miR-18a Inhibitor Suppresses Leukemia Cell Proliferation by Upregulation of PTEN Expression

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCD 1 **Liping Zhang**
BCDE 2 **Lingxia Kong**
ACDEF 1 **Yuzhi Yang**

1 Department of Hematology, Dezhou People's Hospital, Dezhou, Shandong, P.R. China
2 Department of Respiratory Medicine, Dezhou People's Hospital, Dezhou, Shandong, P.R. China

Corresponding Author: Yuzhi Yang, e-mail: yyz03080410@sina.com
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Background: Leukemia is common in aging adults and has very high mortality worldwide. The present study was designed to investigate the therapeutic efficacy of miR-18a inhibitor against WEHI-3 and THP-1 leukemia cells.

Material/Methods: The changes in miR-18a inhibitor-transfected WEHI-3 and THP-1 cell proliferative potential was measured by use of the Cell Counting Kit-8 assay. Apoptotic changes were analyzed by electron microscopy, and evaluation of PI3K, AKT, mTOR, and PTEN expression was assessed by RT-qPCR assay.

Results: Transfection of miR-18a inhibitor significantly ($P<0.05$) suppressed the proliferative potential of WEHI-3 and THP4 cells. The WEHI-3 cells showed the presence of characteristic apoptotic bodies on transfection with miR-18a inhibitor at 48 h. Flow cytometry showed that miR-18a inhibitor transfection significantly ($P<0.05$) increased the WEHI-3 cell percentage in G1 phase. The transfection of miR-18a inhibitor significantly ($P<0.05$) promoted apoptosis in WEHI-3 cells. In WEHI-3 cells, miR-18a inhibitor transfection markedly suppressed the expression of PI3K, AKT, and mTOR mRNA. The expression of PTEN mRNA was significantly ($P<0.05$) upregulated by miR-18a inhibitor transfection in WEHI-3 cells.

Conclusions: The present study investigated the therapeutic efficacy of miR-18a inhibitor against WEHI-3 and THP1 leukemia cells. The study demonstrated that miR-18a inhibitor suppressed the proliferative potential of WEHI-3 and THP1 cells and activated apoptotic process through upregulation of PTEN mRNA expression. Therefore, miR-18a inhibitor can be of therapeutic importance for the treatment of leukemia.

MeSH Keywords: **Apoptosis • Microscopy, Ultraviolet • Neoplasm Metastasis**

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Background

Leukemia is the malignant carcinoma detected in approximately 140 000 people every year throughout the globe [1]. The hematopoietic cells of leukemia patients undergo uncontrolled division and fail to achieve differentiation [2]. Every year acute leukemia is diagnosed in around 1.4 million people throughout the world [3]. The most commonly detected type of acute leukemia, accounting for highest rate of mortality worldwide, is acute myeloid leukemia [4]. Among various malignant hematological diseases, acute myeloid leukemia is most difficult to treat and patients have very poor survival [5]. Various studies are being performed to explore the molecular mechanism associated with leukemia [6–10]. The presently used therapies for leukemia by clinicians include radiotherapy, chemotherapy, and transplant of bone marrow [11]. However, these strategies have yielded unsatisfactory results, and a deeper understanding of leukemia is needed for the development of more effective treatment.

miR-18a is a miR cluster oncomir-1, which encodes for 5 major types of miRNAs [12]. The expression of miR17-92 cluster has been found to be markedly higher in several carcinomas, including colon, pulmonary, breast, pancreatic, digestive tract cancers, and lymphomas [12]. It has been revealed that cluster genome locus of the miR17-92 gene is upregulated in hematopoietic malignancies [13]. Activation of the miR17–92 gene is believed to be promoted by members of c-Myc, N-myc, and E2F families [14]. Studies have shown that overexpression of hnRNP Al facilitates Drosha-induced miR-18a processing [15]. miR-17 overexpression has been found to promote breast carcinoma cell metastasis and proliferation through HBP1 inhibition [16]. The downregulation of miR-17 and miR-20a causes suppression of breast carcinoma development by promoting ZBTB4, a tumor suppressor [17]. The miR-19 overexpression suppresses ERbeta1 in breast carcinoma cells by targeting 39-UTR [18].

There are several molecular pathways in carcinoma cells for escaping the process of apoptosis [19]. Therapeutic compounds activate or inhibit several signaling pathways, leading to suppression of tumor progression [19]. The upregulation of the PI3K/AKT/mTOR pathway has been shown to promote development and progression of various cancers [20]. Downregulation of the PI3K/AKT/mTOR pathway through various approaches is an important target for tumor inhibition [21]. It is reported that miR-18a is overexpressed in many cancer cells, such as glioma cells and tumor tissues [22]. In the present study, the anti-leukemic potential of miR-18a inhibitor was investigated against WEHI-3 and THP-1 leukemia cells. We also investigated the mechanism of antileukemia activity of miR-18a inhibitor in WEHI-3 cells *in vitro*.

