

MicroRNA-203a-3p improves bleomycin and pingyangmycin sensitivity by inactivating the PI3K/AKT pathway in hemangioma

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Abstract. MicroRNAs (miRs) have been found to play a fundamental role in the pathology and progression of hemangioma. Of note, miR-203a-3p prevents hemangioma progression via inactivation of the PI3K/AKT pathway. Bleomycin and pingyangmycin are drugs used in sclerotherapy, but certain hemangioma patients experience drug resistance, leading to poor clinical outcomes. The present study aimed to explore the impact of miR-203a-3p on bleomycin and pingyangmycin sensitivity in hemangioma, as well as the involvement of the PI3K/AKT pathway. miR-203a-3p or negative control mimics were transfected into human hemangioma endothelial cells, which were treated with 0-20 μ M bleomycin or pingyangmycin. Subsequently, 740 Y-P, a PI3K/AKT pathway agonist, was added. Cell viability, rate of apoptosis and the expression levels of proteins involved in the PI3K/AKT pathway, including phosphorylated (p)-PI3K, PI3K, p-AKT and AKT, were detected. miR-203a-3p overexpression significantly decreased the half-maximal inhibitory concentration (IC₅₀) values of bleomycin (5.84 \pm 0.87 vs. 14.23 \pm 2.17 μ M; P<0.01) and pingyangmycin (5.13 \pm 0.55 vs. 12.04 \pm 1.86 μ M; P<0.01), compared with untreated cells. In addition, under bleomycin or pingyangmycin treatment, miR-203a-3p overexpression significantly reduced the proportion of EdU positive cells (both P<0.05) and B-cell leukemia/lymphoma-2 (BCL2) protein expression levels (both P<0.05), whilst increasing cell apoptosis rate (both P<0.05) and cleaved caspase 3 protein expression levels (both P<0.05) compared with untreated controls. Furthermore, miR-203a-3p overexpression significantly inhibited the phosphorylation of PI3K and AKT (both P<0.05), an effect that was significantly diminished by 740 Y-P treatment (both P<0.01).

In addition, 740 Y-P significantly increased IC₅₀ values of bleomycin (P<0.01) and pingyangmycin (P<0.001) and also significantly increased the proportion of EdU-positive cells and BCL2 protein expression levels, while decreasing the apoptosis rate and cleaved caspase 3 protein expression levels in cells treated with bleomycin or pingyangmycin (all P<0.05). Of note, 740 Y-P weakened the effect of miR-203a-3p overexpression on the aforementioned cellular characteristics. The present study demonstrated that miR-203a-3p improved the sensitivity of cells to bleomycin and pingyangmycin treatment by inhibiting PI3K/AKT signaling in hemangioma.

Introduction

Hemangioma is the most common type of benign tumor in infants and children and can affect physical appearance and quality of life and may cause functional impairment (1). The incidence of hemangioma in infants is 2-3% (statistics from China, the US, and Germany), and this condition is more prevalent in females compared with males (approximately 2 to 3 times higher) (2-5). The first-line treatments for hemangioma include propranolol, corticosteroids, β -blockers, topical timolol and pulsed dye laser therapy (6-8). However, nearly 10-15% of patients do not respond to treatments and sclerotherapy can be offered as a subsequent treatment due to its efficacy, safety, affordability and non-invasive nature in patients with hemangioma (6,9-14). Bleomycin (BLM) and pingyangmycin (PYM) are drugs used in sclerotherapy that act by preventing DNA replication to interfere with cell division and proliferation (15-17). However, treatment outcomes of BLM and PYM may not be effective in certain patients with hemangioma, which may be due to BLM and PYM resistance in these patients (18,19). For example, a previous study reported that 26% of patients with hemangioma receiving PYM do not achieve a cure, defined as the lesion disappearing completely without recurrence at least 1 year after treatment (19). In addition, another study reported that 45.5% of patients with hemangioma do not exhibit a complete response to BLM and 18.2% of these patients show no response to BLM treatment (18). Thus, exploring potential strategies to improve BLM and PYM sensitivity is key to enhance the management of symptoms of patients with hemangioma.

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microRNA (miR)-203a-3p is located on the 14q32.33 chromosome and contributes to the progression of certain types of malignant tumor, such as cervical, colorectal and thyroid papillary cancer (20-22). Our recent studies reported the involvement of miR-203a-3p in hemangioma (23,24). miR-203a-3p silencing regulates the long non-coding RNA maternally expressed 8-mediated Notch pathway to increase hemangioma cell proliferation and invasion whilst decreasing apoptosis (23). Furthermore, miR-203a-3p over-expression inactivates vascular endothelial growth factor A (VEGFA)-regulated PI3K/AKT pathway signaling to inhibit hemangioma cell proliferation and invasion, as well as increase apoptosis (24). A number of studies have reported the involvement of the PI3K/AKT pathway in sensitizing hemangioma cells to BLM and PYM (25,26). For example, the PI3K/AKT pathway inhibits the anti-tumor effect of PYM on hemangioma cell viability, apoptosis and invasion (26). In addition, inhibition of AKT activity increases sensitivity to BLM, which attenuates hemangioma progression (25). Considering the regulatory role of miR-203a-3p on the PI3K/AKT pathway in hemangioma progression and the involvement of the PI3K/AKT pathway in BLM and PYM resistance (24-26), miR-203a-3p may have the potential to improve sensitivity to BLM and PYM.

The present study aimed to explore the influence of miR-203a-3p on hemangioma cell sensitivity to BLM and PYM treatment, in addition to analyzing potential effects on the PI3K/AKT pathway.

