

Original Article

Targeted regulation of FoxO3a/p27Kip1 by miR-155 for mediating HL-60 leukemia cell proliferation and apoptosis

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Abstract: Leukemia patients had significantly elevated miR-155 expression. P27Kip1 plays a role in regulating cell proliferation, cycle and apoptosis. Down-regulation of p27Kip1 participates in leukemia pathogenesis. Transcription factor FoxO3a plays a role in regulating target gene p27Kip1 and is related with leukemia occurrence. Bioinformatics analysis revealed targeted regulation of FoxO3a by miR-155. This study investigated if miR-155 played a role in regulating FoxO3a/p27Kip1 and leukemia pathogenesis. A total of 48 acute promyelocytic leukemia (APL) patients in our hospital were recruited in parallel with 55 healthy individuals. Expressions of miR-155, FoxO3a and p27Kip1 were measured in peripheral monocytes. *In vitro* cultured APL cell HL-60 was treated with miR-155 inhibitor and/or pIRES2-FoxO3a. qRT-PCR was used to test expressions of miR-155, FoxO3a and p27Kip1 genes, whilst Western blot was used to test protein level of FoxO3a and p27Kip1, followed by flow cytometry to detect Ki-67 expression and cell apoptosis. APL patients had significantly lower expression of FoxO3a or p27Kip1 in peripheral monocytes, plus significantly elevated miR-155 expression. Dual luciferase assay confirmed the targeted regulation of FoxO3a by miR-155. Over-expression of miR-155 inhibited expression of FoxO3a and downstream regulator factor p27Kip1, and meanwhile inhibited HL-60 cell proliferation to facilitate apoptosis. MiR-155 targets and inhibits expression of FoxO3a and downstream factor p27Kip1, facilitates HL-60 cell proliferation and inhibits cell apoptosis.

Keywords: Acute promyelocytic leukemia, MicroRNA-155, FoxO3a/p27Kip1, HL-60 cell, cell cycle, apoptosis

Introduction

Leukemia is one type of malignant clonal disease of hematological stem cells. With economic development, environmental pollution aggravation and transition of life styles, incidence of leukemia is rapidly increasing, especially in children and young people under 35 years [1]. Unfavorable prognosis exists for leukemia, which had no effective preventive and management measures, leading to high mortality [2]. Acute promyelocytic leukemia (APL) is one special type of acute myelocytic leukemia (AML) with severe condition. Its pathogenesis is closely correlated with abnormal cytogenetics, and occupies about 10% of adult AML cases. In bone marrow, there are abundant promyelocytes with abnormally increased granules. APL has severe tendency of hemorrhage, as nearly

20% patients died from early hemorrhage and 60% may suffer from disseminated intravascular coagulation (DIC).

FoxO3a is one transcriptional regulator in FoxO family and locates on chromosome 6q21. As one important signal transduction molecule, it exerts physiological functions via modulating expression of downstream target genes such as p27Kip1 and Bim. Besides the regulation of cell/tissue differentiation, it also plays a role in modulating biological processes including cell cycle, proliferation and apoptosis [3, 4].

MicroRNA (miR) is one type of single stranded non-coding small RNA molecule with 22 nucleotide length and is widely distributed in eukaryotes [5]. Mature miR can bind with target mRNA via complete or incomplete complementary

binding to facilitate degradation of target gene mRNA or inhibit its translation. Current studies [6-8] found the involvement of miR in regulating cell cycle and biological processes including cell proliferation, differentiation, apoptosis and genetic recombination. MiR-155 has been found to be up-regulated in various malignant tumors. It can affect intracellular signal transduction pathway via complementary binding onto specific target gene, thus affecting tumor pathogenesis, progression and prognosis [9, 10]. O'Connell et al found significantly elevated miR-155 expression in monocytes of bone marrow from AML patients [11]. Bioinformatics analysis revealed complementary binding sites between miR-155 and 3'-UTR of FoxO3a. This study investigated if miR-155 could affect leukemia pathogenesis via targeted regulation on FoxO3a/p27Kip1.

