

Overexpression of miR-21 is involved in acute monocytic leukemia-associated angiogenesis by targeting IL-12

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Abstract. Angiogenesis is important in pathophysiological processes, including the pathogenesis of acute monocytic leukemia (AML). MicroRNA-21 (miR-21) is overexpressed and exhibits oncogenic activity in cancer. However, the biological mechanism underlying the effect of miR-21 in AML remains to be fully elucidated. In the present study, the expression levels of miR-21 and vascular endothelial growth factor (VEGF) were determined in 26 patients with AML and 28 healthy individuals. The secretion of VEGF was also measured following the transfection of THP-1 cells with miR-21 mimic or inhibitor. The supernatants of the THP-1 cells, which were transfected with miR-21 mimic, inhibitor or small interfering RNA (si)VEGF, respectively, were used to incubate human umbilical vein endothelial cells (HUVECs), following which tube formation of the HUVECs was measured. miR-21 targets were predicted using a biological target prediction website and confirmed using a luciferase assay. The effects of interleukin (IL)-12 were investigated by examining the tube formation of HUVECs and the secretion of VEGF following recombinant human (rh) IL-12 pretreatment. The results revealed that miR-21 and VEGF expression was significantly increased in the peripheral blood monocytes of the patients, compared with the healthy controls. There was negative correlation between the expression of IL-12 and miR-21 in the serum of patients with AML. Furthermore, supernatant VEGF levels from the miR-21 mimic-transfected THP-1 cells were increased, whereas a decreasing trend was observed in the miR-21 inhibitor group. The angiogenic ability of the HUVECs pretreated with supernatant from the THP-1 cells transfected with miR-21 mimic was higher, and was lower in THP-1 cells co-transfected with miR-21 mimic and siVEGF, compared with the miR-21 mimic only group. A

luciferase assay demonstrated that IL-12 was the direct target of miR-21, and the level of IL-12 in the supernatant of THP-1 cells transfected with miR-21 mimic was increased. IL-12 pretreatment increased VEGF expression and angiogenic ability in HUVECs. The inactivation of miR-21 or activation of its target gene may be a potential therapeutic strategy in human AML.

Introduction

Acute myeloid leukemia (AML) is a clonal expansion disorder of myeloid precursors, which is characterized by the deregulation of important regulators in hematopoiesis (1,2). The mechanisms underlying the formation and development of AML remain to be fully elucidated. Therefore, further investigations on the molecules and signaling pathways, which predict the metastasis and recurrence of AML, are essential.

In previous years, microRNAs (miRNAs) have emerged as novel regulators in myelopoiesis and monocytic leukemia. The overexpression of miRNA (miR)-22, which modulates PU.1, contributes to monocyte/macrophage differentiation and acute monocytic leukemia (3). The enforced expression of miR-34a in T-cell immunoglobulin and mucin domain-containing protein-3-positive leukemia stem cells inhibits its clonogenic expansion and metastasis (4), whereas miR-142-3p and miR-29a promote myeloid differentiation (5). The abnormal expression of miRNAs is involved in AML, serving as useful diagnostic and prognostic indicators (6-8).

Studies have suggested that miR-21 is involved in the initiation and progression of several types of cancer in previous years. In a glioblastoma animal model, the anticancer effect of the R3V6 peptide was mediated by the delivery of an anti-miR-21 antisense-oligodeoxynucleotide (9). The serum expression level of miR-21 is a potential diagnostic marker in colorectal cancer (10) and esophageal carcinoma (11). In addition, miR-21 in peripheral blood mononuclear cells can serve as a novel biomarker in the diagnosis and prognosis of prostate cancer (12). The genetic deletion of miR-21 suppresses the proliferation, migration and invasion in colorectal cancer cells (13), gastric cancer cells (14) and breast cancer cells (15).

In tumors, cancer cells recruit new blood vessels for their growth, maintenance and metastasis. Angiogenesis is a complex process, which depends on the interaction between growth factors, cytokines and a number of components

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of the extracellular matrix (16). Of note, miR-21 can act as either a negative modulator or a positive modulator in different pathways. As a negative modulator of angiogenesis, the overexpression of miR-21 reduces human umbilical vein endothelial cell (HUVEC) proliferation, migration and angiogenic capacity (17). By contrast, miR-21 has also been reported to promote angiogenesis in critical limb ischemia by targeting Hsc70-interacting protein to enhance the activity of hypoxia-inducible factor-1 α (18).

