

ORIGINAL ARTICLE

## Apatinib enhances chemosensitivity of acute myeloid leukemia HL60 cells to cytarabine by inducing apoptosis

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### Summary

**Purpose:** To investigate the effect of apatinib combined with cytarabine on acute myeloid leukemia (AML) HL60 cells and its relevant mechanisms.

**Methods:** HL60 cells were treated with control, apatinib alone, cytarabine alone and apatinib combined with cytarabine. Cell proliferation in each group was detected via methyl thiazolyl tetrazolium (MTT) assay, changes in the cell cycle and mitochondrial transmembrane potential in each group after treatment were detected via flow cytometry, and apoptosis was detected via Annexin V-PI double labeling. Moreover, changes in cell cycle-related proteins and apoptosis-associated proteins in each group after treatment were detected via Western blotting.

**Results:** MTT assay revealed that the sub-lethal dose of apatinib combined with cytarabine had a higher inhibitory rate on tumor cells than cytarabine alone. Cell cycle assay showed that apatinib combined with cytarabine could effectively arrest HL60 cells in G0/G1 phase in the combination group.

In combination group, the expression level of the positive regulator cyclin D1 was decreased, while the expression levels of the negative regulators p21 and p27 were significantly up-regulated compared with those in single application groups. Results of apoptosis assay manifested that in the combination group, the mitochondrial transmembrane potential of HL60 cells could be synergistically destroyed, and the proportion of apoptotic cells was also obviously increased. Results of Western blotting demonstrated that the levels of apoptosis-associated proteins cleaved caspase-9, cleaved caspase-3, cleaved PARP and Bax in the combination group after treatment were remarkably up-regulated, while the Bcl-2 protein level was significantly down-regulated.

**Conclusion:** Apatinib combined with cytarabine resists acute myeloid leukemia through synergistically regulating cell cycle and promoting apoptosis.

**Key words:** apatinib, cytarabine, acute myeloid leukemia, cell cycle, apoptosis

### Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous malignant clonal disease of hematopoietic stem cells, as well as a type of acute leukemia with the highest incidence rate in adults, seriously threatening human life [1]. Different subtypes of AML have different morphological, immunophenotypic, cytogenetical and molecular biological characteristics [2,3]. In the treatment of AML with traditional chemotherapy regimens,

AML relapses easily with a relatively low survival rate [4,5]. Increasingly more molecular targeted drugs have been used in AML, thus improving the clinical efficacy on AML.

Apatinib, a new generation of small-molecule tyrosine kinase inhibitors, highly selectively inhibits the activity of tyrosine kinase and blocks the signal transduction after binding to vascular endothelial growth factor (VEGF), thus strongly in-

hibiting the tumor angiogenesis [6,7]. Apatinib can induce apoptosis in a variety of tumor cells, and is clinically used in advanced lung cancer, gastric cancer, liver cancer and breast cancer [8]. Studies have proved that apatinib can inhibit growth and promote apoptosis of myeloid leukemia HL-60 and K562 cell lines [9]. Cytarabine is a deoxycytidine nucleoside analog and pyrimidine antimetabolite mainly acting in the S phase, which can enter cells through the translocator and be metabolized into arabinosylcytosine triphosphate (Ara-CTP) through phosphorylation of deoxycytidine kinase (DCK) [10]. Ara-CTP is an active metabolite of cytarabine, which can competitively inhibit DNA polymerase, thus inhibiting DNA synthesis and leading to cell death [11,12]. There have been no related reports on the combined application of apatinib and cytarabine. Therefore, the present experimental work aimed to study the lethal effect of apatinib combined with cytarabine on AML HL-60 cell line and its molecular mechanism.