Materials and methods

Equipment and materials

APL cell line HL-60 was purchased from Cell Bank, China Academy of Science. DMEM culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (US). Mononuclear cell separation buffer was purchased from Haoyang Bio (China). Trizol and Lipofectamine 2000 liposome transfection reagent were purchased from Invitrogen (US). Reverse transcription kit and SYBR Green dye were purchased from Toyobo (Japan). Mouse anti-human FoxO3a, p27Kip1 and beta-actin antibody was purchased from Cell signaling (US). Rabbit anti-mouse enzymatic labelled secondary antibody was purchased from Sigma (US). Annexin V/PI apoptotic kit was purchased from Yusheng (China).

Patient information

A total of 48 APL patients who received treatment in the First Affiliated Hospital of Zhejiang Chinese Medical University from June 2015 to February 2016 were recruited, including 28 males and 20 females, with average age at 46.5 ± 4.8 years. All samples were diagnosed with cytology and genetics examination. Another cohort of 36 healthy individuals were recruited as the control group, including 20 males and 16 females, aging between 23 and 67 years (average = 47.8 ± 5.1 years). All samples have obtained informed consents of

patients. This study was approved by the ethical committee of the First Affiliated Hospital of Zhejiang Chinese Medical University.

Peripheral blood collection and mononuclear cells separation

4 mL fasted blood was collected from elbow veins in the morning. Monocytes were separated following the manual instruction of separation kit. In brief, 2 mL blood was mixed with 2 mL PBS, and was added on 5 mL separation buffer carefully. After centrifugation at 300 g for 30 min, the white membrane was collected and washed twice in PBS to obtain peripheral monocytes. Phenol-chloroform extraction method was used to extract RNA. In brief, 1 mL Trizol was mixed well with monocytes for 5 min room temperature incubation. 200 μ L chloroform was added for 15 s vortex, followed by 10 min room temperature incubation. After 12000 rpm centrifugation at 4°C for 15 min, supernatant was saved and precipitated by isopropanol to be washed in 75% ethanol. RNA pellet was air dried and solved in DEPC water, and was kept in -80°C. 100 μ L RIPA lysis buffer was added in monocyte solution for 30 min iced incubation and 12000 rpm centrifugation for 10 min at 4°C. Proteins were extracted and stored at -80°C for further use.

Cell culture

APL cell was cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin in 37°C incubator with 5% CO₂. Fresh medium was changed every other day. Cells were passed based on growth conditions. Cells at log-growth phase with satisfactory status were used for experiments.

Luciferase reporter gene assay

Lipofectamine 2000 was used to transfect HEK293 cells with 500 ng pGL3-FoxO3a-3'UTR plasmid, 30 nmol miR-155 oligonucleotide fragments (or negative control), and 30 ng pRL-TK. After 6 h transfection, normal DMEM medium containing 10% FBS and 1% streptomycin-penicillin was used. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, cells were washed twice in PBS, with the addition of 100 μ L PLB lysis buffer. With vortex at room temperature for 30 min, the mixture was centrifuged at 1000 rpm for 10 min. 20 μ L

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cell lysate was mixed with 100 μ L LAR II. Fluorescent value I was measured in a micro-plate reader. The enzymatic reaction was stopped in 100 μ L Stop & Glo, followed by quantification of fluorescent value II. The relative expression level of reporter gene was calculated as the ratio of fluorescent value I/ fluorescent value II.

Construction of over-expression plasmid and cell transfection

Using pIRES as eukaryotic expression plasmid, and Xho I and BamH I as restriction digestion enzyme, amplification primer of FoxO3a was synthesized using primer 6.0 based on mRNA sequence of FoxO3a in Gene Bank (Forward, 5'-ATCCT CGAGA TGGCA GAGGC ACCGG CTTC-3'; Reverse, 5'-ATGGA TCCTC AGCCT GGCAC CCAGC TCTG-3'). FoxO3a gene was amplified based on cDNA template. Agarose gel electrophoresis was used to determine targeted fragments (2022 bp length), which were extracted by gel extraction kit. After ligated with vector, recombinant plasmid was used to transfect competent cell JM109. Ampicillin-containing culture dish was used to collect positive bacterial strain, which was further amplified and extracted for recombinant plasmid containing targeted fragment. Gene sequencing was performed to confirm the correct insertion of target sequence into the plasmid. Lipofectamine 2000 was used to transfect inhibitor NC, miR-155 inhibitor, non-sense controlled plasmid (pIRES2-Scramble), or over-expression plasmid (pIRES2-FoxO3a) was transfected into HL-60 cells, which were divided into five groups: inhibitor NC, miR-155 inhibitor, pIRES2-Scramble, pIRES2-FoxO3a and miR-155 inhibitor+pIRES2-FoxO3a group. 72 h later cells were collected for extracting RNA to confirm transfection efficiency, and were extracted for protein and other assays.