In the present study, the potential function of miR-21 in AML was investigated, and the results indicated that miR-21 was overexpressed in the peripheral blood monocytes of patients with AML. The abnormal expression of miR-21 promoted angiogenesis by repressing the release of IL-12, and these results assist in elucidating the underlying mechanism by which miR-21 promotes the development of AML.

Patients and methods

Patients. A total of 26 newly diagnosed patients with AML were recruited between July 2013 and September 2015 from the Military General Hospital of Beijing PLA (Beijing, China). A total of 28 age- and sex-matched healthy individuals were included as controls. The exclusion criteria included individuals with hypertension and diabetes, and those who have received any other surgery. The present study was approved by the Medical Ethics Committee of the Military General Hospital of Beijing PLA and every patient provided written informed consent. The characteristics of the 26 patients with AML and the 28 healthy individuals are listed in Table I.

Peripheral blood monocyte isolation. Samples of ~0.5 ml blood were harvested from the patients and were dissolved in PBS (1:1). The samples were added to 1 ml gradient centrifugation liquid 10771 (Sigma; Merck Millipore, Darmstadt, Germany), and centrifuged at 300 x g at 4°C for 25 min. The monocytes were present in a layer between the PBS and 10771, and this cell layer was harvested. The primary monocytes were incubated with a rat anti-human CD14 antibody (1 μ g/ml; cat. no. 367116; BioLegend, Inc., San Diego, USA) at 37°C for 30 min. The monocytes were identified as CD14-positive cells using flow cytometry, and the purity of the cells was \geq 90%.

Cell culture and transfection. The human leukemia THP-1 cells were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China). The cells were maintained in medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured at 37°C in humidified air containing 5% CO₂. miR-21 mimics, inhibitor and negative control were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For transfection, the THP-1 cells were cultured to 80% confluence and transfected with either 50 nM miR-21 mimic (sequence unavailable), 100 nM inhibitor (sequence unavailable), 100 nM negative control (sequence unavailable), 50 μ M small interfering RNA targeting VEGF (siVEGF; 5'-GGAGUACCCUGAUGAGAU CdTdT-3'; Sangon Biotech Co., Ltd., Shanghai, China) or 150 mM recombinant human (rh) IL-12 (PrimeGene; R&D Systems China Co., Ltd., Shanghai, China) using a Ribo

Table I. Demographic and clinical data of patients with AML and control subjects.

Variable	AML	Healthy subject
Age (years)	47 \pm 7	50 \pm 5
Sex (male/female)	15/11	18/10
FAB classification		
M4 (n)	16	-
M5 (n)	10	-
WBC (x10 ⁹ /l)	4.48 \pm 3.83	5.60 \pm 4.22
PB blast (%)	51.75 \pm 30.65	18.00 \pm 6.75
PB monocyte (%)	21.01 \pm 20.24	3.65 \pm 0.67
RBC (x10 ¹² /l)	3.05 \pm 0.86	4.15 \pm 0.43
Hb (g/l)	96 \pm 21.90	145 \pm 36.21
PLT (x10 ⁹ /l)	75.23 \pm 50.45	174.58 \pm 55.47
CRP (mg/dl)	2.84 \pm 0.63	0.51 \pm 0.14

Data are presented as the mean \pm standard deviation. AML, acute monocytic leukemia; FAB classification, French-American-British classification; WBC, white blood cell; PB, peripheral blood; RBC, red blood cell; Hb, hemoglobin; PLT, platelet; CRP, C-reactive protein.