## Methods

### Materials

Human acute promyelocytic leukemia HL-60 cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium required for cell culture were purchased from Gibco (Rockville, MD, USA). Primary antibodies used in Western blotting were purchased from BD (Franklin Lakes, NJ, USA). Horseradish peroxidase (HRP)-labeled secondary antibodies were bought from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Cell cycle and apoptosis detection kits were bought from Shanghai Beyotime Biotechnology (Shanghai, China). Rhodamine 123 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Cell culture

HL60 cells were incubated in the RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100

U/mL streptomycin at 37°C in 5% CO<sub>2</sub> and saturated humidity in a sterile incubator. The solution was replaced every other day. During the experiment, cells were inoculated at a density of 3×10<sup>5</sup>/mL and incubated with the drugs in required concentration.

### Cell proliferation assay

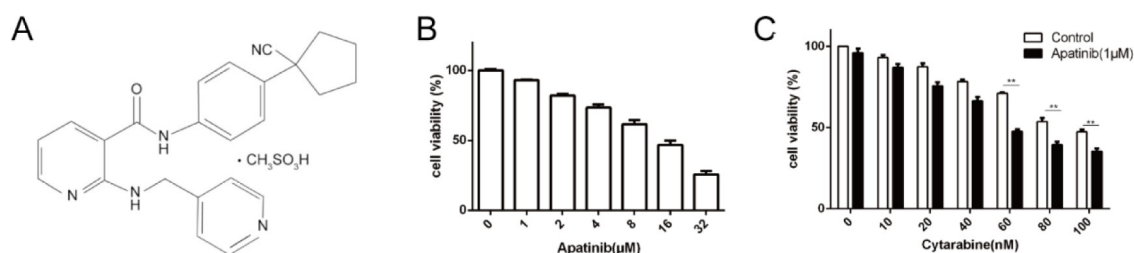
Cell proliferation was detected via methyl thiazolyl tetrazolium (MTT) assay according to the instructions of the reagent. HL-60 cells in the logarithmic growth phase cultured in the incubator with 5% CO<sub>2</sub> at 37°C were inoculated into a 96-well plate (8×10<sup>3</sup>/well). The cell proliferation in each group at 24, 48 and 72 hrs was detected using the MTT kit. The value was read at an absorption wavelength of 450 nm, and the cell growth curve was drawn.

### Cell cycle analysis

HL60 cells were inoculated in the RPMI 1640 medium containing 10% FBS and cultured in the incubator with 5% CO<sub>2</sub> at 37°C. HL60 cells in the logarithmic growth phase were collected and added into a 6-well culture plate, and control group, apatinib group, cytarabine group and apatinib + cytarabine group were set up. After cells were cultured with corresponding drugs for 24 hrs, 1×10<sup>6</sup> cells were taken, washed with cold phosphate buffered saline (PBS) twice and fixed with 70% cold ethanol at 4°C overnight. After cells were washed again with PBS twice, they were incubated with PBS containing 10 mg/mL RNase at 37°C for 30 min, followed by DNA staining with 50 µg/mL propidium iodide (PI). After standing in a dark place at 4°C for 10 min, the cell DNA content was detected using flow cytometer.

### Cell apoptosis assay

After cells in the logarithmic growth phase were collected, they were counted, resuspended in the RPMI 1640 medium containing 10% FBS, inoculated into a 12-well plate (3×10<sup>5</sup>/well) and cultured in the incubator with 5% CO<sub>2</sub> at 37°C. The control group, apatinib group, cytarabine group and apatinib + cytarabine group were set up. After cells were cultured with corresponding drugs for 24 hrs, they were collected and centrifuged at 800 rpm for 5 min. After the supernatant was discarded, cells were washed with cold PBS twice, followed by centrifugation. The supernatant was discarded, and 150 µL



**Figure 1.** Apatinib and cytarabine inhibit the cell viability of HL60 cells. **(A)** Chemical structure of apatinib. **(B)** HL60 cells were treated with indicated concentrations of apatinib for 48 h. **(C)** HL60 cells were treated with indicated concentrations of cytarabine combined with or without 1 µM apatinib for 48 h. Three independent experiments were performed. Data are presented as means ± SD (n=3).\*\*p<0.01.

binding buffer, 10  $\mu$ L Annexin-V and 5  $\mu$ L PI (50 mg/mL) were added into cells in each tube according to instructions of the Annexin V-FITC/PI apoptosis assay kit. The mixture was mixed evenly and reacted in a dark place at room temperature for 15 min, followed by detection using the flow cytometer within 1 hr.

