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Original Article

Targeting miR-9 suppresses proliferation and induces apoptosis of HL-60 cells by PUMA upregulation in vitro

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Abstract: Objective: Recent studies have shown that upregulation of *miR-9* occurs in several types of human cancer, suggesting a tumor promotive function of *miR-9*. *miR-9* has been found to be overexpressed in the acute myeloid leukemia (AML). In the present study, we observed the effect of targeting *miR-9* on proliferation and apoptosis in AML HL-60 cells and explored its mechanisms. Methods: HL-60 cells were transfected with MiR-9-inhibitor and its scrambled siRNA or/and PUMA siRNA for 24-72 h. After transfection with PUMA siRNA or/and miR-9 inhibitor 24-72 h, cell viability and cell apoptosis was detected by MTT assay, flow cytometry and TUNEL assay; qRT-PCR and western blot assay was used to detected PUMA and miR-9 mRNA and protein levels. Results: Targeting miR-9 inhibited proliferation and induced apoptosis of HL-60 cells, accompanying the upregulation of PUMA protein and mRNA. Knockdown of PUMA by siRNA blocked miR-9 inhibitor-induced apoptosis and growth inhibition. Conclusion: These findings suggested that targeting miR-9 inhibited cell growth and induced apoptosis of HL-60 cells via increasing PUMA expression.

Keywords: Acute myeloid leukemia, miR-9, PUMA, apoptosis

Introduction

Leukemia is a cancer which is arisen from uncontrolled proliferation in the hematopoietic lineage [1, 2]. AML is characterized by abnormal proliferation and survival of myeloid progenitor and precursor cells and a premature block in myeloid differentiation [3]. It accounts for roughly 70-80% of all adult acute leukemias and 20% of all childhood acute leukemias [4]. Despite aggressive therapies, AML remains difficult to treat, and many patients will die as a consequence of treatment failure or complications from either treatment-related toxicities or impaired normal hematopoiesis [5]. The majority of AML respond to initial treatment; however relapse is common indicating resistance of malignant cells to chemotherapy [6]. For AML patients who relapse or have resistant disease, therapeutic options are limited. Therefore, the need for effective yet better tolerated new therapies is needed.

MicroRNAs (miRNAs) are regulatory RNAs. It is a -22 nucleotides in length and serves to regulate posttranscriptional expression of target genes [7]. Numerous evidences demonstrated that miRNAs play critical regulatory roles in both normal development and pathogenesis of disease [8, 9]. MicroRNA-9 (miR-9) is emerging as a critical regulator of organ development and neurogenesis. Aberrant miR-9 expression has been reported in several cancers, such as gastric cancer [10], Hodgkin's lymphoma [11], ovarian cancer [12] and acute lymphoblastic leukemia [13], suggesting that miR-9 can act as an oncogene or as a tumor suppressor.

Recent study has found that miR-9 plays an important role in supporting AML cell growth and survival by downregulation of Hes1, suggesting miR-9 has potential as a therapeutic target for treating AML [14]. Chen et al. has reported that enforced miR-9 expression can significantly promote fusion-mediated leukemogenesis in vivo, suggesting that miR-9 could be a target for treating MLL-rearranged AML [15]. Silencing of miR-9 was found to inhibit invasion, proliferation and induce apoptosis in many tumors [16-19]. However, the molecular mechanism of how miR-9 sliencing functions in cancer has not been defined.

Recent study has found that pro-apoptotic PUMA (p53-upregulated-modulator of apoptosis) was induced by miR-221 and miR-222-sliencing, resulting in cell apoptosis [20, 21]. Cazanave et al. has found enforced miR-296-5p levels efficiently reduced PUMA protein expression in Huh-7 cells, while antagonism of miR-296-5p function increased PUMA cellular levels [22]. Adlakha et al. has found that-miR-128 induces apoptosis via induction of PUMA. Pretreatment with PUMA and Bak siRNAs abolished miR-128-induced apoptosis of HCT116 cells [23].

The purpose of this study was to investigate the potential role of miR-9 sliencing on proliferation and apoptosis of HL-60 cells, and explore its mechanisms. We found that knockdown of miR-9 inhibits proliferation and induce apoptosis of HL-60 cells by PUMA upregulation.