Material and Methods

Cell lines and culture

The WEHI-3 and THP-1 cell lines were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Normal monocytes were provided by the Astarte, Inc. (Bothell, WA). The cells were grown in RPMI-1640 medium containing fetal bovine serum (FBS, 10%). The antibiotics penicillin (100 IU/ml) and streptomycin (100 µg/ml) were also mixed with the medium. The culture of cells was carried out in an incubator in a humid atmosphere with 5% CO_2 at 37°C.

Transfection of miR-18a inhibitor or negative control (siRNA)

The segment in human ATM 39-UTR in the region from 3340 to 3540 was prepared from SKBR3 cell DNA using PCR amplification. Then, it was subjected to cloning into pEGFP-C1 and pGL3 vector. The siRNA for depletion of ATM was prepared and then purified (Ribobio, Inc., Guangzhou, Guangdong, China). The sequence of ATM siRNA consisted of TGGTGCTATTTACGGAGCT. The plasmid and siRNAs (10 ng) were transfected into the cells using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions.

Cell viability assay

The viability changes by miR-18a inhibitor in WEHI-3 and THP-1 cells were measured by Cell Counting Kit-8 assay (CCK-8). The cells were placed at a concentration of $3×10^4$ cells per well in 96-well plates, cultured for 24 h in RPMI-1640 medium, and then transfected with miR-18a inhibitor or negative control. After 24, 48, and 72 h, CCK-8 solution (10 µl) was added to the wells and further incubated for 3 h. The absorbance measurements were made by microplate reader (EL-x 800) at 455 nm to determine cell viability.

Transmission electron microscopy

The WEHI-3 cells were maintained in RPMI-1640 medium for 48 h following miR-18a inhibitor or negative control transfection. The harvested cells were fixed with glutaraldehyde (3%) at 4°C for 2.5 h. Then, cells were washed using PBS (0.1 mol/l), followed by fixing with osmium tetroxide (1%) for 3 h at 4°C. Cells were dehydrated using gradient ethyl alcohol and then were embedded in epoxy resin. The epoxy resin-embedded cellular layer was cut into 40-mm sections and stained for 40 min with uranyl acetate (5% aqueous solution). The cells were also stained for 40 min with lead citrate before observing under a transmission electron microscope (JEOL, Tokyo, Japan).

Cell cycle analysis

The WEHI-3 cells were transfected with miR-18a inhibitor or negative control in 6-well plates at a concentration of $2.5×10^5$ cells per well. PBS washing of the cells was followed by harvesting and fixing overnight in ethyl alcohol at 4°C. The cells were subjected to treatment with Trishydrochloric acid buffer mixed with 1% RNase A. Then, staining of the cells was carried out with 5 mg/ml propidium iodide for determination of DNA content using flow cytometry (BD FACSCalibur Flow Cytometer, BD Biosciences).

Analysis of apoptosis

The WEHI-3 cells were transfected with miR-18a inhibitor or negative control in 6-well plates at a concentration of $2.5×10^5$ cells per well. The cells were maintained in RPMI-1640 medium for 48 h, rinsed twice in PBS, followed by re-suspending in binding buffer (350 µl). Then, incubation of cells was performed for 20 min with annexin V-FITC (5 mg/mL) and propidium iodide in the dark. Fluorescence was assessed by flow cytometry (FACSCalibur) combined with argon laser for analysis of apoptosis.

Cell invasion assay

The 24-well Transwell plates (8-mm pore size) were coated with 200 mg/ml Matrigel and then dried under sterile conditions overnight. WEHI-3 cells were transfected with miR-18a inhibitor or negative control and maintained at $2×10^5$ cells per ml concentration in RPMI-1640 medium in the upper chamber. The lower chamber contained RPMI-1640 medium mixed with 20% FBS. Following incubation for 48 h, the non-invasive cells in the upper chamber were wiped off using a cotton swab. The cells were then fixed in methyl alcohol for 15 min at room temperature, followed by staining with hematoxylin-eosin for 25 min. A light microscope (Olympus Corporation, Tokyo, Japan) was used for counting the cells that invaded into the lower chamber.

In vitro wound-healing assay

The WEHI-3 cells were placed at $2×10^5$ cells per ml density in a 6-well plate and allowed to attain 100% confluence by incubation at 37°C. The cells were starved for 24 h and then a 100-ml plastic pipette tip was used to scratch a wound (straight cell-free) through middle of the wells. The wells were washed with PBS 2 times followed by transfection with miR-18a inhibitor or negative control. After fixing and staining with 3.5% ethyl alcohol containing 1.5% crystal violet dye for 15 min, the cells were observed for migration potential. An inverted light microscope (Nikon Corporation) was used to observe the cells in 5 randomly selected fields.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR analysis was carried out on WEHI-3 cells following transfection with miR-18a inhibitor or negative control for evaluation of PTEN, PI3K, AKT, and mTOR levels. The total RNA from miR-18a inhibitor or negative control-transfected cells was isolated using TRIzol reagent. The synthesis of cDNA from RNA (1 µg) samples was performed at 37°C using a PrimeScript RT reagent kit for 15 min. The Roche LightCycler®96 RT-PCR system in combination with a SYBR Premix EX Taq II kit was used for RT-PCR assay. The reaction mixture involved a 20-µl sample consisting of 10 µl SYBR Premix EX Taq II, 0.8 µl backward primer, 0.8 µl forward primer, 2 µl cDNA, and 6.4 µl sterilized H_2O. The amplification was performed by pre-denaturation for 2 min at 93°C, then 38 cycles of denaturation for 5 s at 93°C, followed by annealing for 10 min at 60°C. The levels of mRNA expression were measured using $2^{-\Delta\Delta Cq}$ method with GAPDH as the loading control.

