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MicroRNA-199 inhibits proliferation and promotes apoptosis in children with acute myeloid leukemia by mediating caspase-3

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Abstract. – OBJECTIVE: The aim of this study was to detect the expression level of microRNA-199 in acute myeloid leukemia (AML). Meanwhile, we also investigated whether microRNA-199 could inhibit the proliferation and promote apoptosis of AML cells by regulating caspase-3.

PATIENTS AND METHODS: The expre[illegible] level of microRNA-199 in peripheral bloo[illegible] ples of AML patients and healthy contro[illegible]as determined using Real Time-quantitative [illegible]- merase Chain Reaction (RT-qPCR). Simila[illegible] microRNA-199 expression was [illegible]lso detect[illegible] in AML cells and human me[illegible]ells. Th[illegible] overexpression plasmid of [illegible]oR[illegible]99 was constructed and transfec[illegible]nto ce[illegible] Subsequently, the regulatory e[illegible]s of m[illegible]199 on the proliferation, [illegible]ll [illegible]pto[illegible] vasion and migrati[illegible]ere a[illegible]d. The relationship between [illegible]oRNA-19[illegible] caspase-3 expression was[illegible]r elucidate[illegible] Western blot and RT-q[illegible].

RESULTS: M[illegible]croRNA[illegible] was lowly expressed in periph[illegible] blood of A[illegible]atients and AML cell line[illegible]an normal contr[illegible]he overexpression [illegible]icroR[illegible]-199 signi[illegible]antly decreased pro[illegible]ative, i[illegible]sive and migratory capacities of A[illegible]lls, [illegible]ereas [illegible]rkedly increased apoptotic [illegible] [illegible]estern [illegible] results showed that [illegible]oRN[illegible] inc[illegible]ed caspase-3 expression i[illegible] cells[illegible] experiments demonstrated tha[illegible]croRN[illegible] inhibited malignant progressio[illegible] AML by targeting caspase-3.

CONCLUSIONS: MicroRNA-199 is lowly expressed in AML patients. Furthermore, it inhibits the malignant progression of AML by targeting caspase-3.

Key Words
MicroRNA-199, Caspase-3, Acute myeloid leukemia (AML), Malignant progression.

Introduction

[illegible]cute leuken[illegible](AL) is a malignant hematolo[illegible]d disease c[illegible]ed by excessive proliferation of i[illegible]poie[illegible]lls and infiltration into various tissues [illegible]ns. AL can eventually result in a series of clinical manifestations[1]. Currently, it is [illegible]t common childhood malignant tumor in [illegible]. The incidence rate of males is higher than that of females[2]. AL cells can infiltrate into various tissues and organs, causing various symptoms and signs. This includes liver swelling, splenomegaly and lymphedema, bone and joint pain, headache, nausea and vomiting[3-5]. Acute myeloid leukemia (AML) is a common type of pediatric AL[4]. It has been found that infections, bleeding, anemia and vital organ failure secondary to extramedullary infiltration are important causes of death in leukemia patients. Therefore, the infiltration characteristics of leukemia cells have been well concerned[3-5].

The early diagnosis and treatment of AML depend on an in-depth understanding of its mechanism[6]. However, due to the complexities of the occurrence and development of AML, the specific mechanism has not been fully clarified. A large number of genes and pathways are involved in the pathogenic progression of AML[7,8]. Specific roles and significances of related factors may contribute to AML development[9,10]. In recent years, studies have shown that microRNAs are closely related to the occurrence and development of a variety of malignant tumors. Due to pronounced biological functions of microRNAs, they have been well studied[11].

MicroRNA is an endogenous, non-coding small RNA consisting of 18-22 nucleotides. It is a

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single-stranded RNA precursor containing a hairpin structure of about 70 bases in length, which is processed by Dicer. A 5'-terminal phosphate group and a 3'-hydroxyl group are positioned at the 3'- or 5'-end of RNA precursor, respectively[12-15]. MicroRNA was first discovered in 1993. Previous studies[16,17] have shown that it is widely expressed in humans, fruit flies, plants and other organisms. More than 1,000 microRNAs have been found in the human genome. MicroRNAs have highly conserved sequences, which are capable of regulating gene expression by inhibiting or degrading mRNA of target genes. Functionally, they can regulate multiple biological processes, such as early development, cell proliferation, apoptosis and differentiation[18-22]. Furthermore, potential roles of microRNA in tumors have been reported, including chromosomal recombination regulation, gene imprinting, epigenetic regulation, nuclear transport, mRNA splicing and translation[20-22].