Materials and methods

Cell culture. Human hemangioma endothelial cells (HemECs) were purchased from the BeNa Culture Collection, sourced the cells from Otwo Biotech (cat. no. HTX2171). HemECs were cultured in endothelial cell medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (HyClone; Cytiva) and 1% penicillin/streptomycin (Sangon Biotech Co., Ltd.) in 5% CO₂ at 37°C. The present study was approved by the Ethics Committee of the Affiliated Hospital of Hebei University of Engineering [Handan, China; approval no. 2019(K)016].

Cell transfection. miR-203a-3p (5'-GTGAAATGTTTAGGA CCACTAG-3') and negative control (NC) mimics (5'-CAGTAC TTTTGTGTAGTACAA-3') were purchased from Changchun Changsheng Gene Pharmaceutical Co., Ltd. HemECs were seeded in 6-well plates (3x10⁵ cells/well) and incubated overnight at 37°C, and then incubated with 50 nM miR-mimic or NC-mimic for 48 h using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). Following transfection, HemECs were collected for detection of miR-203a-3p expression with RT-qPCR as previously described (24). In brief, total RNA was isolated using the RNeasy Mini kit (Qiagen GmbH). Then, the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used for RT according to the kit's instructions. qPCR was performed using SYBR[®] Green PCR Master Mix (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min,

followed by 40 cycles of 95°C for 10 sec and 61°C for 30 sec (this was a two-step PCR, and the annealing and extension steps were combined). The primer sequences were as follows: miR-203a-3p forward (F), 5'-ACACTCCAGCTGGGGTGA AATGTTTAGGAC-3' (adapter sequence, ACACTCCAG CTGGG) and reverse (R), 5'-TGTCGTGGAGTCGGCAAT TC-3'; and U6 F, 5'-GCTCGCTTCGGCAGCACATA-3' and R 5'-AATATGGAACGCTTCACGAATTTGC-3' (23,27,28). The result was analyzed with 2^{-ΔΔC_q} method (29).

BLM and PYM treatment. Sensitivity of HemECs to BLM and PYM (both MedChemExpress) was measured using Cell Counting Kit-8 (MilliporeSigma). In brief, transfected HemECs were seeded into 96-well plates (3x10³ cells/well) and incubated with 0, 4, 8, 12, 16 or 20 μM BLM or PYM for 24 h at 37°C. The concentrations of BLM and PYM were determined according to preliminary experiments. Next, HemECs were incubated with CCK-8 buffer for 2 h at 37°C. The optical density (450 nm) of samples was recorded using an enzyme immunoassay analyzer (BioTek China) and used for calculating cell viability at various treatment concentrations. Half-maximal inhibitory concentration (IC₅₀) of BLM and PYM was calculated using sigmoidal dose-response function (30).

Transfected HemECs were seeded in 6-well plates (3x10⁵ cells/well) and incubated overnight at 37°C. BLM (14 μM) and PYM (12 μM) were added, then cells were incubated for 24 h at 37°C. The concentration of BLM and PYM was determined according to the IC₅₀. EdU staining, annexin V-FITC/PI apoptosis assay and western blotting assays of apoptotic proteins were subsequently performed.

740 Y-P treatment. HemECs were transfected with miR-mimic or NC-mimic as aforementioned, then stimulated with 740 Y-P (20 μM; APeXBIO Technology LLC), a cell-permeable phosphopeptide activator of PI3K (31), for 24 h at 37°C to investigate the potential effects of miR-203a-3p on the PI3K/AKT pathway. The phosphorylated level of PI3K and AKT were assessed with western blotting and the drug sensitivity of cells to BLM and PYM were measured as aforementioned. In addition, after stimulating with 740 Y-P, HemECs were incubated with BLM (14 μM) and PYM (12 μM) for 24 h at 37°C and EdU staining, annexin V-FITC/PI apoptosis assays and western blotting assays of apoptotic proteins were performed.

EdU staining. EdU staining was performed to analyze cell proliferation using the BeyoClick[™] EdU kit (Beyotime Institute of Biotechnology). Briefly, treated HemECs (as described in 'BLM and PYM treatment' and '740 Y-P treatment' subsections) were seeded in 6-well plates (3x10⁵ cells/well) and incubated overnight at 37°C. EdU (10 μM) was added to each well and cells were incubated for 2 h at 25°C. HemECs were fixed in 4% paraformaldehyde at room temperature, permeabilized by 0.3% Triton X-100 and stained with Click Additive Solution (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Cells were imaged using a fluorescence microscope (Olympus Corp.) under a magnification of x200. The proportion of EdU-positive cells was calculated as follows: (Red fluorescent spot number/blue fluorescence spot number) x100.