FECT™ CP Transfection kit (Guangzhou RiboBio Co, Ltd.) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of miR-21 was determined using a 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total cellular RNA and miRNAs were isolated from the peripheral blood monocytes and cell lines using an RNeasy/miRNeasy Mini kit (Qiagen AB, Limburg, The Netherlands) according to the manufacturer's protocol. The RT reactions were performed using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). The qPCR analysis was performed using SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a final volume of 20 μ l, comprising of 2 μ l cDNA, 10 μ l master mix, 1 μ l ROX and primers (0.4 pmol/ μ l each; 0.8 μ l) and 6.2 μ l ddH₂O. qPCR cycling conditions were as follows: 95°C for 5 sec, followed by 40 cycles of 60°C for 30 sec, 95°C for 15 sec and 60°C for 15 sec, and 95°C for 15 sec. The expression levels of miRNAs and RNAs were calculated using the 2^{- $\Delta\Delta$ C_q} method (19) using U6 as an internal reference. The primer sequences were as follows: miR-21 forward, 5'-TAGCTTATCAGACTGATGTTGA-3' and reverse, 5'-AACGCTTCACGAATTGCGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCA CA-3 and reverse, 5'-AACGCTTCACGAATTGCGT-3'; PDCD4 forward, 5'-AGGCCGAGGTGGGCGGATCACTTGA-3' and reverse, 5'-GCCACCATGRCCTGGCTACT-3'. The RT and PCR primers used for miR-21 and U6 were purchased from Guangzhou RiboBio Co., Ltd.

ELISA for IL-12 and VEGF. The protein levels of IL-12 and VEGF in the serum and in the supernatants of the transfected cells were measured using ELISA kits. ELISA was performed using the human IL-12 and VEGF Quantikine

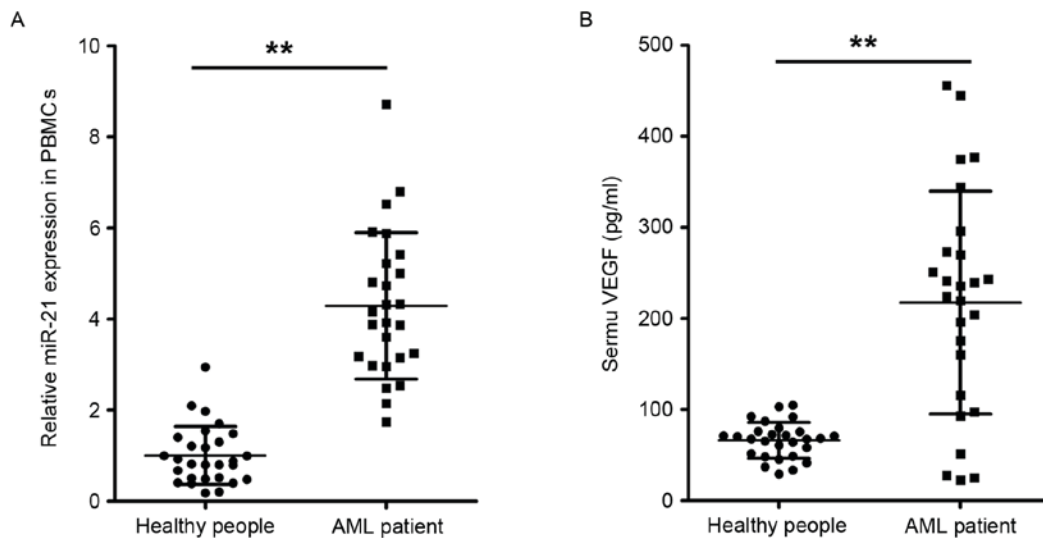


Figure 1. miR-21 and VEGF are significantly increased in patients with AML. (A) Expression of miR-21 in peripheral blood monocytes. (B) Expression of VEGF in serum. ** $P < 0.01$. VEGF, vascular endothelial cell growth factor; AML, acute monocytic leukemia; PBMCs, peripheral blood mononuclear cells.

kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's protocol. The final concentration was calculated according to the standard curve.

Luciferase assay. The wild-type 3'untranslated region (3'UTR) of IL-12 was cloned into the Renilla luciferase gene. (Shanghai GeneChem Co., Ltd, Shanghai, China). The THP-1 cells were cotransfected with vectors carrying wild-type 3'UTR and miR-21 mimic or negative control. The cells were collected 48 h following transfection and analyzed using the Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). The luciferase activity values were normalized relative to that of the Renilla luciferase internal control.