Luciferase target assay

The binding sites in 3'-UTR of PTEN for miR-18a inhibitor were determined using the predicting databases for miRNA target (Miranda, TargetScan, and PicTar). The segment of PTEN 3'-UTR in the region from 400 nt to 1700 nt was put into the pmirGLO vector (PTEN500) after cloning. The binding of miR-18a inhibitor to PTEN 3'-UTR was assessed by luciferase reporter assay. Briefly, WEHI-3 cells at $5×10^4$ cells in 150 µl of medium were distributed in 96-well plates and incubated overnight. The Firefly luciferase vector and mimic of miR-18a inhibitor were transfected into the cells with Effectene Reagent (Qiagen) according to the manufacturer's instructions. The luciferase reporter system (Promega) was used for measurement of activities for Firefly and Renilla luciferase at 48 h of transfection.

Statistical analysis

The data are presented as mean±standard deviations. Data were analyzed using SPSS (version 18.0; SPSS Inc., Chicago, IL, USA). Determination of statistically significant differences was made by one-way analysis of variance (ANOVA) and Tukey's test. The $P<0.05$ values were taken to represent statistically significant differences.

Results

miR-18a was overexpressed in WEHI-3 and THP1 leukemia cells

The level of miR-18a in WEHI-3 and THP-1 cells was markedly higher compared to normal monocytes cells (control) using
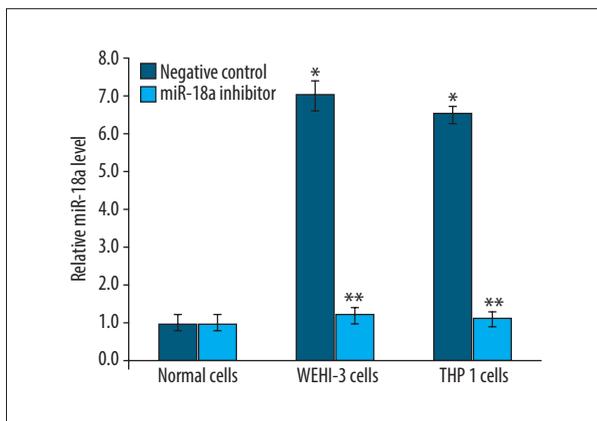
Figure 1. Overexpression of miR-18a in WEHI-3 and THP-1 cells. The miR-18a expression in WEHI-3 and THP-1 cells was assessed by real-time PCR. P<0.05 and * P<0.02 *vs.* normal cells.

real-time PCR (Figure 1). However, transfection of miR-18a inhibitor significantly suppressed miR-18a in WEHI-3 and THP-1 cells.

miR-18a inhibitor suppressed WEHI-3 and THP-1 cell growth *in vitro*

The CCK-8 assay was used to measure the effect of miR-18a inhibitor or negative control on WEHI-3 and THP-1 cell proliferation (Figure 2). Transfection of miR-18a inhibitor significantly (P<0.05) suppressed the proliferative potential of WEHI-3 and THP-1 cells in a time-dependent manner. The anti-proliferative potential of miR-18a inhibitor against the tested leukemia cell lines was greatest at 48 h. However, WEHI-3 and THP-1 cell proliferation was not reduced in negative control-transfected cultures.

miR-18a inhibitor changed WEHI-3 cell ultrastructure

Electron microscopy was used for analysis of ultra-structural changes in WEHI-3 cells after miR-18a inhibitor or negative
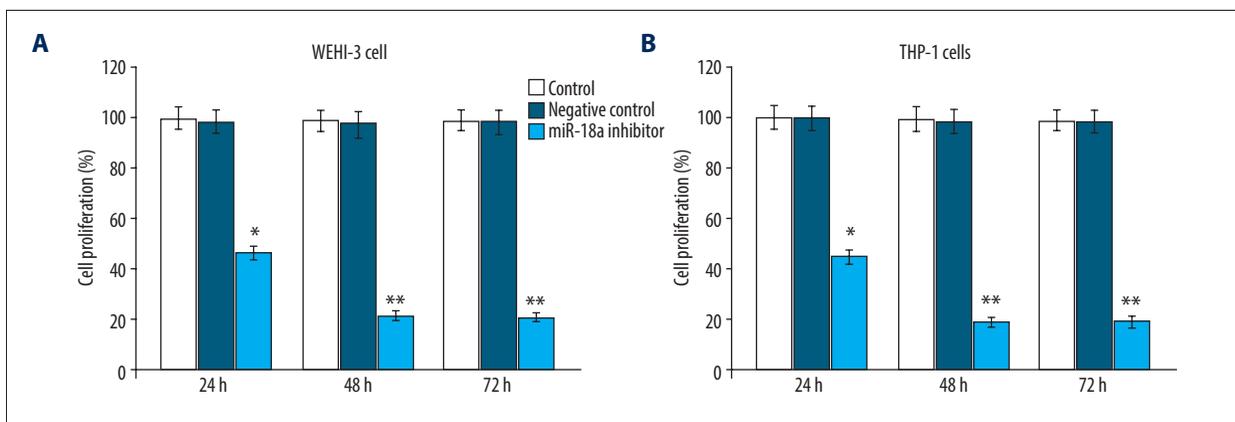