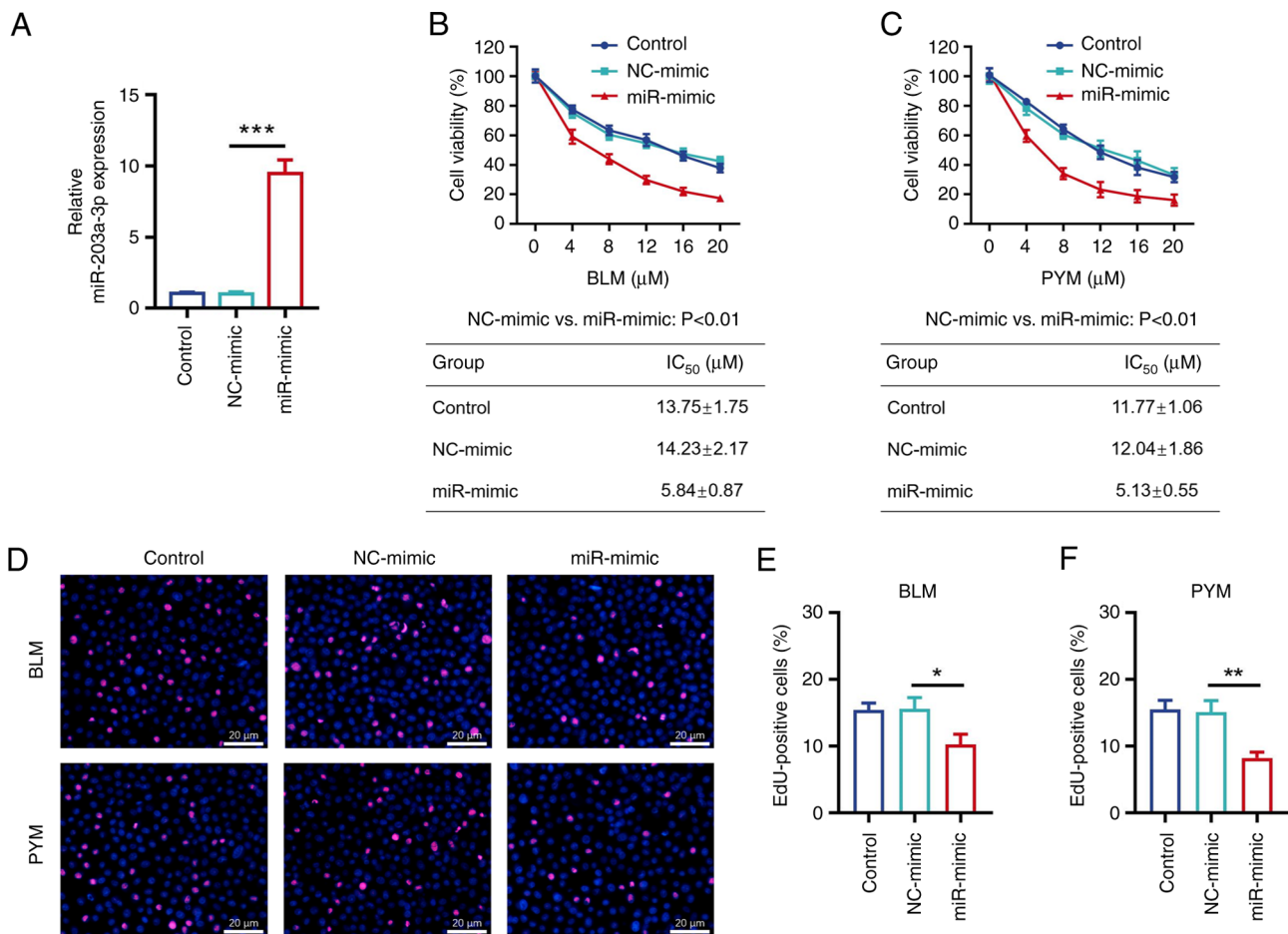


Figure 1. Effect of miR-203a-3p on cell viability following BLM or PYM treatment. (A) Relative miR-203a-3p expression levels in cells transfected with miR and NC mimics. Cell viability and IC₅₀ values of transfected cells treated with (B) BLM or (C) PYM (the comparison between groups was conducted based on the IC₅₀ values). (D) Representative EdU staining (scale bars, 20 μ m) and the proportion of EdU-positive transfected cells treated with (E) BLM (F) or PYM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NC-mimic vs. miR-mimic. miR, microRNA; BLM, bleomycin; PYM, pingyangmycin; NC, negative control; IC₅₀, half-maximal inhibitory concentration.

Apoptosis assay. The apoptosis rate of HemECs was measured using Annexin V-FITC/PI kit (Beyotime Institute of Biotechnology). In brief, treated HemECs were washed and resuspended in Annexin V-FITC binding buffer (5×10^5 cells/ 195μ l). Annexin V-FITC (5 μ l) and PI (10 μ l) were added for 10 min at 37°C. Flow cytometric detection was performed within 1 h using a FACSCanto II (BD Biosciences). The data was analyzed by FlowJo 10.0.7 (Becton, Dickinson and Co.).

Western blot analysis. HemECs were lysed using precooled RIPA reagent (MilliporeSigma) for protein extraction. The proteins were quantified by bicinchoninic acid kit (Beyotime Institute of Biotechnology), 20 μ g of which were separated by 4-20% SDS-PAGE and transferred onto nitrocellulose membranes (Pall Life Sciences). Following blocking with 5% BSA (MilliporeSigma) at 37°C for 1.5 h, membranes were incubated with primary antibodies overnight at 4°C, then incubated with secondary antibodies for 1 h at 37°C. ECL-PLUS reagent (Thermo Fisher Scientific, Inc.) was used for visualization of protein bands. The protein bands were analyzed by Image J 1.8 (National Institutes of health). The specific antibodies were as follows: BCL2, cleaved caspase 3,

phosphorylated (p)-PI3K, PI3K, p-AKT, AKT (all 1:1,000 dilution; cat. nos. PA5-22209, PA5-114687, PA5-105113, MA5-14870, 44-621G and MA5-14916; all Invitrogen; Thermo Fisher Scientific, Inc.), GAPDH (1:5,000 dilution; cat. no. GB15004-100) and Horseradish Peroxidase conjugated goat anti-rabbit secondary antibodies (1:20,000 dilution; cat. no. GB23303; both Wuhan Servicebio Technology Co., Ltd.).

