinoma, colorectal cancer, and breast cancer [15]. In this study, the expression of miR-26 was lower in AML cell lines than that in normal myeloid cell lines, and miR-26 inhibited AML cell proliferation *in vitro* through the induction of apoptosis and cell cycle arrest. Besides, the results showed that MTHFR was directly targeted and down-regulated by miR-26, so it was determined as one of the target genes for miR-26 in AML cells.

The abnormal activation of the intracellular signal transduction pathway confers malignant properties on cancer cells. The JAK/STAT pathway is considered one of the major molecular pathways for the progression of AML [19]. The STAT proteins, including STAT1-6, can promote or inhibit the antiviral response, inflammation, and tumorigenesis, among which STAT-5 can promote liver damage [20]. Knockout of lncRNA UCA1 inhibits viability, migration, and invasion, and promotes apoptosis of AML cells *in vitro*. LncRNA UCA1 can bind to miR-126 and down-regulate the expression of miR-126. At the same time, the anti-growth and anti-metastasis effects of lncRNA UCA1 knockdown are reversed by inhibiting miR-126. The Ras-related C3 botulinum toxin substrate 1 (RAC1) is a target gene of miR-126, and its overexpression eliminates the anti-myeloid leukemia effect of miR-126. Moreover, the PI3K/AKT and JAK/STAT signaling pathways are blocked by miR-126 overexpression and activated by RAC1 overexpression. In this study, the effects of miR-26 on expression levels of the JAK/STAT pathway-associated proteins (p-JAK, p-STAT, JAK, and STAT) were detected. The results manifested that the protein expression levels of p-JAK and p-STAT were significantly down-regulated, while those of JAK and STAT exhibited no obvious changes after overexpression of miR-26.

CONCLUSION

Changes in the proliferation ability of AML cells after overexpression of miR-26 are regulated via JAK/STAT signaling pathway due to miR-26 targeting methyltetrahydrofolate reductase. Thus, MTHFR is a possible target for drug development in the management of AML.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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