Materials and methods

Cell line and culture

The HL-60 cell line was purchased from the American Type Culture Collection (ATCC, Shanghai, China). The cell line was maintained in RPMI 1640 (Life, Technologies), supplemented with 10% (v/v) fetal bovine serum, 3 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin.

Synthesis oligo-nucleotide and cell transfection

miR-9 inhibitor and negative control were synthesized in RibobioInc (Guangzhou, China) based on the sequence of miR-9 in miRBase database. The HL-60cells were transfected with oligonucleotides at working concentrations of 20 μM using Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's instruction.

PUMA siRNA transfection

PUMA-siRNA and its scrambled siRNA was purchased from CSC (Shanghai, China). MiR-9-inhibitor and its scrambled siRNA was transfected into the HL-60 using Lipofectamine™2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blotting

For analysis of protein by Western blotting, cells were lysed on ice in lysis buffer, clarified by centrifugation at 6,000×*g* for 5 min in a microcentrifuge at 4°C, separated by SDS-PAGE, and transferred to Hybond-P (Amersham) membranes. Antibodies used for western blot were anti-PUMAand goat anti-β-actin (Santa Cruz). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit serum (BD Biosciences) and rabbit anti-goat serum (DAKO). Chemiluminescence was detected using the ECL reagents.

qRT-PCR analysis of mRNA and miR-9

Quantitative real-time PCR (qRT-PCR) was performed on tumor cDNA using the Taqman Fast System and reagents (Applied Biosystems, Foster City, CA) per manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using random primer and MultiScribe RT (High-Capacity cDNA Archive Kit) for mRNA analysis and miScript Reverse TranscriptionKit for miRNA analysis. PCR was performed with the resulting reverse transcription products using specific oligonucleotide primers. The sequence of the primer for miR-9 was 5'-TAAGGCACGCGGTGAATGCC-3' and 5'-ACTCATAGACCTACATAACGAAACA-3'; The sequence of the primer for PUMA was 5'-GGTGTCGATGCTGCTCTTCT-3', and 5'-GTAACCCGTTGAACCCCATT-3'. The sequence of the primer for β-*actin* was 5'-TGA CTG ACT ACC TCA TGA AGA TCC-3' and 5'-GCG AAG ATA CCG GGG GAC ACT CAT GAG-3'. All reactions were performed in triplicate. Results were analyzed using the $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ method.

Cell viability assay

HL-60 cells viability was assessed by methyl thiazoltetrazolium (MTT) assay. Cells were plated in 96-well plates containing 10% FBS. After transfection with PUMA siRNA or or/and miR-9 inhibitor 24, 48, or 72 hs, cells from each group were collected and plated in 96-well plates at a density of 1.0×10^4 cells/well for MTT assay. The absorbance was measured at 570 nm. Each assay was performed in triplicate. Cell growth (mean absorbance ± standard deviation) was plotted versus time.

Flow cytometry

Apoptosis induction was quantified by Annexin V/PI double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain view, CA) following the manufacturer's instruction. Briefly, after various transfec-