qRT-PCR for gene expression

Total RNA was extracted by phenol-chloroform method and was quantified by spectrometry for further reverse transcription. cDNA was synthesized in a 20 μ L system including 2 μ g total RNA, 4 μ L RT buffer (5X), 1 μ L oligo dT + random primer mix, 1 μ L RT enzyme mix, 1 μ L RNase inhibitor, and 11 μ L ddH₂O. The reaction conditions were: 37°C for 15 min, followed by

98°C 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers to detect target gene mRNA level and miR-155 level. Primer sequences were: miR-155P_F: 5'-ACGCT CAGTT AATGC TAATC GTGAT A-3'; miR-155P_R: 5'-ATTCC ATGTT GTCCA CTGTC TCTG-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; FoxO3aP_F: 5'-TCGCG CACCA ATTCC AAC-3'; FoxO3aP_R: 5'-TCGCT GTGGC TGAGT GAGTC-3'; p27Kip1P_F: 5'-CACTG CAGAG ACATG GAA-3'; p27Kip1P_R: 5'-GCTTC ATCAA GCAGT GA-3'; β -actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β -actinP_R: 5'-TGTC ACGAC GATTT CC-3'; In a PCR system with 20 μ L total volume, we added 10 μ L 2XSYBR Green Mixture, 1.0 μ L of forward/reverse primer (at 2.5 μ M/L), 2 μ L cDNA, and 6.0 μ L ddH₂O. PCR conditions were: 95°C 5 min, followed by 95°C for 15 s and 60°C for 1 min. The reaction was performed on ABI 7500 fluorescent quantitative PCR cyclor for 40 cycles to collect fluorescent data.

Western blot

HL-60 cells were lysed in RIPA lysis buffer for 30 min on ice. After centrifugation at 1000 rpm for 10 min, supernatant proteins were collected and stored at -20°C for further use. BAC method was employed to quantify protein concentration. 50 μ g protein samples were separated by 12% SDS-PAGE for 3 h, and were transferred to PVDF membrane for 1.5 h. The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-FOXO3a at 1:300, anti-p27Kip1 at 1:200 or anti- β -actin at 1:1000) incubation at 4°C overnight. By PBST washing (5 min \times 3 times), enzymatic labelled secondary antibody (1:10 000 dilution) was added for 60 min incubation. After PBST rinsing for three times (5 min each), ECL reagent was added for 2~3 min dark incubation. The membrane was then exposure in dark and scanned for data analysis using Quantity One software.

Flow cytometry for Ki-67 expression

HL-60 cells were collected and were rinsed twice in PBS containing 2% FBS. After fixation in 4% paraformaldehyde for 20 min, cells were treated using PBS containing 0.1% Triton X-100. FITC labelled Ki-67 antibody was added for 4°C