#### Detection of mitochondrial membrane potential

HL60 cells in the logarithmic growth phase were collected and added into a 6-well culture plate. The corresponding drugs were added using the above methods for incubation for 24 hrs. Then cells were collected, incubated with 1 mM rhodamine 123 at 37°C for 30 min, washed twice with PBS and resuspended, followed by detection using the flow cytometer.

#### Western blotting

After the lysis solution was added to extract the total protein in both groups, 30  $\mu$ l total protein was loaded into each well, followed by electrophoresis using the spacer gel under 80 V for 40 min and electrophoresis using the separation gel under 100 V for 2 hrs. The protein was transferred onto a membrane using the routine wet method, sealed with 5% skim milk powder for 2 hrs, p21, p27, and incubated with cyclin D, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), caspase-9, caspase-3 and poly ADP-ribose polymerase (PARP) primary antibodies (1:200), and goat anti-rabbit secondary antibody (1:1000) for 2 hrs, followed by quan-

titative determination of the protein bands using the gel imaging system and Quantity One1-D analysis software. The relative expression level of the target protein equalled the measured value of target protein/measured value of actin.

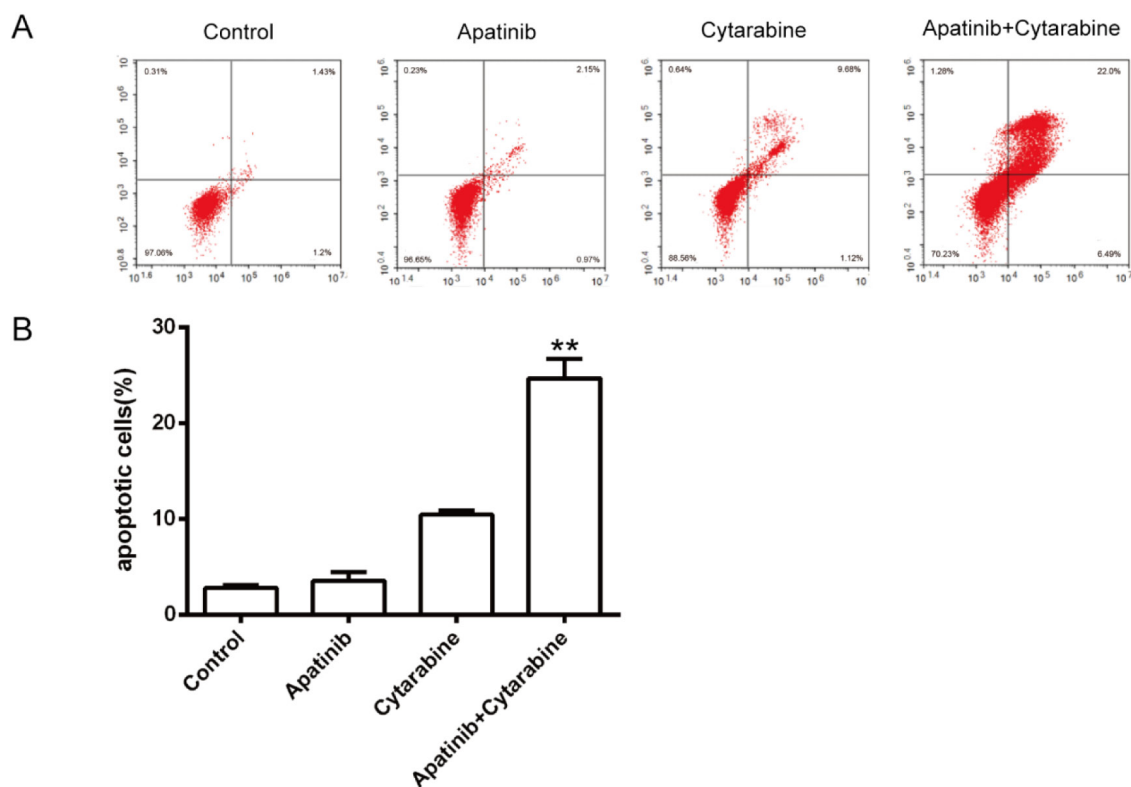