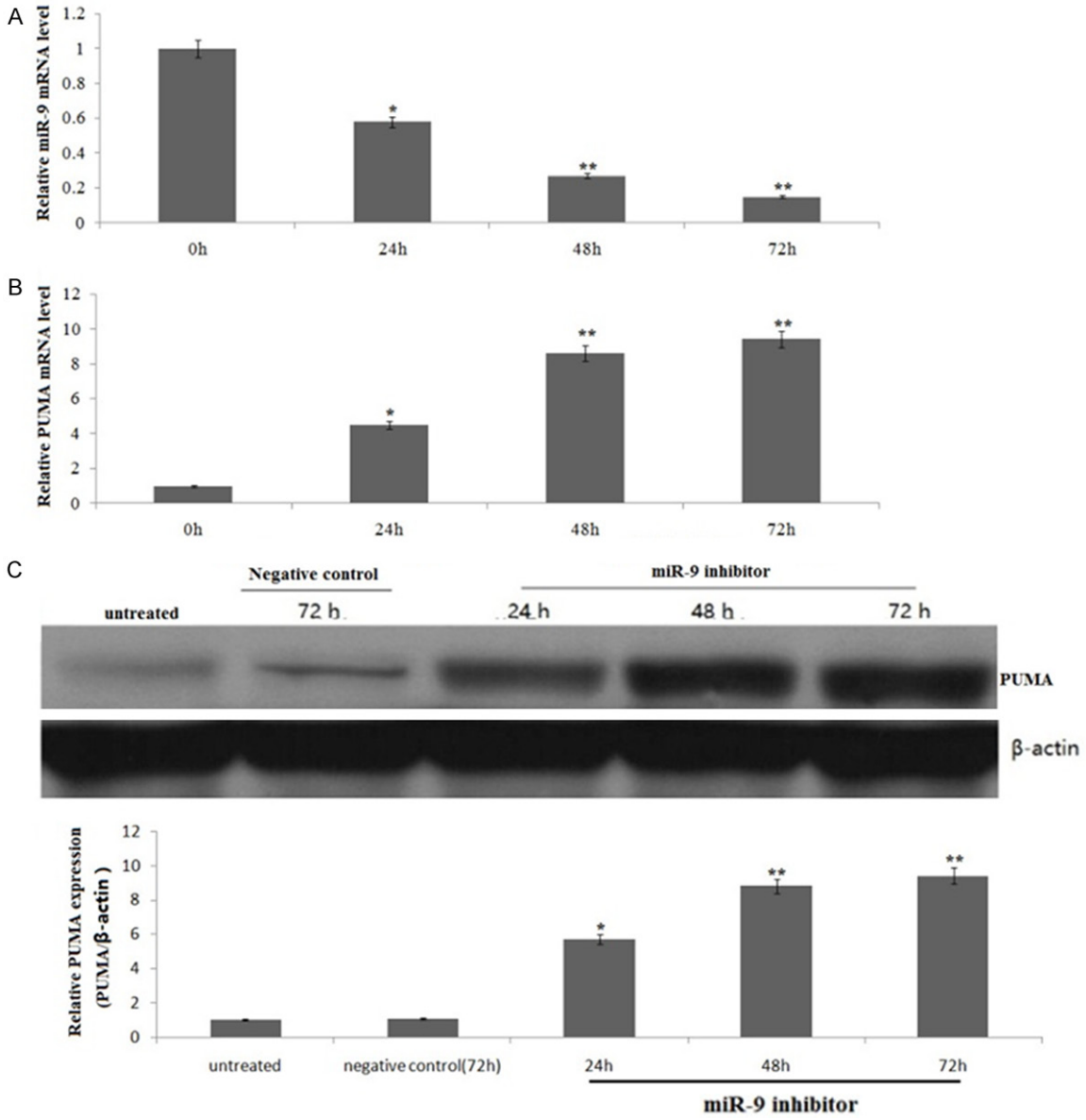

Figure 1. Effect of miR-9 inhibitor on miR-9 and PUMA expression in HL-60 cells. HL-60 cells were transfected with miR-9 inhibitor on negative control for 24, 48 and 72 h. A. miR-9 mRNA expression was detectd by qRT-PCR assay; B. PUMA mRNA expression was detected by qRT-PCR assay. C. PUMA protein expression was detected by western blot assay. The results are expressed as mean ± SD (n=3), vs control, $^{*}P<0.05$, $^{**}P<0.01$.

tion, cells were gently detached by brief trypsinization, and then washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 μL binding buffer containing Annexin V and propidium iodide, and incubated for 5 min at room temperature. Early apoptotic cells were identified as Annexin V positive/PI negative cells, while late apoptotic/necrotic cells were identified as Annexin V positive/PI positive cells using a BD LSR II cell analyzer.

TUNEL analysis

The terminal deoxyribonucleotidyltransferase mediated dUTP nick end labeling (TUNEL) method was conducted with the In Situ Cell Death Detection Kit (Roche Applied Science, Shanghai, China) as the manufacture's instruction.