MicroRNA-199 was first cloned from mouse cells in 2003. It contains two miRNA precursors, namely pre-microRNA-199a-1 derived from chromosome 19 and pre-microRNA-199a-2 derived from chromosome 1. Two different m[illegible]e microRNAs are lysed by Dicer, includi[illegible] croRNA-199a-5p and microRNA-199a-3p. [illegible] to different sequences and mRNA targets, mic[illegible] NA-199a-5p and microRNA-199a-3p have dif[illegible] ent mRNA targets and exert [illegible] biologic[illegible] functions[23-26]. However, whe[illegible] mi[illegible]NA-199 participates in the pathoge[illegible]s of A[illegible] has not been fully elucidated.

In this study, we fi[illegible] det[illegible] oRN[illegible]9 expression in peri[illegible]l blood[illegible] ML patients and healthy con[illegible] Furthermo[illegible] explored the regulatory [illegible] microRNA[illegible]9 on biological perfo[illegible]nances [illegible] ML cells. Here, we aimed to [illegible]idate the po[illegible]l role of microRNA-199 [illegible]he AML develop[illegible].

[illegible]ents [illegible] Methods

[illegible]nts a[illegible] Samples

[illegible]newly d[illegible]osed AML patients who were ad[illegible] the Pediatric Department were se[illegible]d [illegible] study. Enrolled AML patients were [illegible]agnosed according to pathological and clinical [illegible]res. Meanwhile, 45 healthy people were en[illegible]s normal controls. The study has been approved by the Ethics Committee of our hospital. The signed informed consent was obtained from patients and their families before the study.

Cell Culture

6 human AML cell lines (AML2, AML5, AML193, HL-60, Kasumi-1, and U937) and 1 human medullary cell line (HS-5) were pr[illegible]d by the American Type Culture Collecti[illegible] Manassas, VA, USA). Cells were cu[illegible]d in Du[illegible] becco's Modified Eagle's Mediu[illegible]MEM; HyClone, South Logan, UT, USA) c[illegible]ng 10% fetal bovine serum (FBS; C[illegible]o, Gr[illegible]land, NY, USA) in a 37°C, 5% C[illegible]ncubator.

Cell Transfection

We authorized G[illegible] Biote[illegible]logy Co., Ltd (Shang[illegible] Chi[illegible] constr[illegible] overexpression plasm[illegible] microRN[illegible]e corresponding n[illegible]ntrol. Cells [illegible]irst seeded into 6-we[illegible]ates. [illegible] the confluence was up to 70%, cell transfectio[illegible] performed according to t[illegible]uctions of L[illegible]tamine 2000 (Invitr[illegible], Carlsbad, CA, US[illegible]). 48 hours later, the [illegible]sfected cells [illegible]ere harvested for subsequent [illegible]riments.

Ce[illegible]lifer[illegible]n Assay

The [illegible]ted cells were plated into 96-well [illegible]lates at a density of 2000 cells per well. After [illegible]g for 6 h, 24 h, 48 h, and 72 h, respective[illegible], [illegible] of cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well. After 2 hours of incubation in the dark, the optical density (OD) value at the wavelength of 490 nm was measured using a microplate reader.

Flow Cytometry Analysis of Cell Apoptosis

AML cells were pre-seeded into 6-well plates. After specific treatment for 24 h, the cells were washed with phosphate-buffered saline (PBS) twice and re-suspended in the binding buffer for 15 min in the dark. Then the cells were then incubated with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of Propidium Iodide (PI) (Solarbio, Beijing, China) in the dark. Finally, cell apoptosis was detected using flow cytometry.

Flow Cytometry Analysis of Cell Cycle

AML cells were pre-seeded into 6-well plates. After specific treatment for 24 h, the cells were washed with PBS twice and fixed with 70% precooled ethanol overnight. On the next day, the cells were washed with PBS for discarding the remaining ethanol, followed by incubation with 0.6

mL of PI in the dark for 30 min. The cell cycle was finally detected using flow cytometry.