#### Statistics

Experimental data were presented as mean  $\pm$  standard deviation. SAS statistical software was used for the statistical analyses of data, and t-test was used for statistical processing of data.  $p < 0.05$  suggested that the difference was statistically significant.

## Results

### Apatinib and cytarabine synergistically inhibited the proliferation of HL60 cells

The chemical structure of apatinib is shown in Figure 1A. First, the effects of apatinib and cytarabine on the proliferation of AML HL60 cells were observed. HL60 cells were incubated with apatinib in different gradient concentrations. As has been demonstrated in existing studies, apatinib displayed an inhibitory effect in a dose-dependent manner (Figure 1B). To further investigate whether apatinib could synergistically promote the sensitivity of HL60 cells to cytarabine, HL60 cells were



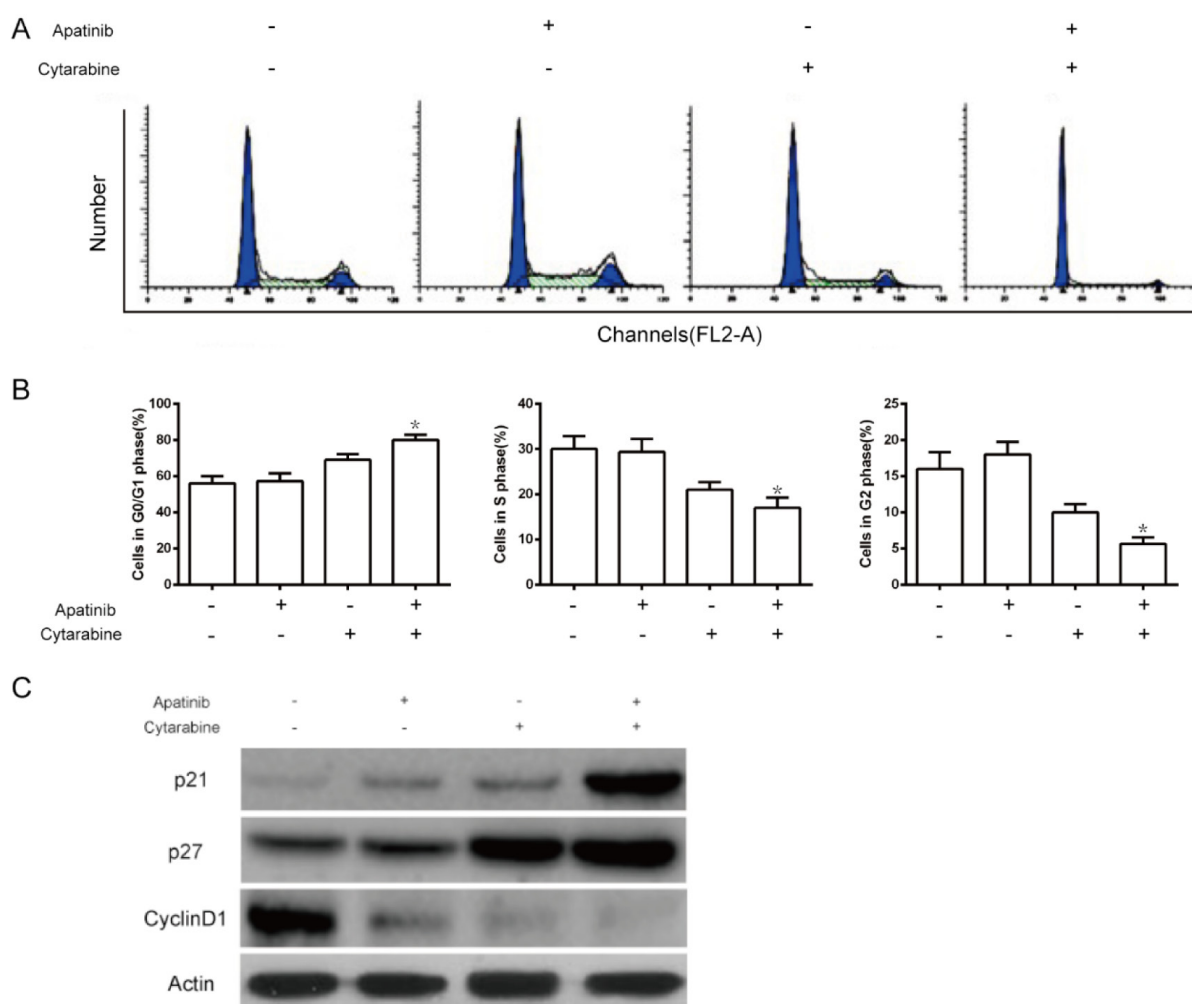
**Figure 2.** Apatinib sensitizes HL60 cells to cytarabine-induced apoptosis. **(A,B)** HL60 cells were treated with RPMI1640, 1  $\mu$ M apatinib, 60 nM cytarabine or the combination of apatinib and cytarabine for 24 h. Cells were analyzed by Annexin V/PI staining. Data shown are representative of 3 independent experiments. Data are presented as means  $\pm$  SD (n=3). \*\* $p < 0.01$ .