Figure 2. Effect of miR-18a inhibitor or negative control on leukemia cell proliferation. The (**A**) WEHI-3 and (**B**) THP-1 cell proliferation at 24, 48, and 72 h of miR-18a inhibitor or negative control transfection was assessed by CCK-8 assay. * P<0.02 and ** P<0.01 *vs.* control cells.
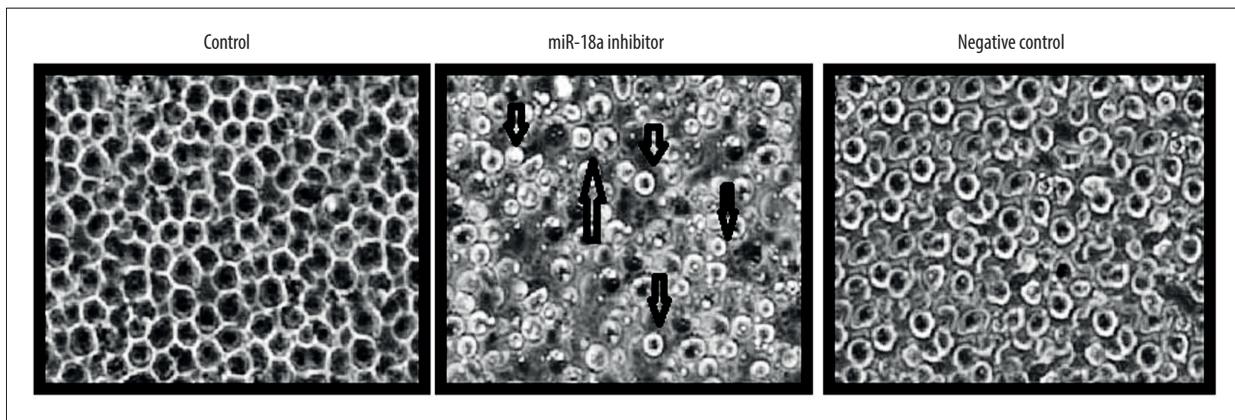

Figure 3. Effect of miR-18a inhibitor on apoptotic changes in WEHI-3 cells. The cells transfected with miR-18a inhibitor or negative control were analyzed by scanning electron microscopy. Magnification 200×.