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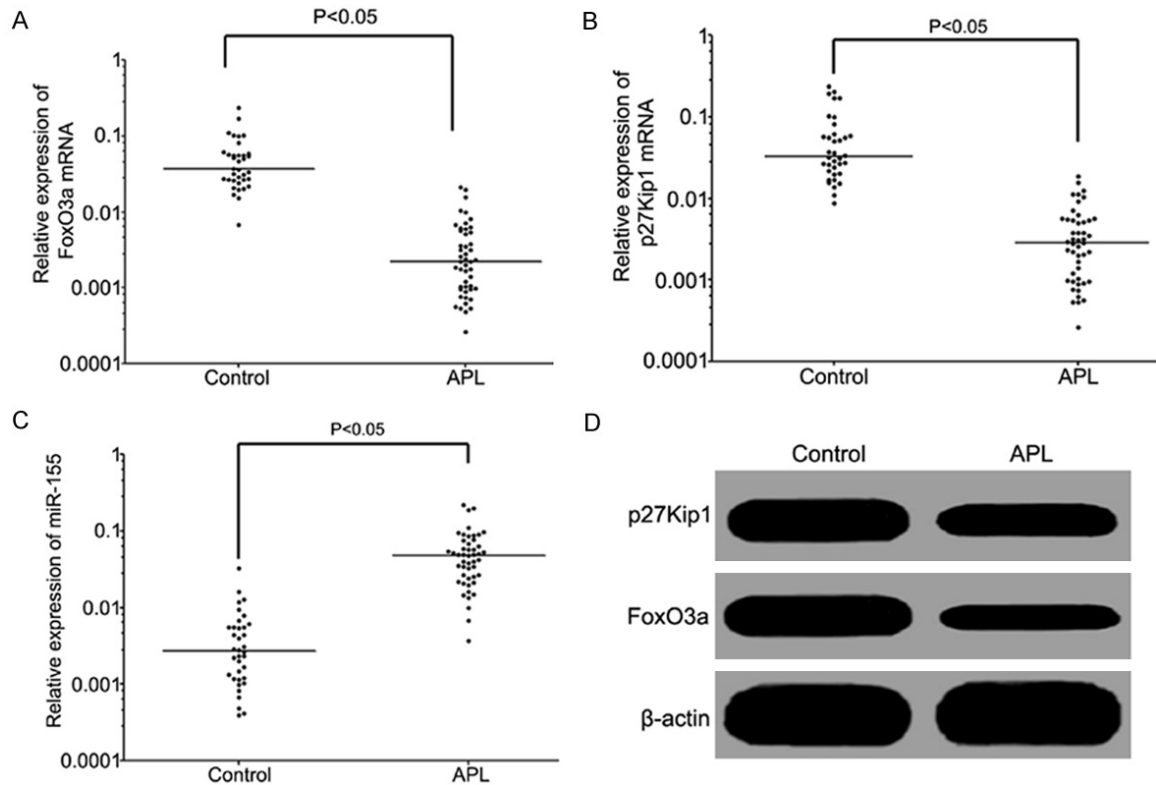


Figure 1. Expressional profile of miR-155, FoxO3a and p27Kip1 in peripheral blood monocytes of APL patients. A. qRT-PCR for FoxO3a mRNA expression; B. qRT-PCR for p27Kip1 mRNA expression; C. qRT-PCR for miR-155 expression; D. Western blot for FoxO3a and p27Kip1 protein expression.

dark incubation for 40 min, followed by twice rinsing in PBS containing 2% FBS. Cells were loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

Flow cytometry for cell apoptosis

HL-60 cells were collected. 100 μ L Binding Buffer was used to re-suspend cells. The mixture was added with 5 μ L Annexin V-FITC and 5 μ L PI staining solution. After gentle mixture, the mixture was incubated in dark for 10 min, with the addition of 400 μ L 1XBinding Buffer, and was immediately loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

Statistical analysis

SPSS18.0 software was used for data analysis. Measurement data were presented as mean \pm standard deviation (SD). Student t-test was used to compare measurement data between groups. A statistical significance was defined when $P < 0.05$.

Results

Expression of miR-155, FoxO3a and p27Kip1 in peripheral blood of APL patients

qRT-PCR results showed significantly lower mRNA expression of FoxO3a and p27Kip1 in peripheral monocytes of APL patients compared to control people (**Figure 1A** and **1B**), whilst miR-155 expression was significantly higher than healthy control group (**Figure 1C**). Western blot results showed remarkably lower FoxO3a and p27Kip1 protein expression in peripheral monocytes of APL patients compared to control people (**Figure 1D**). These results showed the dysregulation of miR-155 and FoxO3a might be involved in APL pathogenesis.