Transwell Assay

Cells were digested and re-suspended in serum-free medium and the cell density was adjusted to 2.0×10^5/mL. Transwell chamber containing Matrigel or not was placed in a 24-well plate. 200 µL of cell suspension and 500 µL of medium containing 10% FBS were added to the upper and lower chamber, respectively. After 48 hours of culture, the chamber was removed. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 15 min. The inner layer cells were carefully removed. 5 fields were randomly selected for each sample, and the number of penetrating cells was counted.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from AML cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (Ta… Otsu, Shiga, Japan). RT-qPCR was perfo… strict accordance with SYBR® Premix Ex T… M (TaKaRa, Otsu, Shiga, Japan), and StepOne … Real-time PCR System (Applied Biosystems, F… ter City, CA, USA). Primers u… this stu… were as follows: microRNA-1… -A… GGGGT-… GGAGGGGAGACTAG-3’… 5’-T… GGGT-… GCTCGCTTCGGCAGC… Taspas… … 5’-TTGGGAACCAGC… CC… … 5’-CCAGGGATCT… CTTG… B-actin: forward: 5’-CCTGG… CCAGCAC… 3’, reverse: 5’-TGCCGTAC… CTTTG-3’.

Western Blot

Total protein was extracted using RIPA cell lysate (Beyotime, Shanghai, China). Protein samples were separated by sodium dodecyl sulphat… acrylamide gel electrophoresis (SDS-P… transferred onto polyvinylidene diflu… (PVDF) membranes (Millipore, Billerica, … USA). After blocking with the blocking solut… 1 h, the membranes were incubated w… prima… body at room temperature for 2… After wash… Tris-Buffered Saline an… ween 2… (TBST), corresponding second… antibo… was used for incubation at room tem… 2 h. I… uno-reactive bands w… visua… by the …anced chemilumines… method (… sher Scientific, Wal… A, USA).

Statistical Analysis

S… l Product an… ice Solutions (SPSS) 22… IBM, Armonk, NY, … SA) was used for all s… stical analyses. Data were expressed as mean ± … dard deviatio… Continuous variables were an… using *t*-te… nd categorical variables were ana… sin… est or Fisher’s exact test. $p<0.05$ was co… statistically significant.

Results

MicroRNA-199 Was Lowly Expressed in AML Tissues and Cell Lines

We first detected microRNA-199 expression in peripheral blood samples of 45 AML patients and healthy controls by RT-qPCR. Compared with healthy controls, the expression of microRNA-199 in peripheral blood of AML patients was significantly lower (Figure 1A). Mi-

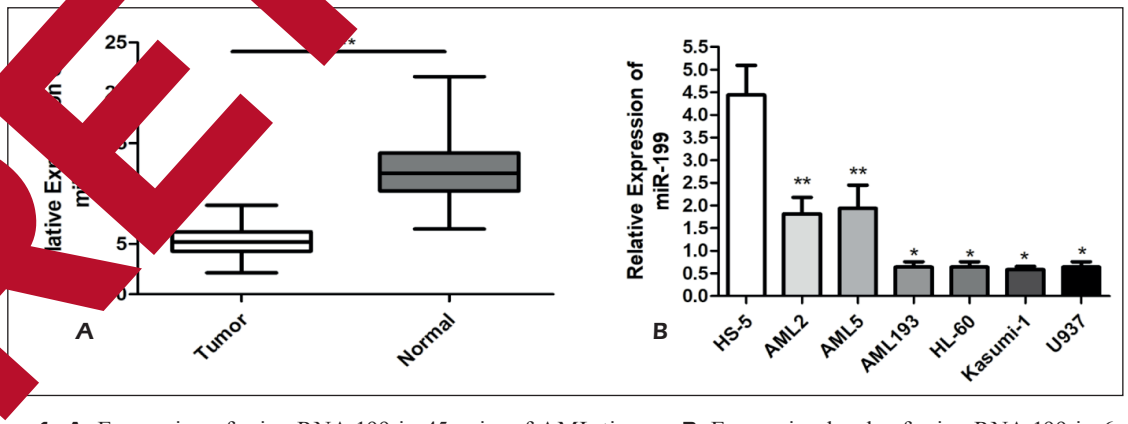

Figure 1. A, Expression of microRNA-199 in 45 pairs of AML tissues. **B**, Expression levels of microRNA-199 in 6 AML cell lines (AML2, AML5, AML193, HL-60, Kasumi-1, U937) and 1 normal marrow cell (HS-5). A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

croRNA-199 expression was also determined in AML cells and human medullary cells. The results showed that the expression of microRNA-199 in AML cells was significantly higher than that of the human medullary cell line. Particularly, AML2 and AML193 cells expressed the highest level of microRNA-199 (Figure 1B). These two AML cell lines were utilized for subsequent experiments.