Statistical analysis

Data were dispicted as mean ± SD. Experiments were performed in triplicate. Comparisons were

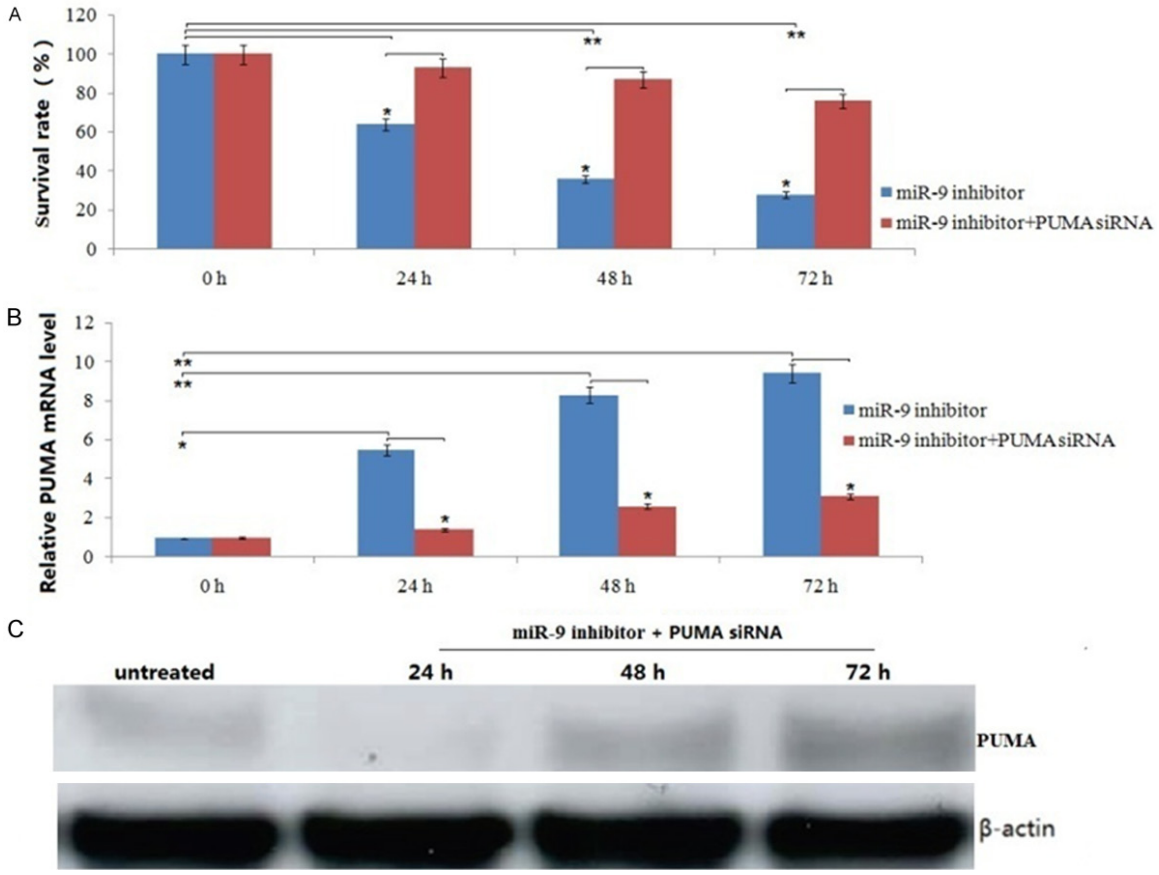

Figure 2. Effect of PUMA on miRNA-9 sliencing-induced growth inhibition. HL-60 cells were transfected with miR-9 inhibitor or/and PUMA siRNA for 24-72 h. A. HL-60 viability was detected by MTT assay; B. PUMA mRNA expression was detectd by qRT-PCR assay; C. PUMA protein expression was detected by western blot assay. The results are expressed as mean ± SD (n=3), vs control, $^{*}P<0.05$, $^{**}P<0.01$.

done with two tailed Student's *t* test. A value of $P<0.05$ was considered statistically significant.

Results

Knockdown of miRNA-9 upregulates PUMA expression

HL-60 cells were transfected with miR-9 inhibitor for 24-72 h, miRNA-9 mRNA (**Figure 1A**) was significantly decreased in a time-dependent method by qRT-PCR assay. However, PUMA mRNA was significantly increased in a time-dependent method by qRT-PCR assay. (**Figure 1B**) and western blot assay (**Figure 1C**).