Statistical analysis. The data in triplet were presented as mean \pm standard deviation and analyzed using GraphPad Prism 7.0 (Dotmatics). One-way ANOVA with Tukey's post-hoc test was used to analyze statistical significance between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-203a-3p enhanced HemEC sensitivity to BLM and PYM. The relative miR-203a-3p expression levels were significantly increased in the miR-mimic group compared with the NC-mimic group ($P < 0.001$; Fig. 1A), which demonstrated that the transfection was successful.

CCK-8 assay demonstrated that relative cell viability was decreased by BLM or PYM treatment in a dose-dependent

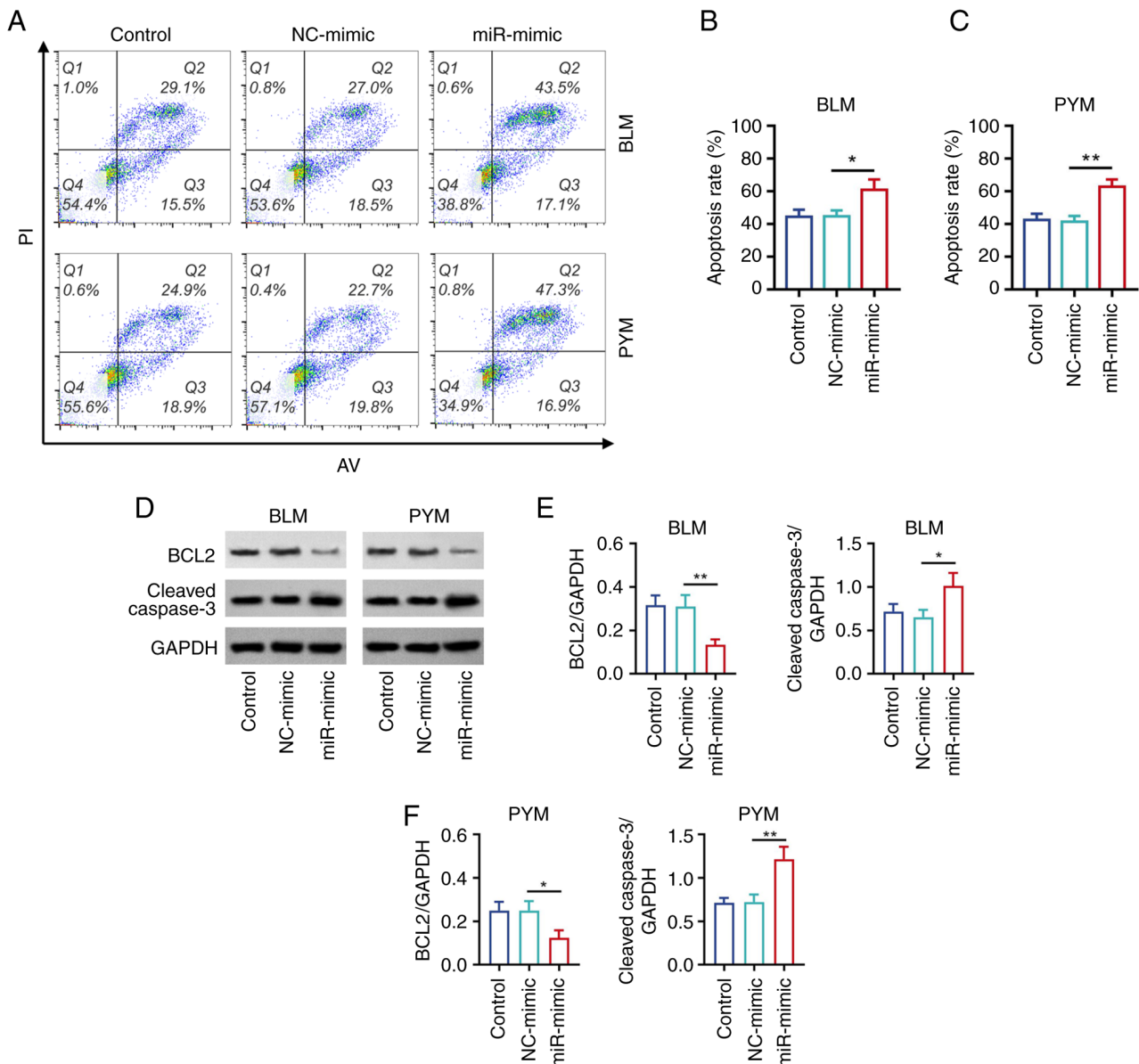


Figure 2. Effect of miR-203a-3p on apoptosis of cells treated with BLM or PYM. (A) Annexin V-FITC/PI detection of apoptosis rate of cells treated with (B) BLM or (C) PYM. (D) Western blotting and relative protein expression of BCL2 and cleaved caspase 3 in cells treated with (E) BLM or (F) PYM. * $P < 0.05$; ** $P < 0.01$, NC-mimic vs. miR-mimic. miR, microRNA; BLM, bleomycin; PYM, pingyangmycin; NC, negative control; AV, Annexin V; PI, propidium iodide.

manner (Fig. 1B and C). IC_{50} values of BLM (5.84 ± 0.87 vs. $14.23 \pm 2.17 \mu M$; $P < 0.01$; Fig. 1B) and PYM (5.13 ± 0.55 vs. $12.04 \pm 1.86 \mu M$; $P < 0.01$; Fig. 1C) were significantly decreased in the miR-mimic group compared with the NC-mimic group.

EdU staining was performed after cells were treated with the IC_{50} dose of BLM ($14 \mu M$) or PYM ($12 \mu M$; Fig. 1D); proportion of EdU-positive cells were significantly decreased in the miR- compared with the NC-mimic cells treated with BLM ($P < 0.05$; Fig. 1E) or PYM ($P < 0.01$; Fig. 1F).