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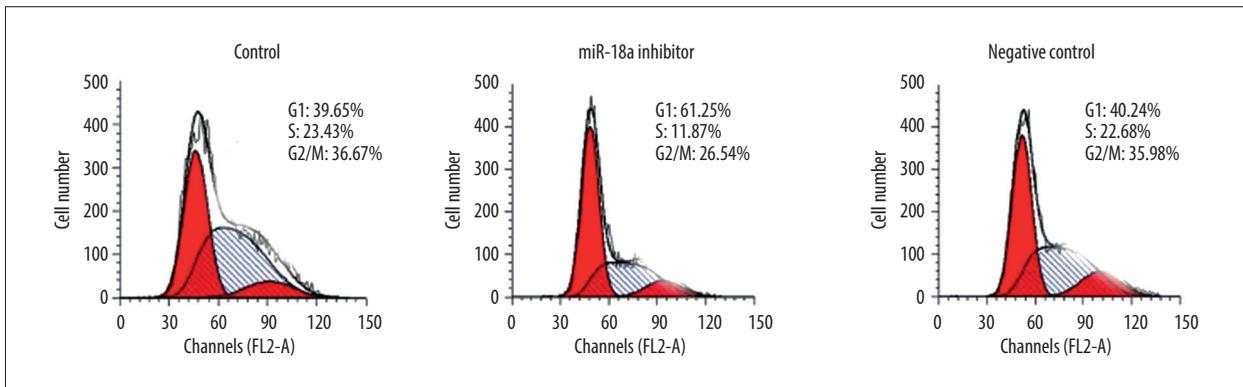
Figure 4. Effect of miR-18a inhibitor transfection on WEHI-3 cell cycle progression. Flow cytometry analysis of miR-18a inhibitor or negative transfected WEHI-3 cells at 48 h.
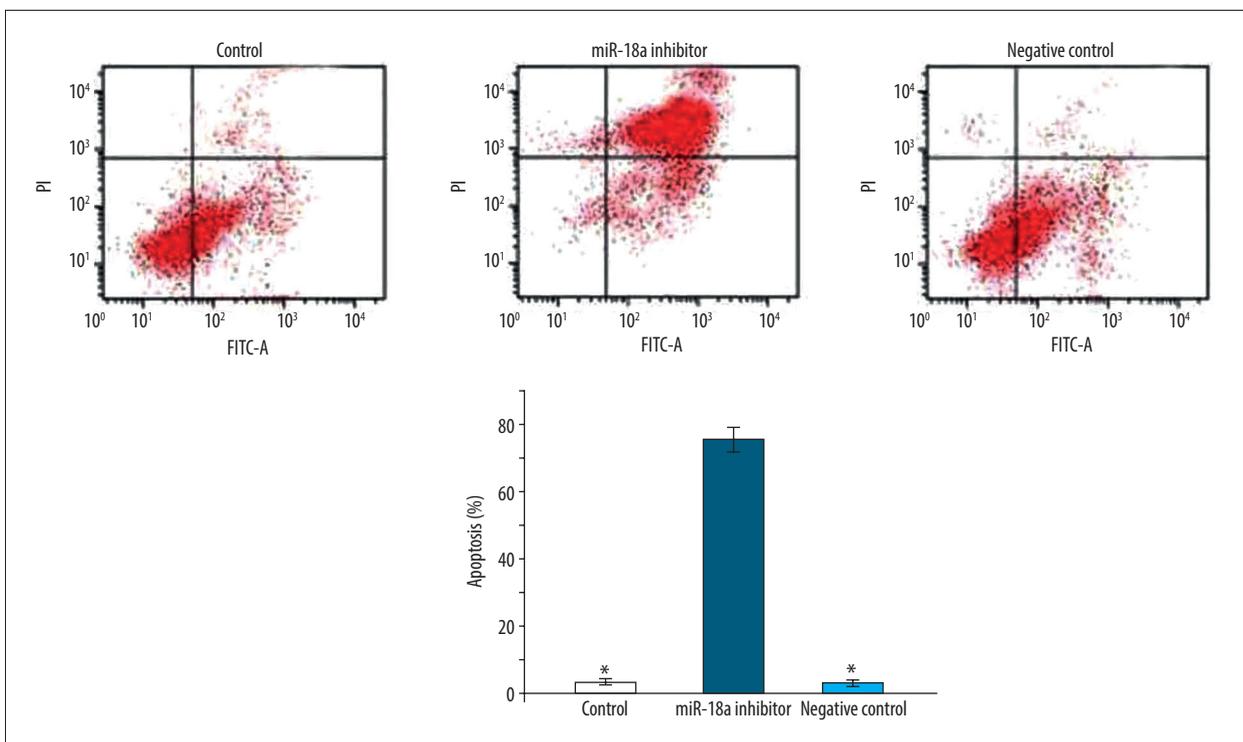

Figure 5. Apoptosis induction by miR-18a inhibitor transfection in WEHI-3 cells. The apoptosis induction in miR-18a inhibitor or negative control-transfected WEHI-3 cells at 48 h was assessed by flow cytometry. * P<0.02 *vs.* control cells or NC.

control transfection (Figure 3). The WEHI-3 cells showed characteristic apoptotic bodies after transfection with miR-18a inhibitor at 48 h. The apoptotic bodies were not seen in WEHI-3 cells transfected with negative control or in control cells.

miR-18a inhibitor arrested WEHI-3 cell cycle progression

Flow cytometry showed that miR-18a inhibitor transfection significantly (P<0.05) increased the percentage of WEHI-3 cells in G1 phase (Figure 4), whereas miR-18a inhibitor transfection decreased the proportion of WEHI-3 cells in S and G2/M phases of the cell cycle. Transfection of WEHI-3 cells with negative

control did not affect the distribution of cells in various phases in comparison to the control.