Overexpression of MicroRNA-199 Inhibited Cell Proliferation

To explore the regulatory effect of microRNA-199 on the proliferative ability of AML cells, we first constructed microRNA-199 overexpression model in AML2 and AML193 cells. Transfection efficacies of the overexpression plasmid of microRNA-199 and negative control in AML cells were determined by RT-qPCR (Figure 2A, 2B). AML cells were divided into three groups, namely NC group (without any treatment), control group (transfected with control plasmid) and overexpression group (transfected with overexpression plasmid of microRNA-199). CCK-8 results elucidated that the proliferative rate of AML cells in microRNA-199 overexpression was remarkably decreased when compared with NC and control groups (Figure 2C, 2D).

Overexpression of MicroRNA-199 Induced Cell Apoptosis in AML Cells

Apoptosis of AML cells was examined using flow cytometry. Compared with the NC group and control group, AML cells in the overexpression group showed significantly higher rate double-staining of Annexin V-FITC/PI. This indicated that the apoptotic rate was markedly enhanced (Figure 3). Therefore, the above results suggested that microRNA-199 overexpression stimulated the apoptosis of AML cells.

Overexpression of MicroRNA-199 Changed Cell Cycle in AML Cells

Cell cycle in AML cells after altering microRNA-199 expression was accessed using flow cytometry as well. The results indicated that the number of cells in the G2/M phase was remarkably higher in the overexpression group when compared with the other two groups. This demonstrated that microRNA-199 overexpression regulated the cell cycle in AML cells (Figure 2F).

Overexpression of MicroRNA-199 Inhibited Cell Invasion

Transwell assay was conducted to evaluate the regulatory effect of microRNA-199 on migratory and invasive capacities of AML cells. As in Figure 3A and 3B, the number of penetrating cells was markedly reduced in the overexpression group than the control and NC group. These findings suggested that the migration and invasion of AML cells were inhibited by microRNA-199 overexpression.

Overexpression of MicroRNA-199 Increased Caspase-3 Expression

To elucidate the potential mechanism of microRNA-199 promoting apoptosis of AML cells, Western blot was conducted to determine the protein expression of caspase-3. The results showed that the overexpression of microRNA-199 increased the protein expression of caspase-3 in AML cells (Figure 4A). Through bioinformatic analysis, we believed that caspase-3 might be closely related to microRNA-199. Subsequently, we detected caspase-3 level in peripheral blood of AML patients and healthy controls. Significantly higher level of caspase-3 was observed in AML patients when compared with that of healthy controls (Figure 4B). Similarly, caspase-3 was also highly expressed in AML cells than that of controls (Figure 4C).

Caspase-3 Regulated MicroRNA-199 Expression in Human AML Cells

We found that the caspase-3 knockdown could upregulate microRNA-199 in AML cells. Subsequently, we selected 16 pairs of peripheral blood samples extracted from AML patients and healthy controls. RT-qPCR was conducted to detect mRNA levels of microRNA-199 and caspase-3 in the blood samples. The results elucidated that microRNA-199 expression was negatively correlated with caspase-3 expression (Figure 4D).

To verify whether microRNA-199 regulated AML development by targeting caspase-3, rescue experiments were conducted. AML cells were co-transfected with overexpression plasmid of microRNA-199 and si-caspase-3. The results indicated that both mRNA and protein levels of caspase-3 were downregulated in co-transfected AML cells (Figure 5A, 5B). Moreover, co-transfected AML cells showed significantly increased proliferative ability and decreased apoptotic rate (Figure 5C, 5D).