Tube formation assay. The THP-1 cells were transfected with either 100 nM negative control, 50 nM miR-21 mimic or 100 nM inhibitor, or were co-transfected with 50 nM miR-21 and 50 μ M siVEGF for 24 h. Following transfection, the supernatants were harvested. HUVECs were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were incubated at 1×10^5 cells/well at 37°C with these supernatants for 24 h, respectively. The angiogenic ability of the HUVECs were detected. Matrigel was used to determine the effects on *in vitro* vascular tube formation. The HUVECs were respectively seeded at 5×10^4 cells per well in a 48-well plate on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated for 6 h at 37°C and evaluated by phase-contrast microscopy, and images were captured. Tubes were defined as straight cellular extensions joining cycle, and were counted in three random digital images (magnification, $\times 200$) for each well.

Statistical analysis. Data are presented as the mean \pm standard deviation. Graphs were drawn using GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using one-way analysis of variance followed by Student's t-test to assess significant differences between

groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-21 and VEGF are significantly increased in patients with AML. To examine the changes in the expression miR-21 in cases of AML, the expression levels of miR-21 were examined in peripheral blood monocyte specimens from 26 patients with AML and 28 healthy individuals. As shown in Fig. 1A, the expression levels of miR-21 were significantly increased in the peripheral blood monocytes of patients with AML, compared with those of healthy controls. These results of suggested an association between miR-21 and AML. The results of the present study confirmed that the serum levels of VEGF were also significantly elevated in patients with AML, compared with those in the healthy controls, as shown in Fig. 1B. It was hypothesized that miR-21 functionally regulated AML; therefore, subsequent experiments were performed focusing on the role of miR-21 in the regulation of angiogenesis.

miR-21 promotes the angiogenic ability of HUVECs. To determine whether AML cells or normal monocytes overexpress miR-21, the present study used peripheral blood monocytes of healthy individuals as a control to compare the expression of miR-21 in different AML cell lines. It was found that the expression levels of miR-21 were relatively higher in the AML cell lines, particularly in THP-1 cells, as shown in Fig. 2A. Based on this expression pattern, the present study selected the THP-1 cell line for the following experiments. Subsequently, the miR-21 mimic or inhibitor was used to confirm whether miR-21 affects the release of angiogenic factors. It was found that the levels of VEGF in the supernatant of the miR-21 mimic-transfected cells were increased, compared with levels in the negative control (Fig. 2B). However, miR-21 inhibitor transfection led to the level of VEGF being significantly decreased, as shown in Fig. 2C. In addition, pretreatment of