miR-18a inhibitor induced apoptosis in WEHI-3 cells

Apoptosis induction in WEHI-3 cells after miR-18a inhibitor transfection was determined by flow cytometry analysis of the FITC-annexin V and PI double-stained cells (Figure 5). The transfection of miR-18a inhibitor significantly (P<0.05) promoted apoptosis in WEHI-3 cells in comparison to the control cells at 48 h. The percentage of apoptotic cells was markedly lower in WEHI-3 cell cultures transfected with negative control.
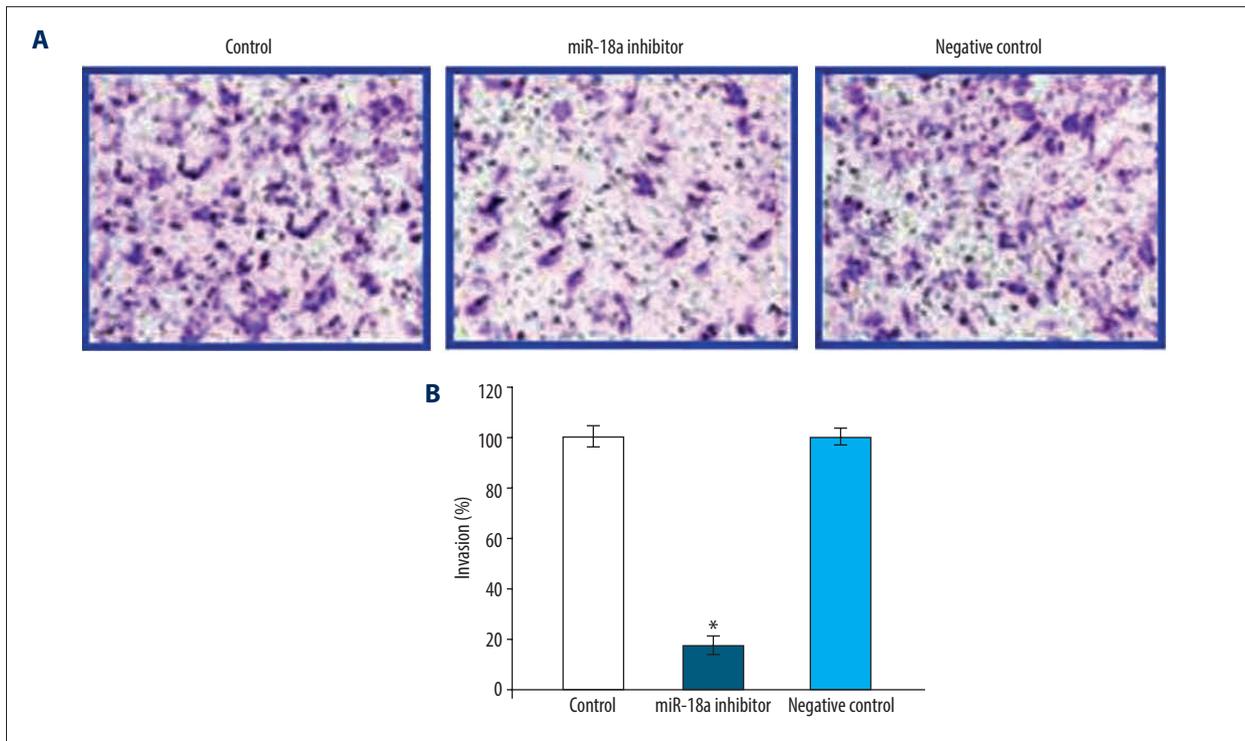
Figure 6. Effect of miR-18a inhibitor on WEHI-3 cell invasion. (**A**) The invasive ability of WEHI-3 cells was detected using Transwell assay following 48 of exposure to miR-18a inhibitor. (**B**) Quantification of the cell invasion data. * P<0.05 and *** P<0.01 *vs.* control cells.
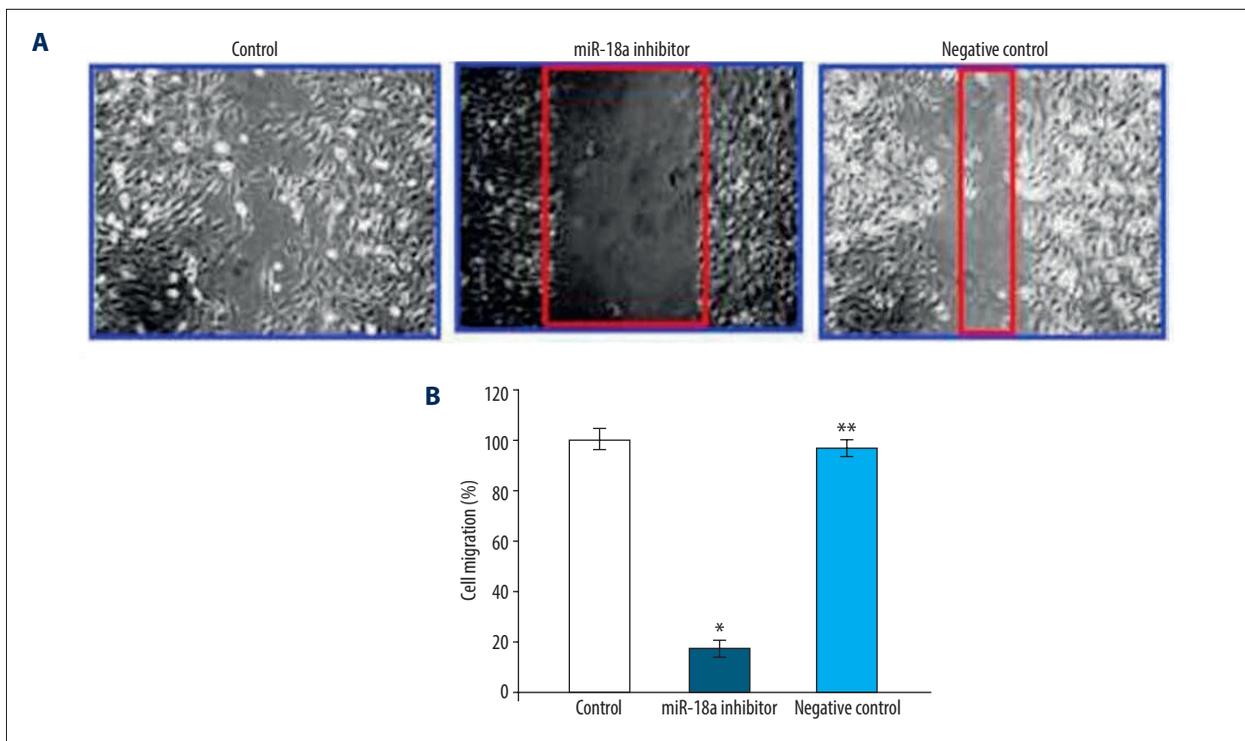