incubated with apatinib in a non-lethal dose (1  $\mu$ M) combined with cytarabine in a gradient concentration for 48 hrs. Interestingly, apatinib and cytarabine displayed a synergistic inhibitory effect on the growth of HL60 cells with the gradual increase of dose. Experimental results revealed that 1  $\mu$ M apatinib combined with 60 nM cytarabine had a higher synergistic inhibitory rate (Figure 1C), indicating that apatinib combined with cytarabine can significantly inhibit the cell proliferation. Based on these results, the mechanism was further explored using 1  $\mu$ M apatinib and 60 nM cytarabine.

#### Apatinib and cytarabine synergistically affected the HL60 cell cycle

The cell cycle includes G0/G1 phase, S phase, G2 phase and M phase. The DNA content shows

periodical changes and differs from phase to phase. In order to study the potential mechanism of apatinib and cytarabine in synergistically inhibiting the proliferation of HL60 cells, the effect of combined application on the cell cycle was further investigated. The regulatory effect of 1  $\mu$ M apatinib combined with 60 nM cytarabine on the HL60 cell cycle was detected via flow cytometry. Experimental results revealed that the proportion of cells in each cycle phase was different after treatment with the corresponding drugs for 24 hrs. In the combination group, the proportion of HL60 cells in G0/G1 phase was significantly increased, while the proportion of cells in S and G2 phase was gradually decreased (Figure 2A and B). The above results demonstrate that apatinib combined with cytarabine effectively arrests HL60 cells in G0/G1 phase,



**Figure 3.** Apatinib and cytarabine induce G0/G1 cell cycle arrest in HL60 cells. **(A)** HL60 cells were treated with apatinib and cytarabine or the two in combination for 24 hrs, followed by flow cytometry to evaluate the distribution of cell cycle phase. The apoptotic rate was 3.12% in the apatinib group, 10.8% in the cytarabine group and up to 28.49% in the combination group. **(B)** The results of distribution in cycle phase were exhibited. The results showed that apatinib combined with cytarabine synergistically promotes the pro-apoptotic effect. **(C)** The expression levels of p21, p27 and cyclin D1 were detected by western blotting and showed that apatinib and cytarabine synergistically upregulated the expression of G0/G1 associated protein in HL60 cells. Data shown are representative of 3 independent experiments and presented as mean  $\pm$  SD (n=3). \*p<0.05.