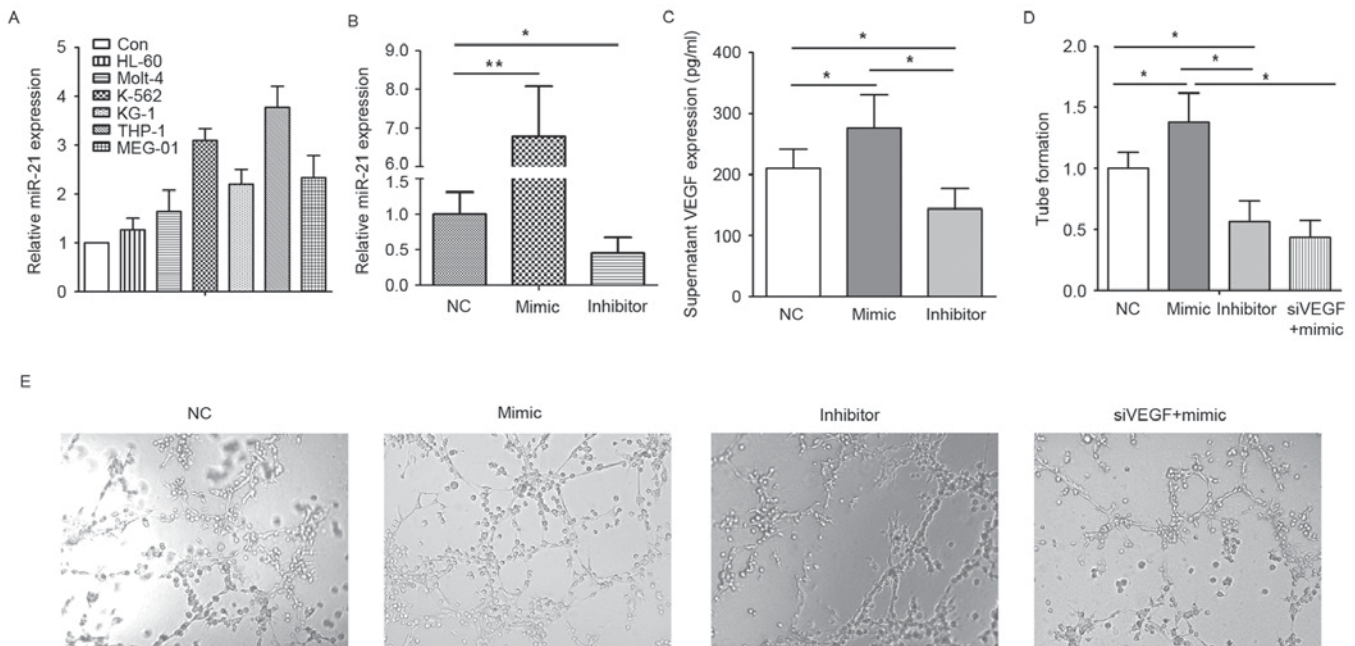


Figure 2. miR-21 promotes the angiogenic ability of HUVECs. (A) Expression of miR-21 in different types of cell. (B) Expression of miR-21 in THP-1 cells following transfection with NC, miR-21 mimic or miR-21 inhibitor. (C) VEGF in the supernatants of THP-1 cells transfected with NC, miR-21 mimic or miR-21 inhibitor. (D) Tube formation of HUVECs pretreated with the supernatant from THP-1 cells transfected with NC, miR-21 mimic, miR-21 inhibitor or siVEGF and miR-21 mimic. (E) Images of tube formation. Magnification, x200. * $P < 0.05$; ** $P < 0.01$. miR, microRNA; Con, control; VEGF, vascular endothelial growth factor; AML, acute monocytic leukemia; PBMCs, peripheral blood mononuclear cells; HUVECs, human umbilical vein endothelial cells; NC, negative control; siVEGF, small interfering RNA targeting VEGF.

the HUVECs with these respective supernatants altered the angiogenic ability of the HUVECs. As shown in Fig. 2D and E, tube formation in the HUVECs pretreated with the supernatant from THP-1 cells transfected with miR-21 mimic was higher, compared with that in the negative control, and was lower in the miR-21 inhibitor-transfected cells. The angiogenic ability of the HUVECs pretreated with supernatant from the THP-1 cells co-transfected with miR-21 mimic and siVEGF were decreased, compared with those in the miR-21 mimic-transfected cells. These data suggested that miR-21 stimulated the monocyte release of VEGF to promote angiogenesis.

miR-21 upregulates angiogenesis by targeting IL-12. To obtain further insight into the molecular mechanism by which miR-21 affect AML cells, the present study performed a search for genes targeted by miR-21 using the biological target prediction website (<http://www.microrna.org/microrna/home.do>), which drew attention to IL-12. To verify the prediction, a luciferase reporter vector was constructed containing the 3'UTR of IL-12. As shown in Fig. 3A, the miR-21 mimic decreased the luciferase activity of the vector carrying the 3'UTR of IL-12. It was also found that the levels of IL-12 in supernatants from the THP-1 cells transfected with miR-21 mimic were decreased, compared with that in the negative control, as shown in Fig. 3B. The present study then used IL-12 to pretreat HUVECs, and found that the level of VEGF in the supernatants was decreased following rh IL-12 pretreatment (Fig. 4A) and the angiogenic ability of the HUVECs was reduced following IL-12 pretreatment (Fig. 4B and C). Patients with <3.5 relative expression of miR-21 were defined as having a low expression of miR-21,

and the present study found that serum levels of IL-12 were significantly decreased in patients with AML with a high level of miR-21, compared with those with a low level of miR-21 (Fig. 5A). To clarify the association between IL-12 and miR-21, the present study further analyzed the levels of serum IL-12 and miR-21, which revealed a negative correlation between the two (Fig. 5B).