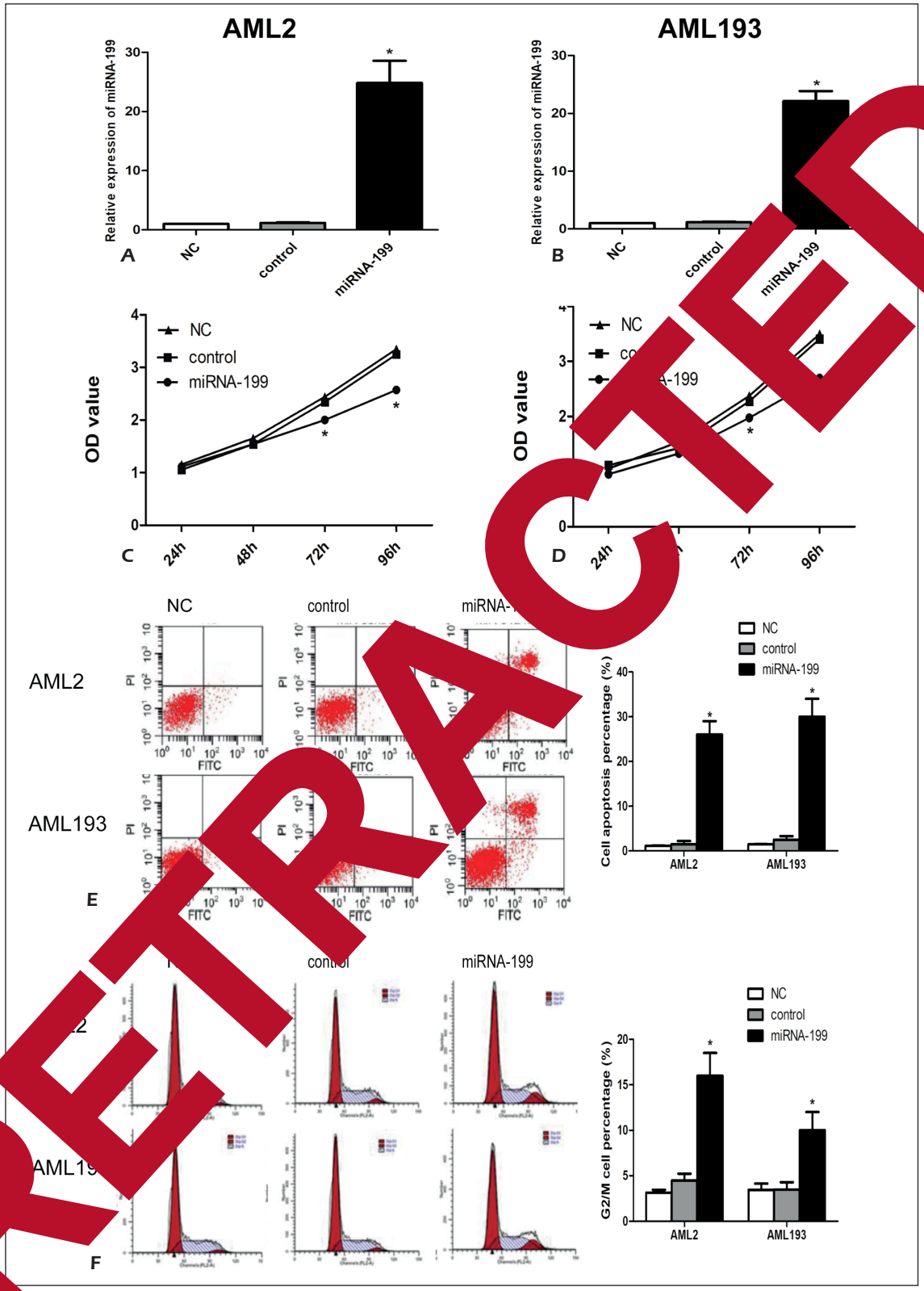

Figure 2. A-B, RT-qPCR was used to verify the efficiency of microRNA-199 overexpression in AML2 and AML193 cell lines. **C-D**, Growth curve analysis of AML2 and AML193 cells with microRNA-199 overexpression. **E**, Efficiencies of cell apoptosis in AML2 and AML193 cells with microRNA-199 overexpression. **F**, Cell cycle in AML2 and AML193 cells with microRNA-199 overexpression. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