Annexin V-FITC/PI kit was used to detect HemEC apoptosis in cells treated with BLM or PYM (Fig. 2A). The apoptosis rate was significantly increased in the miR-mimic group compared with the NC-mimic group treated with BLM ($P < 0.05$; Fig. 2B) or PYM ($P < 0.01$; Fig. 2C). Western blotting (Fig. 2D) demonstrated that protein expression levels of apoptotic marker BCL2 were significantly decreased, but cleaved caspase 3 protein expression levels

were significantly increased in the miR- compared with the NC-mimic groups of cells treated with BLM (Fig. 2E) or PYM (all $P < 0.05$; Fig. 2F).

740 Y-P attenuated the inhibition of miR-203a-3p on the PI3K/AKT pathway in HemECs. Our previous study reported that miR-203a-3p inhibits the PI3K/AKT pathway to facilitate hemangioma progression (24). Therefore, the effect of 740 Y-P, an activator of PI3K, was analyzed in the present study. Western blotting (Fig. 3A) demonstrated that p-PI3K/PI3K and p-AKT/AKT protein expression levels were significantly elevated in the NC-mimic + 740 Y-P group compared with the NC-mimic group (both $P < 0.01$; Fig. 3B). Furthermore, p-PI3K/PI3K and p-AKT/AKT protein expression levels were significantly increased in the miR-mimic + 740 Y-P group compared with the miR-mimic group ($P < 0.01$; Fig. 3C).

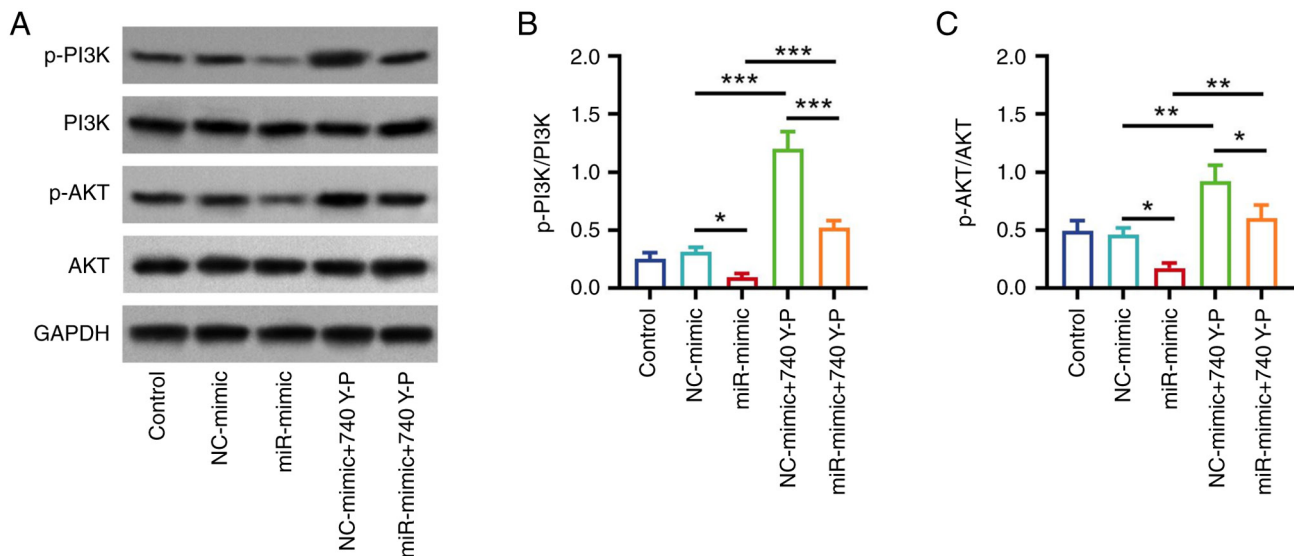


Figure 3. Effect of 740 Y-P on the miR-203a-3p-mediated PI3K/AKT pathway. (A) Detection of p-PI3K and p-AKT protein expression levels by western blotting. (B) p-PI3K/PI3K and (C) p-AKT/AKT protein expression. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as indicated. miR, microRNA; NC, negative control; p, phosphorylated.

740 Y-P attenuated the role of miR-203a-3p in enhancing HemEC sensitivity to BLM and PYM. CCK-8 assay demonstrated that the IC_{50} value of BLM was significantly increased in the NC-mimic + 740 Y-P compared with the NC-mimic group ($P<0.01$). Furthermore, IC_{50} value of BLM was significantly increased in the miR-mimic + 740 Y-P group compared with the miR-mimic group ($P<0.001$; Fig. 4A). The same trend was demonstrated in HemECs treated with PYM (both $P<0.001$; Fig. 4B).

EdU staining (Fig. 5A) demonstrated that, when treated with BLM, the proportion of EdU-positive cells significantly increased in the NC-mimic + 740 Y-P group compared with the NC-mimic group ($P<0.05$; Fig. 5B). Additionally, the proportion of EdU-positive cells was significantly elevated in the miR-mimic + 740 Y-P group compared with the miR-mimic group ($P<0.05$). The same trend was demonstrated in HemECs treated with PYM (both $P<0.01$; Fig. 5C).

Annexin V-FITC/PI staining (Fig. 6A) demonstrated that, in cells treated with BLM, the apoptosis rate was significantly decreased in the NC-mimic + 740 Y-P group compared with the NC-mimic group ($P<0.05$; Fig. 6B). In addition, the apoptosis rate was significantly decreased in the miR-mimic + 740 Y-P group compared with the miR-mimic group ($P<0.01$). The same trend was demonstrated in HemECs treated with PYM (both $P<0.01$; Fig. 6C).