Discussion

AML is the most common type of acute leukemia affecting adults, and its incidence increases with age. Although AML is a relatively rare disease, accounting for ~1.2% of cases of cancer-associated mortality, its incidence is expected to increase as the population ages (20). Several miRNAs are located inside or close to chromosomal fragile sites, which are frequently lost or amplified in cancer, and the correlation between miRNAs and cancer has become a focus for the diagnosis and therapy of cancer (21,22). In the present study, it was demonstrated that the expression of miR-21 was significantly increased in peripheral blood monocytes of patients with AML, compared with healthy controls. The peripheral blood monocytes of healthy individuals were then used as a control to compare the expression of miR-21 in different AML cell lines. It was found that the expression levels of miR-21 were relatively higher in the AML cell lines. These data indicated that the excessive expression of miR-21 was from AML cells, not from the normal monocytes.

miRNAs can act as oncogenes or tumor suppressors and are involved in numerous cellular processes, being key in tumorigenesis by regulating angiogenic processes (23,24). The genetic deletion of miR-21 suppresses the proliferation,

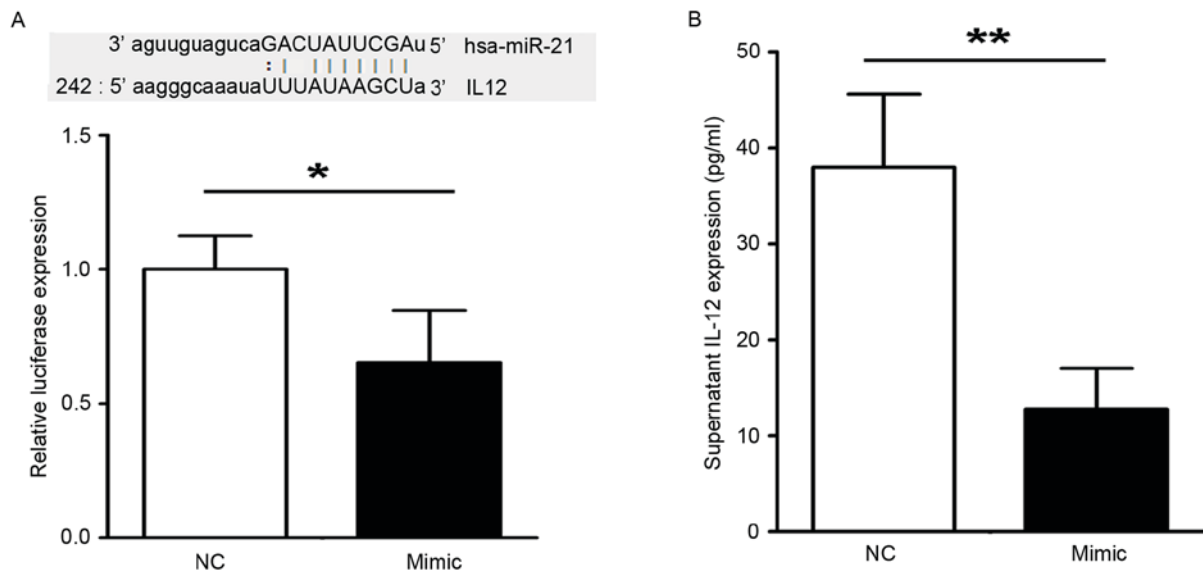


Figure 3. IL-12 is a direct target of miR-21. (A) Luciferase reporter assay in THP-1 cells co-transfected with NC or miR-21 mimic and WT IL-12 3'untranslated region. (B) IL-12 in the supernatant of THP-1 cells transfected with NC or miR-21 mimic. *P<0.05; **P<0.01. miR, microRNA; IL-12, interleukin-12; WT, wild-type; NC, negative control.