MiR-155 targeted and regulated FoxO3a expression

Pictar and TargetScan software were used to predict target gene and found satisfactory targeting relationship between miR-155 and

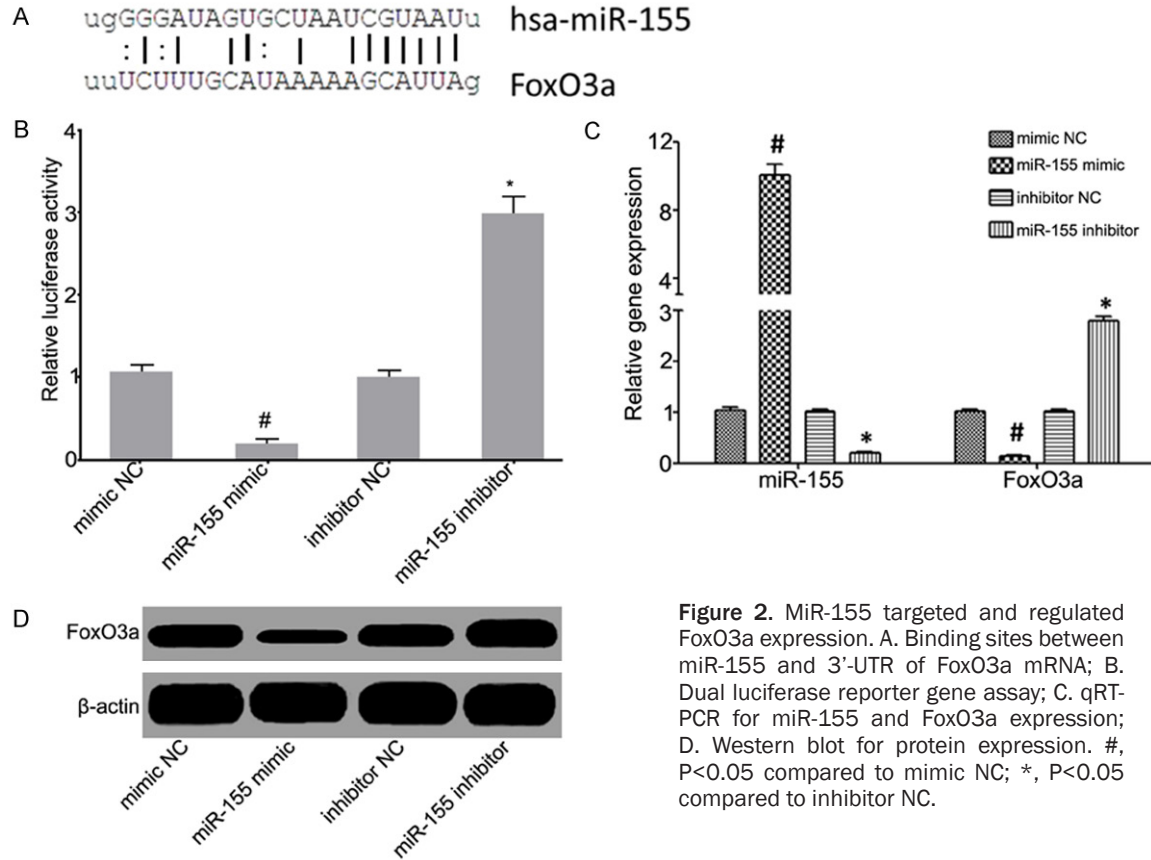


Figure 2. MiR-155 targeted and regulated FoxO3a expression. A. Binding sites between miR-155 and 3'-UTR of FoxO3a mRNA; B. Dual luciferase reporter gene assay; C. qRT-PCR for miR-155 and FoxO3a expression; D. Western blot for protein expression. #, P<0.05 compared to mimic NC; *, P<0.05 compared to inhibitor NC.

3'-UTR of FoxO3a mRNA (**Figure 2A**). Dual luciferase reporter gene assay showed the transfection of miR-155 mimic or miR-155 inhibitor decreased or increased relative luciferase activity inside HEK293 cells (**Figure 2B**), and decreased or increased FoxO3a expression of HL-60 cells (**Figure 2C** and **2D**), demonstrating that miR-155 targeted 3'-UTR of FoxO3a mRNA and regulated its expression.

MiR-155 inhibition suppressed HL-60 cell proliferation and facilitated apoptosis

The transfection of miR-155 inhibitor and/or FoxO3a over-expression plasmid significantly elevated FoxO3a mRNA and protein expression in HL-60 cells (**Figure 3A** and **3B**), in addition to remarkably increased expression of downstream target gene p27Kip1 (**Figure 3A** and **3B**), plus lower cell proliferation (**Figure 3C**) and more apoptosis (**Figure 3D**).