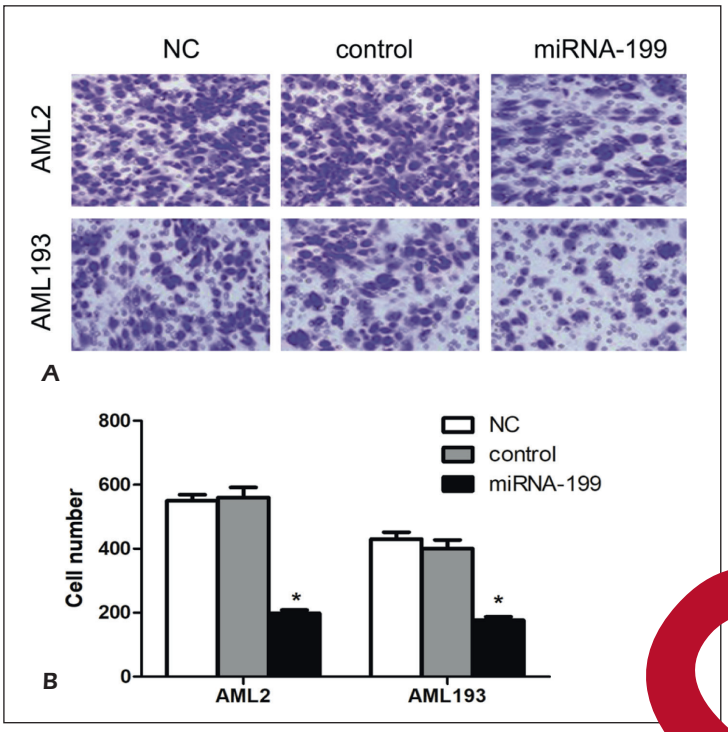

Figure 3. A-B, AML2 and AML193 cells with microRNA-199 overexpression showed significantly lower invasive capacity. A representative data set was displayed as mean ± SD values. *p<0.05, **p<0.01.

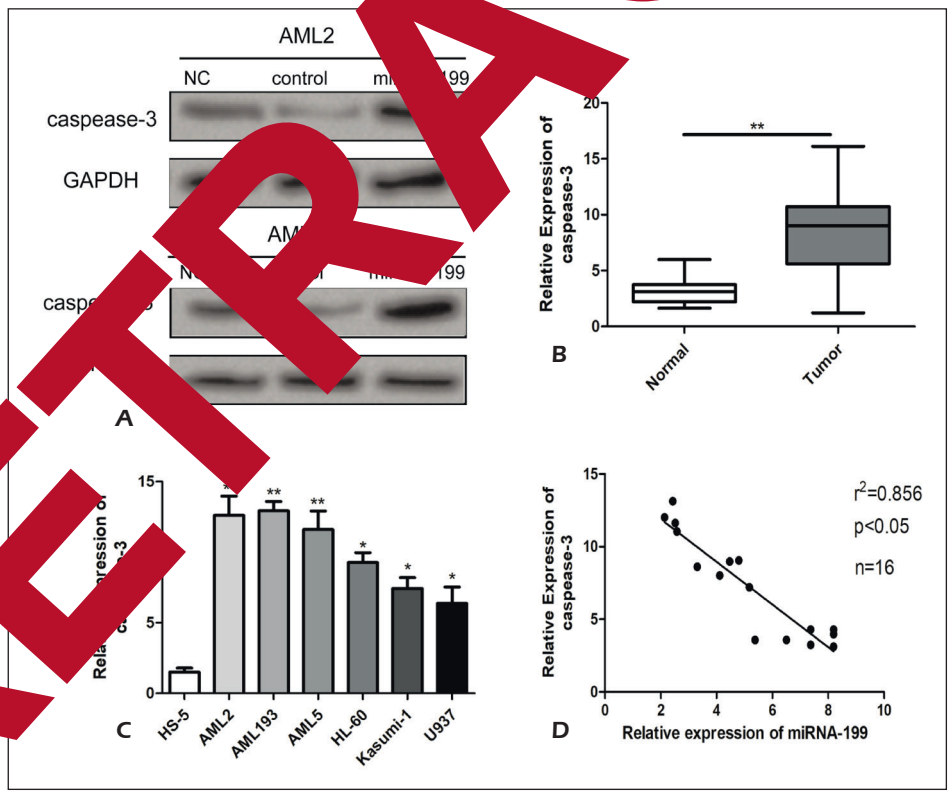

Fig. 4. A, Overexpression of microRNA-199 significantly increased the protein expression level of caspase-3. **B-C**, The mRNA expression level of caspase-3 relative to GAPDH in AML tissues and corresponding normal tissues, and cell lines were detected using RT-qPCR. **D**, A negative correlation was found between microRNA-199 and caspase-3 in tumor samples. A representative data set was displayed as mean ± SD values. *p<0.05, **p<0.01.