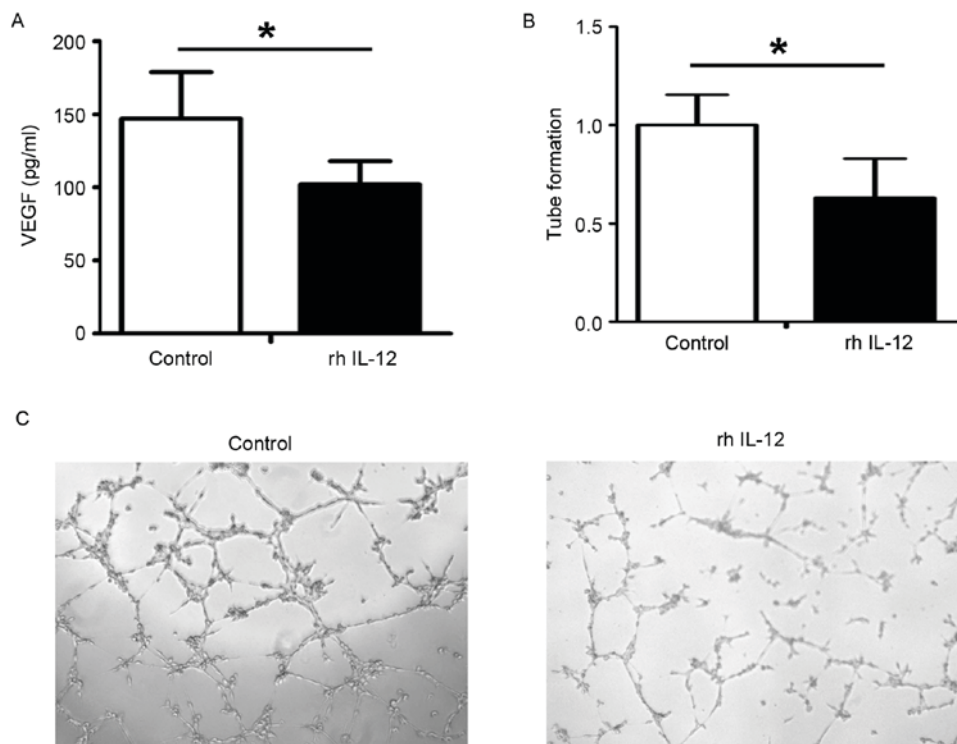


Figure 4. IL-12 downregulates angiogenesis by inhibiting VEGF release. (A) VEGF in the supernatant of HUVECs pretreated with or without rh IL-12. (B) Tube formation of HUVECs pretreated with or without rh IL-12. (C) Images of tube formation (magnification, x200). *P<0.05. miR, microRNA; rh IL-12, recombinant human interleukin-12; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.

migration and invasion in colorectal cancer cells (13), gastric cancer cells (14) and breast cancer cells (15). Furthermore, the expression of miR-21 has been associated with angiogenesis (17,18). However, the effect of miR-21 on the development and metastasis of AML remains to be fully elucidated. Studies have shown that VEGF is a growth factor and an angiogenic inducer, and suppression of the secretion of VEGF and/or inhibition of the activity of VEGF can

attenuate the tumor-induced development of novel blood vessels (25). In the present study, it was found that supernatant levels of VEGF from the miR-21 mimic-transfected cells were increased, compared with those in the negative control. Following miR-21 inhibitor-transfection, the level of VEGF was significantly decreased. The present study also compared tube formation in HUVECs pretreated with supernatant from THP-1 cells transfected with the miR-21 mimic. The tube