Knockdown of miRNA-9 inhibits viabilty of HL-60 cells by PUMA-dependent signal

HL-60 cells were transfectedwith miR-9 inhibitor for 24-72 h. As shown in **Figure 2A**, knockdown of miR-9 resulted in 31%-79% cell growth inhibition compared to the untreated HL-60 cells ($^{*}P<0.05$ and $^{**}P<0.01$). To determine the role of PUMA on miR-9 inhibitor-induced growth inhibition of HL-60 cells, HL-60 cells were transfected with PUMA siRNA for 24 h, then treated with miR-9 inhibitor for 24-72 h. The results showed that miR-9 inhibitor-induced PUMA upregulation was reversed by qRT-PCR (**Figure 2B**) and western blot assay (**Figure 2C**). In addition, miR-9 inhibitor-induced growth inhibition was reversed (**Figure 2A**).

Knockdown of miRNA-9 induced apoptosis of HL-60 cells by PUMA-dependent signal

As shown in **Figure 3A**, following miRNA-9 inhibitor transfection, HL-60 cells showed much apoptotic cells by Annexin V/PI staining. However, when the HL-60 cells were transfected with PUMA siRNA for 24 h, then treated with miR-9 inhibitor for 24-72 h, miR-9 inhibitor-induced apoptosis of HL-60 cells was reversed

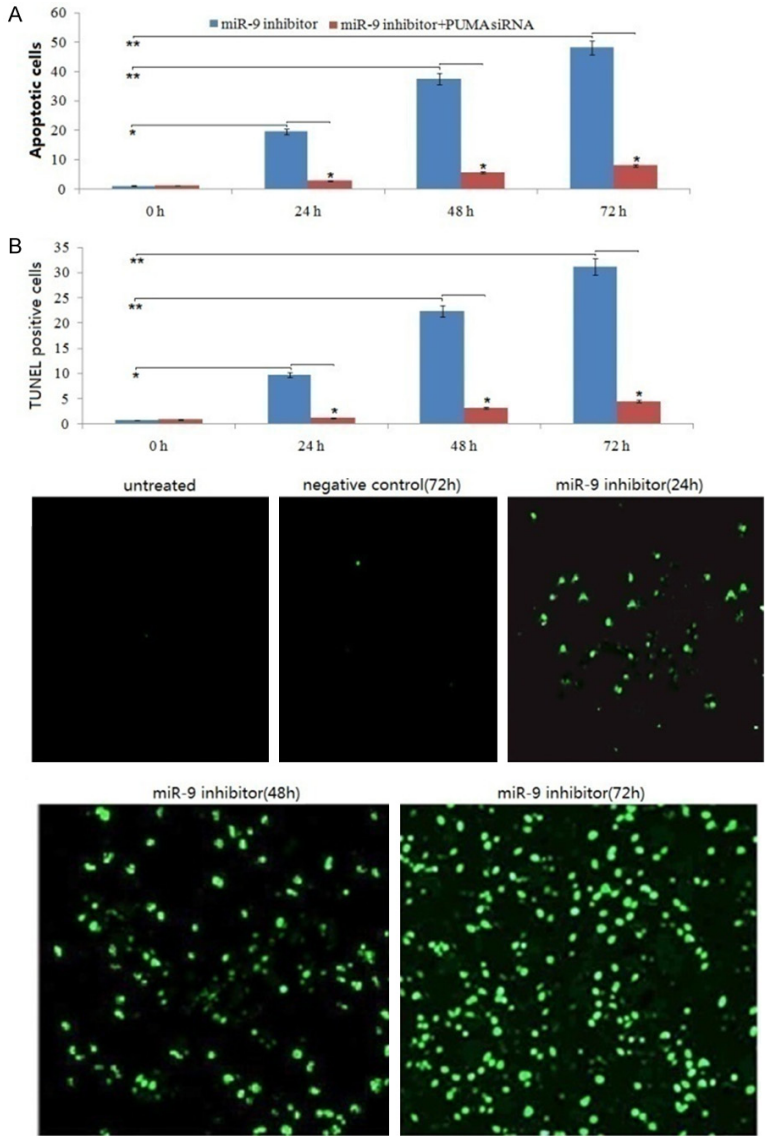

Figure 3. Effect of PUMA on miRNA-9 sliencing-induced apoptosis. HL-60 cells were transfected with miR-9 inhibitor or/and PUMA siRNA for 24-72 h. A. Cell apoptosis was detected by flowcytometry assay. B. TUNEL analysis was used to test cell apoptosis. Histogram showing the number percentage of TUNEL-positive cells in total cell population, and bar graph represents the average value from there independent experiments; The results are expressed as mean ± SD (n=3), $^{*}P<0.05$, $^{**}P<0.01$.