Western blotting (Fig. 6D) demonstrated that, in cells treated with BLM, BCL2 protein expression levels were significantly increased but protein expression of cleaved caspase 3 was decreased in the NC-mimic + 740 Y-P group compared with the NC-mimic group (both $P<0.05$; Fig. 6E). Additionally, BCL2 protein expression was significantly increased and cleaved caspase 3 protein expression levels were decreased in the miR-mimic + 740 Y-P group compared with the miR-mimic group (both $P<0.05$). The same trend of increased protein expression of BCL2 and decreased protein expression levels of cleaved caspase 3 was demonstrated in PYM-treated HemECs ($P<0.05$), with the exception of a marked increase in

BCL2 protein expression levels in the miR-mimic + 740 Y-P group compared with the miR-mimic group ($P>0.05$; Fig. 6F).

Discussion

A number of miRs, such as miR-556-3p, miR-139-5p and miR-382-5p, participate in the progression of hemangioma (32-35). For example, miR-556-3p targets VEGF C to suppress hemangioma cell proliferation and increase apoptosis (32). miR-206 promotes extracellular matrix accumulation and decreases hemangioma cell proliferation by downregulating DNA methyltransferase 3A expression (36). In addition, miR-497-5p inhibits hemangioma cell proliferation and induces ferroptosis by regulating Notch receptor 2 (37). miR-200c-3p facilitates hemangioma cell proliferation by targeting the Notch pathway (35). Our previous study reported that miR-203a-3p knockdown regulates the Notch pathway to facilitate progression of hemangioma (23). Furthermore, our previous study reported that miR-203a-3p inhibits hemangioma cell proliferation and invasion but facilitates apoptosis by inactivating the PI3K/AKT pathway (24).

The present study hypothesized that miR-203a-3p may influence the drug sensitivity of hemangioma cells. The present study demonstrated that miR-203a-3p increased the sensitivity of hemangioma cells to BLM and PYM. This may be due to regulation of the PI3K/AKT pathway, phosphodiesterase 4D or snail family transcriptional repressor 2 gene (21,24,38). The latter factors (the PI3K/AKT pathway, phosphodiesterase 4D and snail family transcriptional repressor 2 gene) are involved in the regulation of BLM and PYM sensitivity (26,39,40). However, this hypothesis requires further experimental validation. In addition, the impact of miR-203a-3p on drug sensitivity in certain types of cancer has been previously reported (41-43) and the findings of the present study were in accordance with aforementioned studies.

The PI3K/AKT pathway is a crucial regulator in hemangioma (24,44,45). Notably, a number of studies have reported

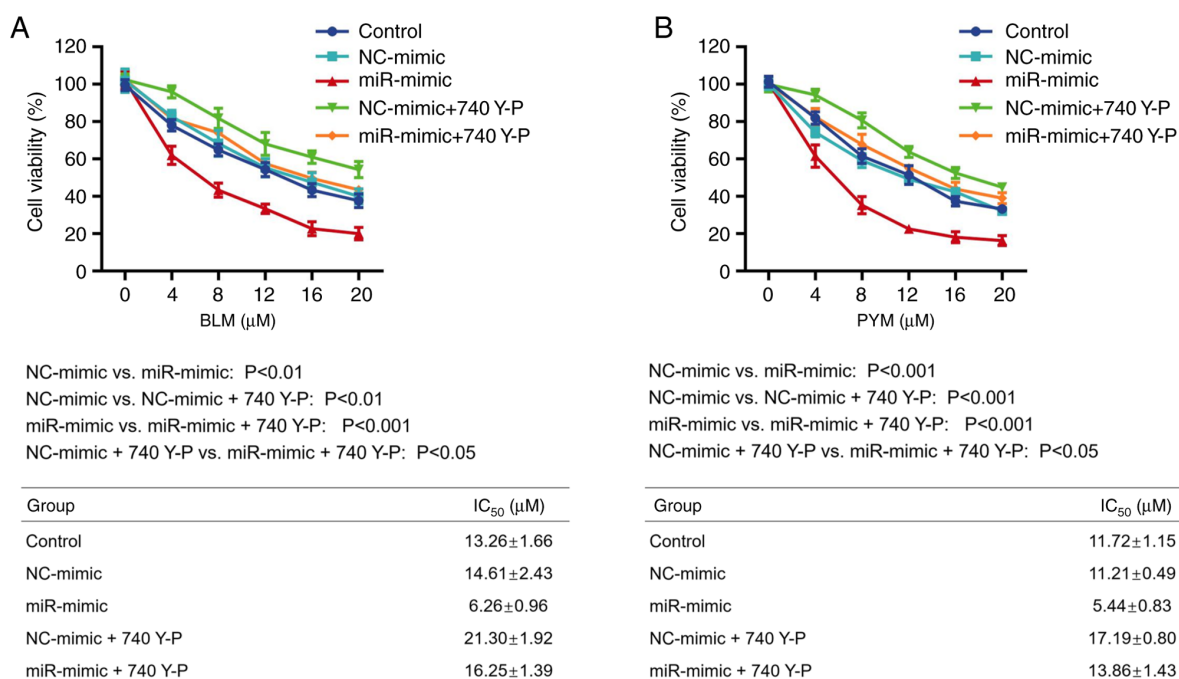


Figure 4. Effect of 740 Y-P on miR-203a-3p-mediated viability of cells (detected by CCK-8) treated with BLM or PYM. IC₅₀ values in cells treated with (A) BLM and (B) PYM (the comparison between groups was conducted based on the IC₅₀ values). miR, microRNA; BLM, bleomycin; PYM, pingyangmycin; NC, negative control; IC₅₀, half-maximal inhibitory concentration.