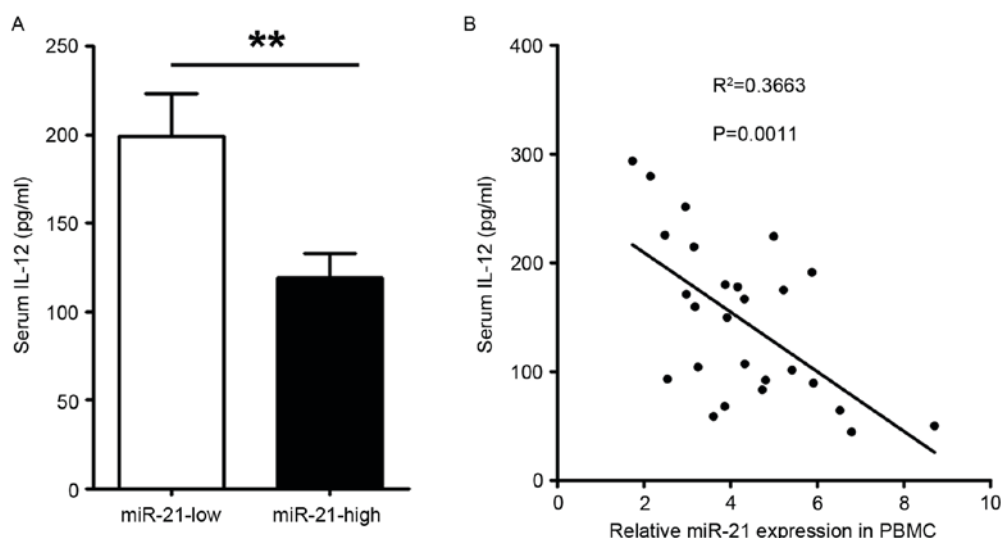


Figure 5. IL-12 is reduced in patients with AML and negative correlated with the expression of miR-21. (A) Expression of IL-12 in patients with low and high levels of miR-21-21. (B) Serum levels of IL-12 are negatively correlated with miR-21 in patients with AML. ** $P < 0.01$. miR, microRNA; IL-12, interleukin-12; AML, acute monocytic leukemia; PBMCs, peripheral blood mononuclear cells.

formation was increased, compared with that in the negative control and was decreased by miR-21 inhibitor transfection. Following neutralization of the effect of VEGF with siVEGF, the angiogenic promotion of miR-21 was eliminated. These results indicated that aberrant increases in the level of miR-21 may have the ability to stimulate the secretion of VEGF to promote angiogenesis.

Certain miRNAs coordinate large numbers of target genes (26). Several miR-21 targets have been reported, including phosphatase and tensin homolog (27), programmed cell death protein 4 (28) and small mothers against decapentaplegic 7 (29). To investigate the molecular mechanism by which miR-21 affects AML cells, the present study performed a search for possible mRNA targets using biological the target prediction website (<http://www.microrna.org/microrna/home.do>), which identified IL-12. IL-12 is a potent immunostimulatory cytokine, which exhibits antitumoral activity. Intratumoral IL-12 gene therapy can stimulate the immune system and decreases angiogenesis in dogs with spontaneous cancer (30). In the present study, it was found that the serum levels of IL-12 were significantly decreased in the patients with AML and high expression levels of miR-21, compared with those with low expression levels of miR-21, and there was a negative correlation between serum IL-12 and miR-21. The miR-21 mimic also decreased the luciferase activity of the vector carrying the 3'UTR of IL-12, and the level of IL-12 in supernatants from THP-1 cells transfected with the miR-21 mimic was increased, compared with that in the negative control. These data indicated that IL-12 was a direct target of miR-21. In a previously reported murine model of breast cancer, following 7 days of IL-12 treatment, the protein levels of VEGF in the tumor decreased markedly and were undetectable at 14 days (31). In the present study, it was also found that supernatant levels of VEGF were decreased, and that the angiogenic ability of the HUVECs expanded following rh IL-12 pretreatment. These results suggested that IL-12 regulated angiogenesis by inhibiting the pro-angiogenic VEGF release.

In conclusion, the present study demonstrated that miR-21 was upregulated in patients with AML. It was also found that miR-21 regulated angiogenesis targeting IL-12. Therefore, the inactivation of miR-21 or activation of its target gene may be a potential therapy for human AML.

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Availability of data and materials

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

Authors' contributions

X-PH designed the experiment and was a major contributor in conducting the experiments and writing the manuscript. PC and KY collected and analyzed the PCR data, and contributed to cell culture and transfection. BL and YZ analyzed and interpreted the patient data. FW detected VEGF levels in serum. ZG, X-DL, J-XL and H-RC performed the molecular assays. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the Military General Hospital of Beijing PLA and every patient provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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