(**Figure 3A**). The number of TUNEL-positive cells also increased with the duration of miRNA-9 inhibitor transfection for 24-72 h, PUMA siRNA inhibited miR-9 inhibitor-induced apoptosis of HL-60 cells. TUMA staining has the same result asAnnexin V/PI staining (**Figure 3B**).

Discussion

During the past few years, it has become clear that miRNAs have crucial roles in the regulation and maintenance of normal and malignant cells. Several miRNAs have been associated with hematopoietic differentiation, and their deregulation was reported in leukemia [24]. It has shown that the miRNAs specifically dysregulated in *MLL*-rearranged AML are predominantly up-regulated ones, in particular, miR-9 is the most specifically and consistently up-regulated miRNA in *MLL*-rearranged AML compared with normal controls and other AMLs [25].

Both tumor suppressor and oncogenic roles of miR-9 have been reported in various solid tumors [16-19, 26-28]. These conflicting findings on miR-9 expression (down-regulation or overexpression in cancer cells) may be due to differential mature miR-9 processing from three different primary precursors where down-regulation of miR-9 due to aberrant hypermethylation of any of the miR-9-1, miR-9-2, and miR-9-3 precursor regions have been reported in independent studies in different cancer types [29-31]. Our findings that endogenous miR-9 levels are higher in HL-60 cells, which was similar to previous reports of high levels of miR-9 associated with leukemia-rearranged leukemia [15], colorectal cancer [16] and bladdercancer [19].

Changes in cell proliferation and apoptosis are key phenotypes observed in malignant transformation [32]. Our results demonstrate that targeting miR-9 decreases cell viability and increases apoptosis in HL-60 cells. The pro-proliferative and anti-apoptotic effects of miR-9 are supportive of a tumor promotor-like role of miR-9 in the context of leukemia cell proliferation and apoptosis. However, the mechanisms underlying the miR-9 in regulation of HL-60 cell apoptosis still remains largely unknown.

Pro-apoptotic PUMA (p53 upregulated modulator of apoptosis) is a potent pro-apoptotic protein belonging to the Bcl-2 protein family. PUMA binds to the pro-survival proteins Bcl-2, Bcl-XL, and Mcl-1, thereby allowing Bax and Bak to promote apoptosis [33]. We et al. has reported that targeting SLUG could induce PUMA expression and restore the drug sensitivity of resistant leukemic cells to conventional chemotherapeutic agents [34]. Liu et al. has found that up-regulation of PUMA sensitized Cyt-induced apoptosis in HL-60 cells [35]. Some study has reported that activation of both extrinsic and intrinsic apoptotic pathways was involved in LG-induced AML cell apoptosis, accompanied by dissipation of mitochondrial membrane potential, downregulation of anti-apoptotic proteins (Bcl-2, Mcl-1, and Bcl-xL) and upregulation of pro-apoptotic protein PUMA [36]. Zhang et al has recently reported that PUMA was the essential and major regulators of AML cell survival [37].

Our findings showed that targeting miR-9 inhibited cell proliferation and induced apoptosis, followed by PUMA upregulation. However, knockdown of PUMA by siRNA transfection reversed miR-9 sliencing-induced apoptosis and proliferation inhibition in HL-60 cells, suggesting that the identification of PUMA as a miR-9 target gene, may explain, why targeting miR-9 suppressed the proliferation and induced apoptosis of HL-60 cells.

Conclusion

Our study highlights targeting miR-9 induced PUMA expression, thereby reduced cell viability and enhancing apoptosis in the HL-60 cells. We therefore suggested that miR-9 could be an effect targets for acute myeloid leukemia therapeutics.

Disclosure of conflict of interest

None.

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