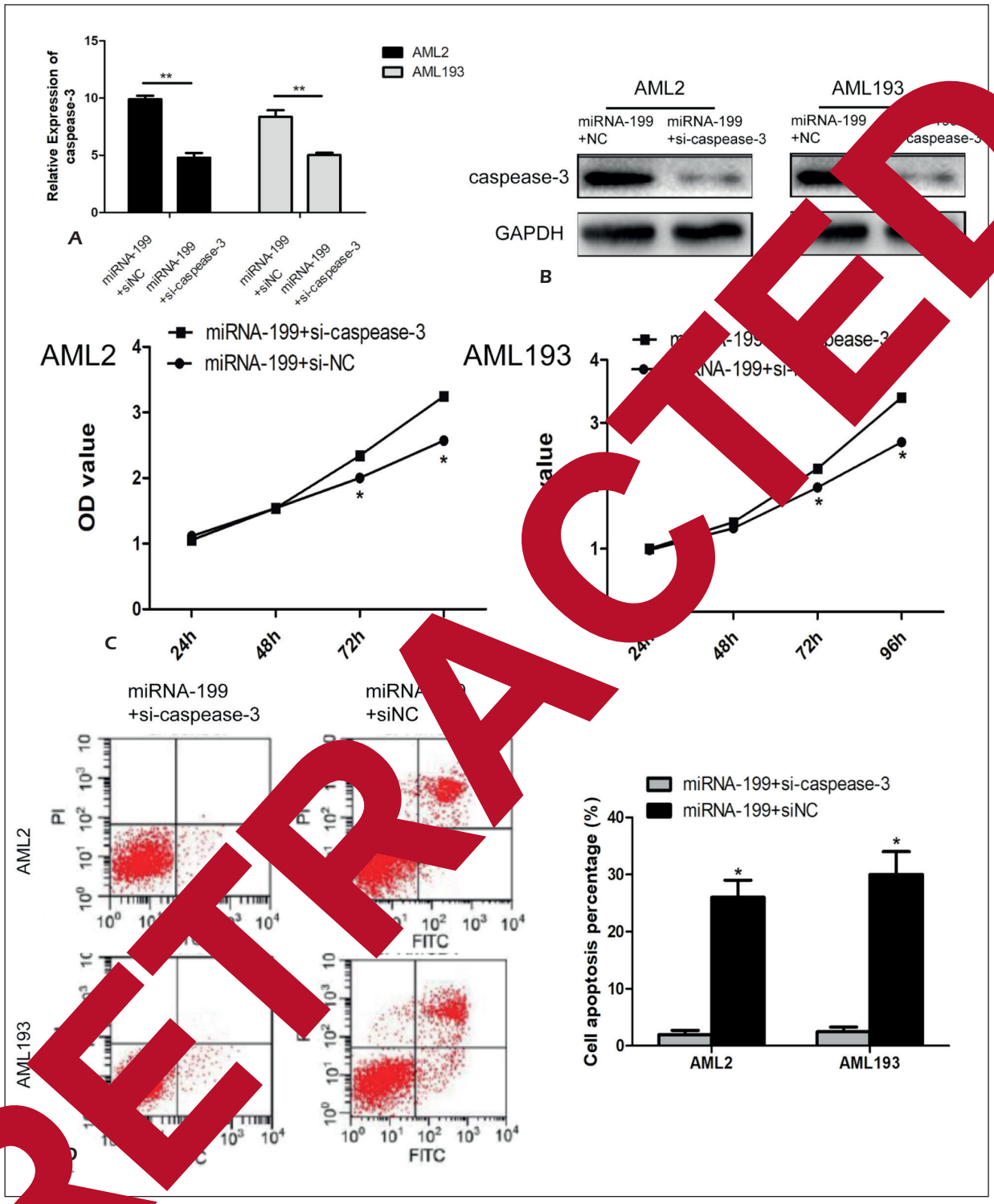

Figure 3. The expression of caspase-3 in co-transfected cell lines was verified by RT-qPCR. **B**, Western blot was used to verify the protein expression of caspase-3. **C**, The roles of microRNA-199 and caspase-3 in the regulation of AML cell proliferation were examined by CCK-8. **D**, The efficiencies of cell apoptosis in co-transfected AML2 and AML193 cells. A representative data set was displayed as mean ± SD values. *$p<0.05$, **$p<0.01$.