Figure 7. Effect of miR-18a inhibitor on WEHI-3 cell migration. (**A**) The WEHI-3 cell migration ability was analyzed using wound-healing assays at 48 h of treatment with miR-18a inhibitor. (**B**) Quantification of the cell invasion data. * P<0.05 and *** P<0.02 *vs.* control cells.
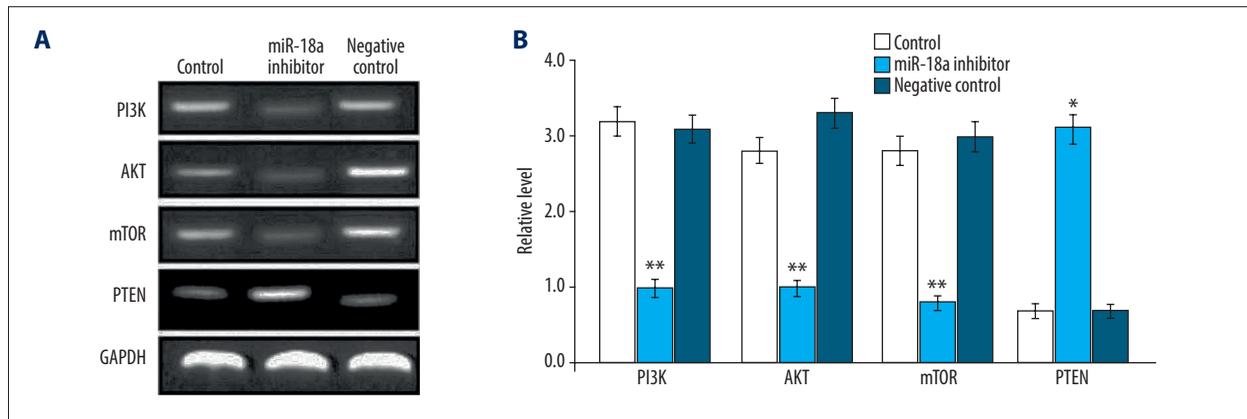
Figure 8. (**A, B**) Effect of miR-18a inhibitor transfection on PI3K/AKT/mTOR signaling pathway. The WEHI-3 cells were analyzed after miR-18a inhibitor transfection by RT-PCR assay for expression of mTOR, PI3K, AKT, and PTEN mRNA. * P<0.05 and ** P<0.02 *vs.* control cells.

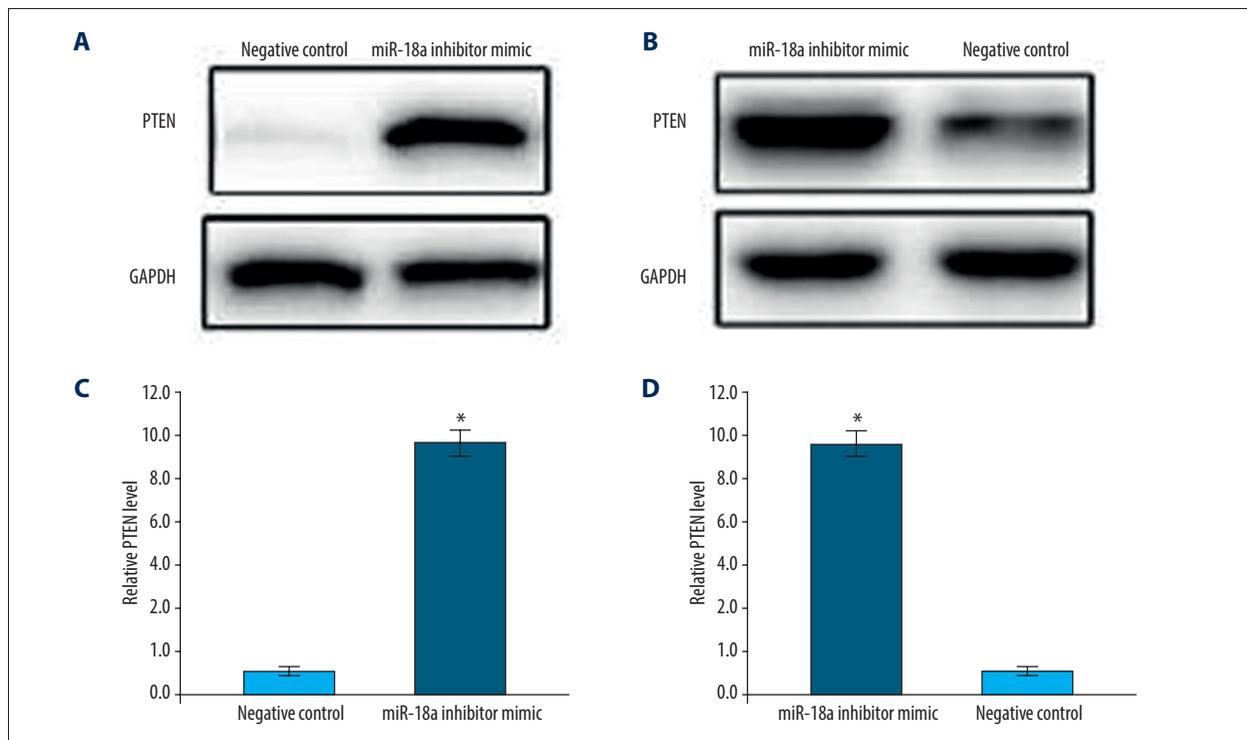**Figure 9.** PTEN was directly activated by miR-18a inhibitor. (**A–D**) The miR-18a inhibitor mimic elevated luciferase activity in WEHI-3 cells containing luciferase reporter vector bearing 3'-UTR of PTEN compared to cells with miRNA control. * P<0.02 *vs.* control cells.