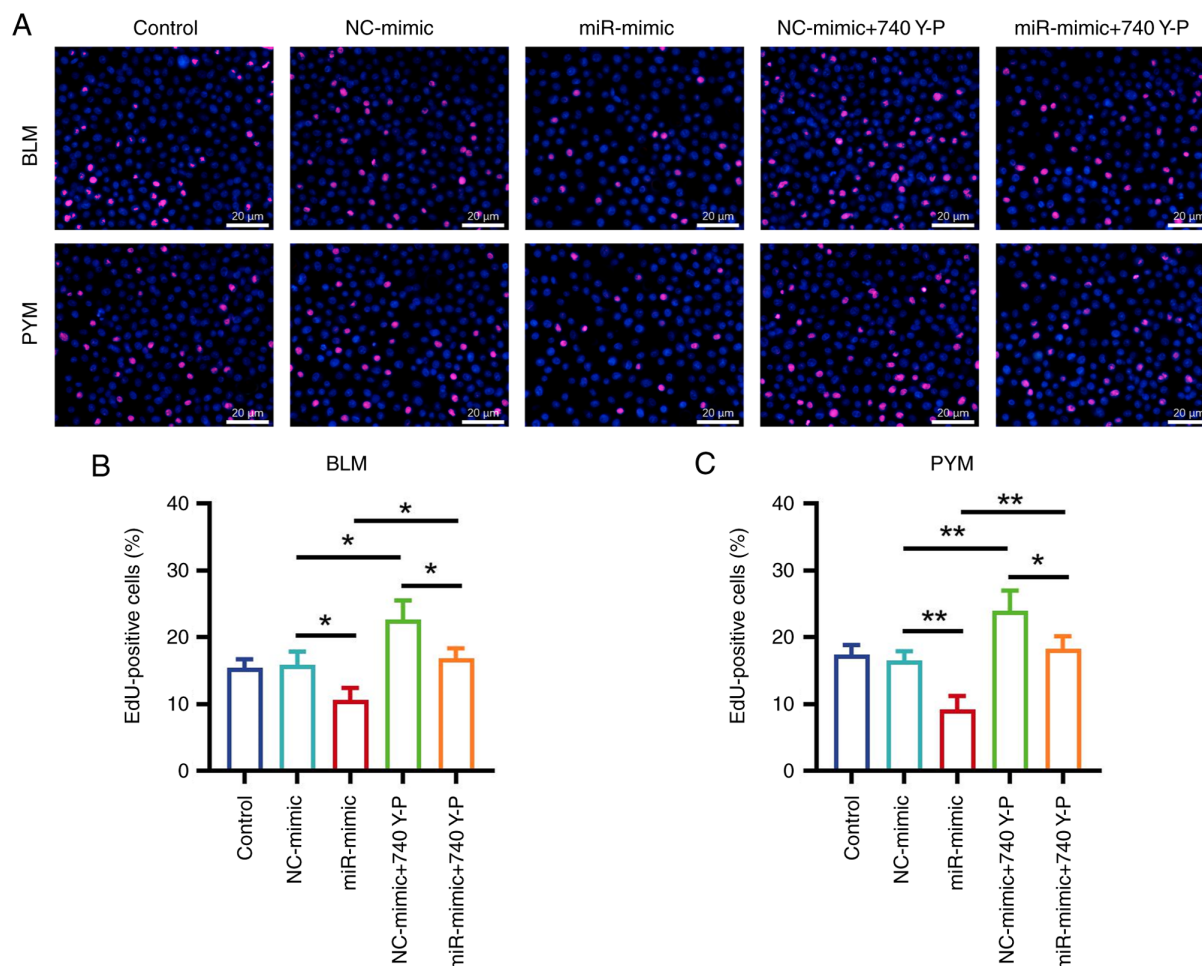


Figure 5. Effect of 740 Y-P on miR-203a-3p-mediated viability of cells (detected by EdU staining) treated with BLM or PYM. (A) Representative EdU staining (scale bars, 20 μm). Proportion of EdU-positive cells treated with (B) BLM or (C) PYM. * $P < 0.05$; ** $P < 0.01$ as indicated. miR, microRNA; BLM, bleomycin; PYM, pingyangmycin; NC, negative control.