Discussion

APL is one special type of AML, and has been identified as M3 subtype by FAB. With unclear

pathogenesis, APL is believed to be related with abnormal cytogenetics, mainly including breakage and translocation of chromosome 15 and chromosome 17, causing the fusion of promyelocytic leukemia (PML) gene with retinoic acid receptor α (RAR α) gene. Moreover, minority of patients also showed the fusion between promyelocytic leukemia zinc finger (PLZF) genes on chromosome 11 with RAR α gene. These fusion proteins can affect wild type protein function, and interfere with cell differentiation, leading to abundant accumulation of abnormal promyelocytes in bone marrow. Furthermore, fusion protein also extends leukocyte proliferation and survival via interrupting tumor inhibition and apoptotic functions by wild type proteins. APL is the leading malignant disease in young population, with common occurrence of hemorrhage in more than 70% patients, leading to higher early phase mortality. Recent years, the introduction of induced differentiation treatment by retinoic acid and arsenic reagent largely improved treatment efficacy and prognosis, although certain recurrent hemorrhage and drug resistance still exist in some patients.

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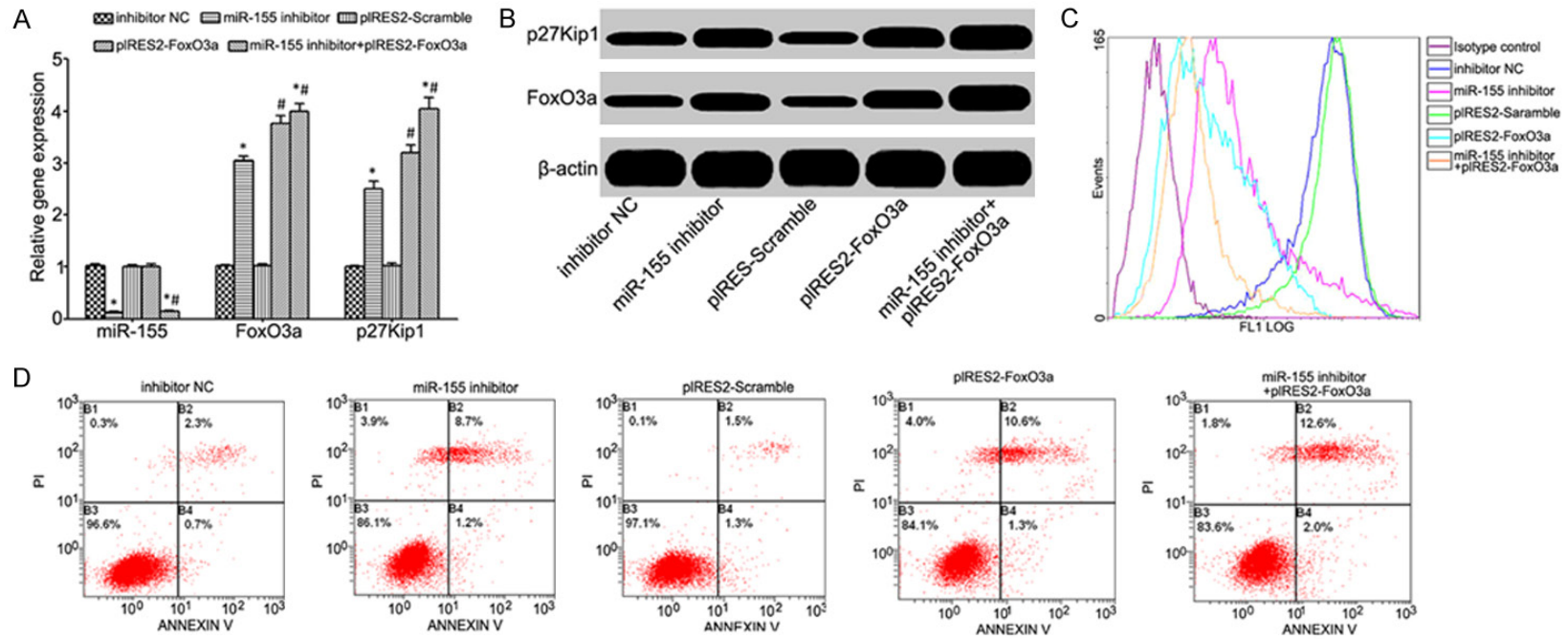