Discussion

AL is a clonal malignant disease with abnormal hematopoietic stem cells. Leukemia cells lose the abilities of clone, differentiation and maturation, thus arresting at different stages of cell development[1-3]. In bone marrow and other hematopoietic tissues, a large number of proliferative leukemia cells infiltrate into other organs and tissues. This can eventually impair the normal function of hematopoiesis. The clinical manifestations of AL include anemia, hemorrhage and infection. Meanwhile, AL is the most common malignancy in childhood[4,5]. According to the French-American-British (FAB) classification systems and cell morphology and cytochemical staining characteristics, AL is divided into ALL and AML[6]. Researches on leukemia immuno-phenotyping and cytogenetic typing have been greatly improved due to advanced technologies. This provides strong evidence for the diagnosis and typing of AL[9,10]. At present, great progress has been made in immunological typing and cytogenetic typing research as well[27]. These new typing methods are highly sensitive, specific, and reproducible. The abovementioned advantages make up for the shortcomings of FAB typing, especially in poorly differentiated and mixed leukemia that are indistinguishable in morphology[27,28]. A current research[29] on the diagnostic classification of AL aims to accurately reflect the genetic biological characteristics of leukemia subtypes. Eventually efficiently improving the outcomes of patients. MiRNAs play important role in a variety of diseases, including malignant tumors. Abnormally expressed miRNAs in AML may serve as biomarkers for diagnosis, treatment and prognosis of AML[11]. Therefore, it is of great significance to analyze the differentially expressed miRNA to improve the diagnostic and therapeutic efficacies of AML.

MicroRNAs can regulate biological functions by inhibiting target genes[12-15]. Bioinformatics analysis suggests that one microRNA may directly regulate the expression of hundreds of genes, thereby regulating multiple important cellular activities and functions[16]. For example, microRNAs regulate cell division, differentiation, proliferation and apoptosis. They also participate in the development of various diseases, such as malignant tumors and cardiovascular diseases[17-19]. Furthermore, microRNAs can influence tumor cell proliferation, apoptosis, sensitivities to chemotherapy and radiotherapy, and even define the phenotype of cancer stem cells[18]. Therefore, revealing the role and mechanism of microRNAs are helpful to understand the complex molecular mechanism of AML. This also provides targets for the development of AML drugs[11]. In this study, we detected the clinical features of microRNA-199a in AML, and investigated its underlying mechanism. The results indicated that microRNA-199a was lowly expressed in AML, which might serve as a tumor-suppressor gene. To further explore the biological function of microRNA-199a in AML, overexpression lentivirus of microRNA-199 was constructed and transfected into cells. Further *in vitro* experiments indicated that microRNA-199 significantly inhibited the cell viability and progression of AML.

Apoptosis is a form of cellular senescence and death. It has important biological significance for cell differentiation, proliferation and body development[30,31]. Apoptosis is a complex process, which can be divided into caspase-dependent and non-caspase-dependent apoptosis[32]. It is believed that it is a process induced by caspase protease cascade. Apoptosis signal first activates the promoter caspase, then activates the effector caspase. The substrate protein is stimulated to induce protein degradation, thereafter leading to apoptosis[33]. In the caspase family, the caspase-3 pathway is the only pathway for apoptosis proteases cascade[34]. Caspase-dependent apoptosis is ubiquitous in a variety of mammalian cells. Caspase-3 exerts a crucial role in neuronal apoptosis. It is activated in the cell cytoplasm, which is immediately transferred to the nucleus[34,35]. It is suggested that the caspase-3 activation in apoptosis is closely related to the AML development[35]. Since caspase-3 is a key enzyme in mammalian apoptosis, the detection of the activation type is widely used as labeling of apoptosis, especially in tumor cells[36]. Relative studies have found that the overexpression of caspase-3 promotes tumor cell metastasis not only by regulating adhesion molecule expressions, but also by initiating metastatic pathways. In the present study, microRNA-199a could regulate caspase-3 expression and metastasis in AML cells. Our findings might help to improve AML treatment.

Conclusions

We observed that microRNA-199 is lowly expressed in AML patients. Moreover, it inhibits the malignant progression of AML by targeting caspase-3.

Conflict of Interests

The authors declared no conflict of interest.

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