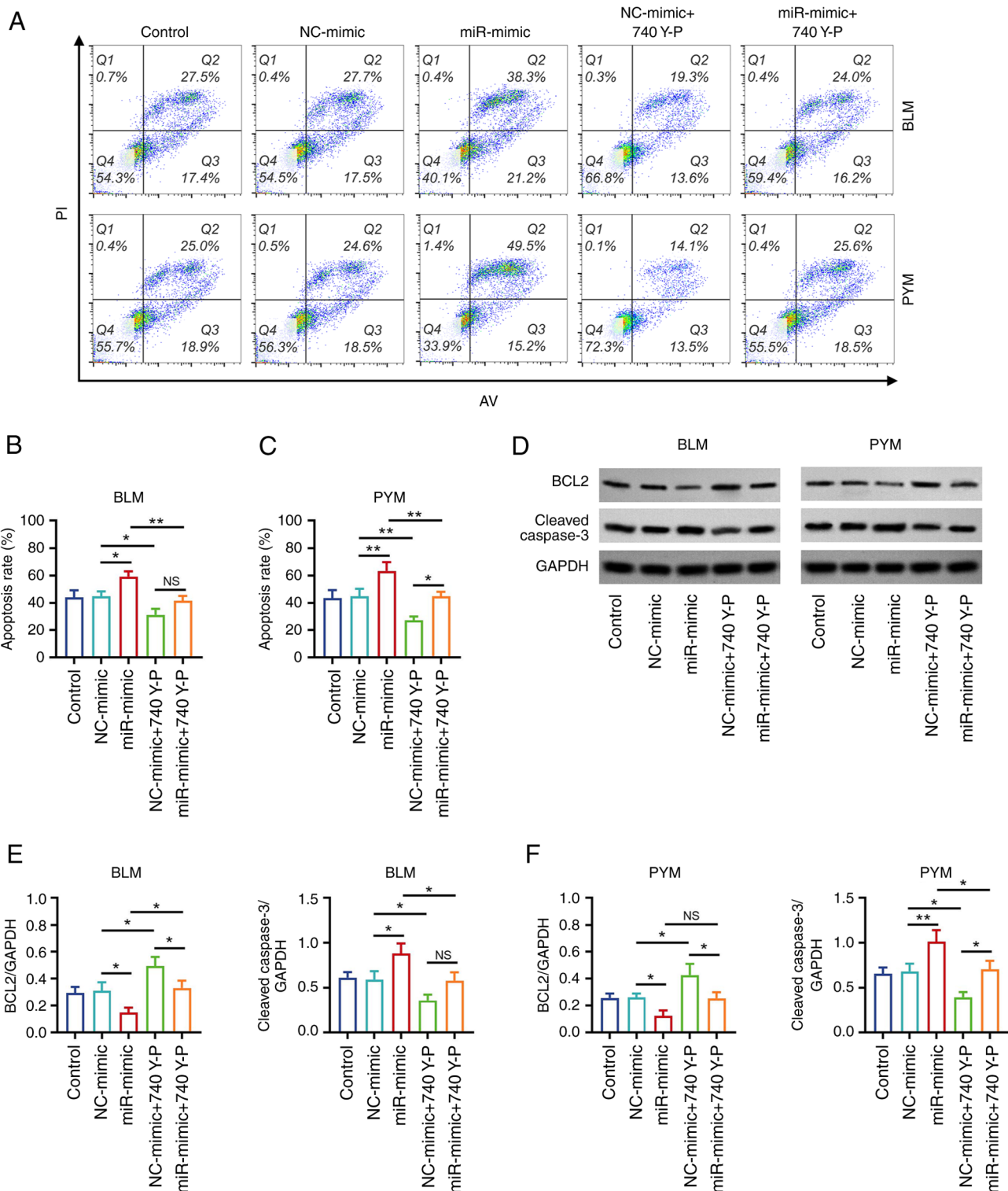


Figure 6. Effect of 740 Y-P on miR-203a-3p-mediated apoptosis of cells treated with BLM or PYM. (A) Annexin V-FITC/PI detection of apoptosis rate of cells treated with (B) BLM or (C) PYM. (D) Western blotting of BCL2 and cleaved caspase 3 in cells treated with (E) BLM or (F) PYM. * $P < 0.05$; ** $P < 0.01$ as indicated. NS, not significant; miR, microRNA; BLM, bleomycin; PYM, pingyangmycin; NC, negative control; AV, Annexin V; PI, propidium iodide.

the involvement of the PI3K/AKT pathway in BLM or PYM sensitivity in hemangioma (25,26). For example, the PI3K/AKT pathway is involved in PYM mediation of hemangioma cell proliferation, invasion and apoptosis (26). To explore the downstream effects of miR-203a-3p regulation of BLM and PYM sensitivity in hemangioma, the impact of 740 Y-P was analyzed in the present study. It was demonstrated that

miR-203-3p negatively regulated the PI3K/AKT pathway in HemECs and 740 Y-P diminished the impact of miR-203a-3p on the PI3K/AKT pathway in HemECs. This finding was in line with our previous study (24). Additionally, 740 Y-P attenuated the effect of miR-203a-3p on BLM or PYM sensitivity in hemangioma cells. This may have been due to miR-203a-3p modulation of the expression of genes, such as VEGFA and

PTEN, to inhibit the PI3K/AKT pathway, which further contributed to increased sensitivity of hemangioma cells to BLM and PYM (24,46).

A number of studies have reported the involvement of the PI3K/AKT pathway in BLM and PYM resistance in hemangioma (25,26). The methods used by the aforementioned studies were different compared with those used in the present study. For example, the cell viability was analyzed using MTT assay in a previous study (26), whereas a CCK-8 assay was used in the present study. In addition, expression of the apoptotic marker BCL2 was studied using immunofluorescence in a previous study (25), while BCL2 protein expression levels were detected by western blotting here. Overall, the findings demonstrated in the present study were in line with those of aforementioned studies (25,26), which indicated the reliability.

The present study demonstrated that miR-203a-3p down-regulated PI3K/AKT pathway signaling to improve the BLM and PYM sensitivity in hemangioma; however, whether the regulation of miR-203a-3p on the PI3K/AKT pathway is direct or indirect should be validated. Furthermore, miR-203a-3p may also regulate other cellular pathways to sensitize hemangioma cells to BLM and PYM.

To conclude, miR-203a-3p improved hemangioma sensitivity to BLM and PYM by inactivating the PI3K/AKT pathway. This demonstrated the potential of miR-203a-3p as a future co-treatment option for patients with hemangioma receiving BLM or PYM treatment.

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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

LZ and ZH contributed to study conception and design. Material preparation and data collection were performed by LZ, ZH and JC. Data analysis was performed by LZ, ZH, JC, QG and JG. The manuscript was written by LZ, ZH and JC, and the manuscript was revised by JC, QG and JG. LZ and ZH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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