miR-18a inhibitor inhibited WEHI-3 cell invasion and migration

The data from Matrigel Transwell assay showed a significant (P<0.05) decrease in invasion of WEHI-3 cells after exposure to miR-18a inhibitor (Figure 6). The invasive ability of WEHI-3 cells was decreased by 8-fold after exposure to miR-18a inhibitor. The migration potential of WEHI-3 cells was also significantly (P<0.05) reduced after exposure to miR-18a

inhibitor (Figure 7). Wound-healing assay revealed a significant reduction of WEHI-3 cell migration potential after administration of miR-18a inhibitor.

miR-18a inhibitor altered PI3K/AKT/mTOR signaling pathway gene expression in WEHI-3 cells

In WEHI-3 cells, miR-18a inhibitor transfection markedly suppressed the expression of PI3K, AKT, and mTOR mRNA (Figure 8).

The expression of PI3K, AKT, and mTOR mRNA was markedly higher in the negative control-transfected or control cells. The expression of PTEN mRNA was significantly ($P<0.05$) up-regulated by miR-18a inhibitor transfection in WEHI-3 cells.

miR-18a inhibitor directly activated PTEN

The expression of PTEN was markedly higher in WEHI-3 cells transfected with mimic for miR-18a inhibitor (Figure 9). Moreover, PTEN expression was suppressed in WEHI-3 cells by transfection with miRNA control. This suggests that miR-18a inhibitor promotes expression of PTEN in WEHI-3 cells.

Discussion

We investigated the therapeutic efficacy of miR-18a inhibitor against leukemia cells, showing that miR-18a inhibitor suppressed the proliferative potential of WEHI-3 and THP-1 cells and activated apoptosis by upregulation of PTEN mRNA expression. It has been reported that several kinds of carcinoma cells are eliminated by chemotherapeutic agents through induction of autophagy and apoptosis [23,24]. In some carcinoma cells and tumor tissues, miR-18a expression is markedly elevated compared to control cells [22]. The process of cell apoptosis induced by anti-neoplastic drugs proceeds in a programmed manner to inhibit proliferation [25]. In the present study, miR-18a expression was found to be markedly higher in WEHI-3 and THP-1 leukemia cells compared to normal monocytes. However, miR-18a inhibitor transfection markedly reduced WEHI-3 and THP-1 leukemia cell proliferative ability in comparison to the negative control-transfected cells or control cells. In WEHI-3 cells, apoptosis was markedly induced by transfection with miR-18a inhibitor compared to the negative control-transfected cells. The induction of apoptosis was evident by the appearance of apoptotic bodies under the scanning electron microscope. The findings of electron microscopy related to induction of WEHI-3 cell apoptosis following miR-18a inhibitor transfection were also confirmed using flow cytometry analysis. PI3K/AKT/mTOR is one of the most common pathways upregulated in many types of cancer cells [26]. The PI3K is stimulated by G-protein coupled receptor or tyrosine kinase receptor, and subsequently phosphorylates phosphatidylinositol 4,5-bisphosphate in the plasma membrane. PI3K/AKT/mTOR pathway dysregulation has been reported in several human cancers [27,28]. It was reported that activating mutations, such as that in tyrosine kinase and c-kit tyrosine kinase receptor, are associated with regulation of the PI3K/AKT/mTOR pathway [29]. The activated PTEN suppresses tumors by negatively regulating the PI3K/AKT/mTOR pathway [30]. There is upregulation of ATP-binding cassette transporter G2 after PTEN inhibition, which causes downregulation of the PI3K/AKT/mTOR pathway [31]. Therefore, targeting PTEN expression is appears to play an important role in leukemia treatment [32]. In the present study, miR-18a inhibitor transfection lead to a marked decrease in PI3K, AKT, and mTOR mRNA expression in WEHI-3 cells. The level of PTEN mRNA expression in WEHI-3 cells was promoted by transfection with miR-18a inhibitor. These findings suggest that miR-18a inhibitor transfection promotes PTEN mRNA expression, which subsequently inhibits PI3K, AKT, and mTOR mRNA, thereby inhibiting proliferation of leukemia cells.

Conclusions

The present study showed that miR-18a inhibitor is cytotoxic to leukemia cells through inhibition of PI3K, AKT, and mTOR expression and upregulation of PTEN expression. Thus, miR-18a inhibitor appears to have therapeutic significance for the treatment of leukemia.

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