Figure 3. MiR-155 down-regulation inhibited HL-60 cell proliferation and facilitated cell apoptosis. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. Flow cytometry for Ki-67 expression; D. Flow cytometry for cell apoptosis. #, P<0.05 compared to piRES2-Scramble; NC; *, P<0.05 compared to inhibitor NC.

miRNA is one group of non-coding single stranded RNA with 22 nucleotides length to be coded by endogenous gene, and is widely distributed in eukaryotes. Major functions of miRNA include negative regulation on gene expression at post-transcriptional level. Current studies have confirmed the correlation between abnormal miRNA expression and tumor pathogenesis and progression, as it can participate in regulating tumor cell proliferation, differentiation and apoptosis, thus affecting tumor prognosis and outcome [12]. MiR-155 is abnormally expressed in various malignant tumors including pulmonary carcinoma, breast cancer and leukemia, and affects tumor occurrence, progression and prognosis. In pulmonary cancer tissues, miR-155 expression is significantly higher than tumor adjacent tissues. Cell study found the over-expression of miR-155 can affect cell proliferation via regulating Rb1 expression, thus playing a role in lung cancer occurrence, progression and prognosis [13]. Jiang et al found the over-expression of miR-155 could potentiate proliferation and metastasis of breast cancer cells via inhibiting SOCS1 protein expression [14]. Various studies also confirmed that abnormal expression of miR-155 can affect prognosis of acute/chronic leukemia via regulating different signal pathways [15, 16]. MiR-155 can modulate cell proliferation, differentiation and apoptosis via down-regulating tumor suppressor genes such as PU.1 and C/EBP- β , further accelerating occurrence and progression of leukemia [17-19]. It can also affect cell proliferation and differentiation via inhibiting expression of oncogene (MYC, GFI1) and anti-apoptotic factor (KRAS, SGK3), thus inducing cell apoptosis and exerting anti-cancer effects [10, 20]. This study found significantly elevated peripheral blood miR-155 level in APL patients compared to healthy controlled people, as consistent with previous results, indicating that abnormal expression of miR-155 might participate in occurrence of APL.

FoxO3a plays important roles in regulating cell cycle, cell proliferation, apoptosis and tumor occurrence/progression [3, 21]. FoxO3a can exert anti-cancer effects via modulating p27Kip1 and BIM expression to inhibit cell proliferation, facilitate cell apoptosis [22]. This study found lower mRNA/protein level of FoxO3a in peripheral monocytes of APL patients

compared to healthy controls. Dual luciferase reporter assay attributed FoxO3a as the target gene of miR-155. Over-expression of miR-155 in HL-60 cells decreased mRNA/protein expression level of FoxO3a, whilst inhibition miR-155 obtained opposite effects, confirming that miR-155 could target and regulate FoxO3a expression. As one of negative regulatory factor for cell cycle, p27Kip1 expression level alters with progression of cell cycle. It exerts an important role in regulating cell cycle via arresting cells at G1 phase, thus inhibiting cell proliferation [23, 24]. Previous study found that p27Kip1 could also regulate cell migration, apoptosis, autophagy, and occurrence and outcome of various tumors in addition to regulating cell cycle and interfering with cell proliferation [25, 26]. This study found lower p27Kip1 expression in peripheral blood of APL patients compared to healthy controlled people. The inhibition of miR-155 or over-expression of FoxO3a in HL-60 cells could elevate mRNA/protein expression levels of FoxO3a, and increase expression of downstream p27Kip1, thus inhibiting HL-60 cell proliferation, and facilitating cell apoptosis simultaneously. Our results indicated that miR-155 probably regulate cell proliferation and apoptosis via targeted inhibition on FoxO3a expression plus its downstream signal molecule p27Kip1, thus affecting occurrence of APL and outcome.

Conclusion

MiR-155 could facilitate HL-60 cell proliferation and inhibit cell apoptosis via targeted inhibition on expression of FoxO3a and downstream factor p27Kip1.

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Disclosure of conflict of interest

None.

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