and their combined application can synergistically affect the DNA replication and synthesis in HL60 cells, inhibit cell mitosis, arrest cells in G0/G1 phase, prevent cells from entering S phase, thus inhibiting cell proliferation. To clarify the mechanism of combined application in inducing the transition of HL60 cells from G1 phase to S phase, the effects of combined application on the expression levels of regulatory proteins (p21, p27 and cyclin D1) at the check point of the transition of HL60 cells from G1 to S phase were studied via Western blotting. Experimental results showed that in the combination group, the expression level of the positive regulator cyclin D1 was decreased, while the expression levels of the negative regulators p21 and p27 were significantly up-regulated compared with those in the single-application groups (Figure 3C). The above results indicate that the combined application can interfere in the cycle and arrest cells in the G0/G1 phase through up-regulating the expression of cyclin-dependent kinase (CDK) inhibitors p21 and p27 and down-regulating the cyclin D1 expression.

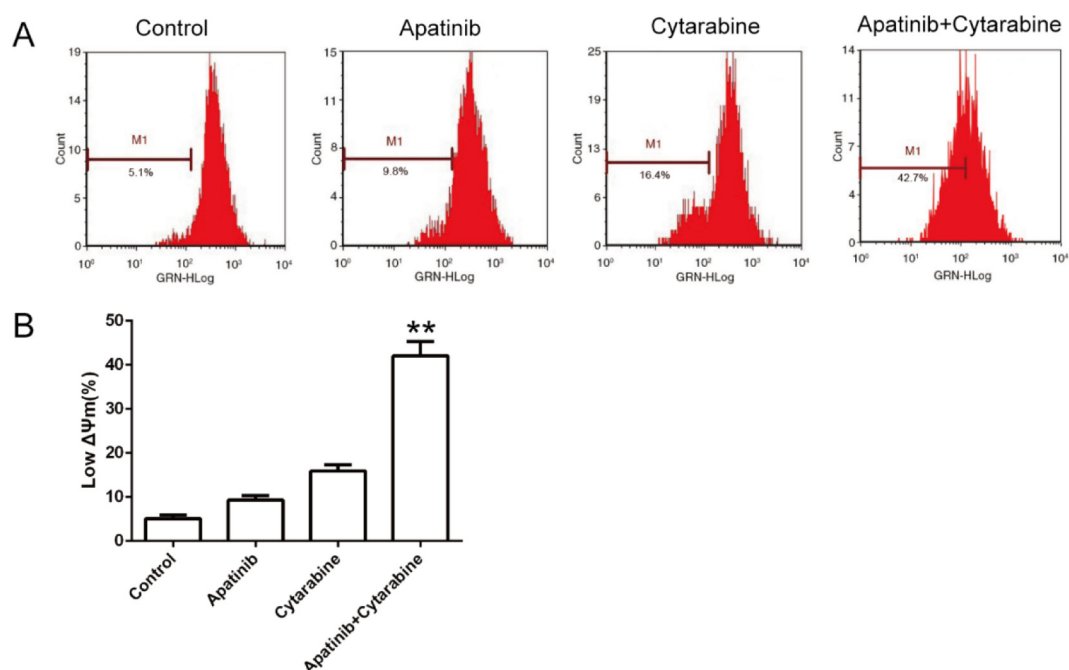
#### *Apatinib and cytarabine synergistically promoted apoptosis of HL60 cells*

The apoptotic rates in the control group, apatinib group, cytarabine group and apatinib + cytarabine group were detected via flow cytometry (Figure 3A). The results showed that the apoptotic

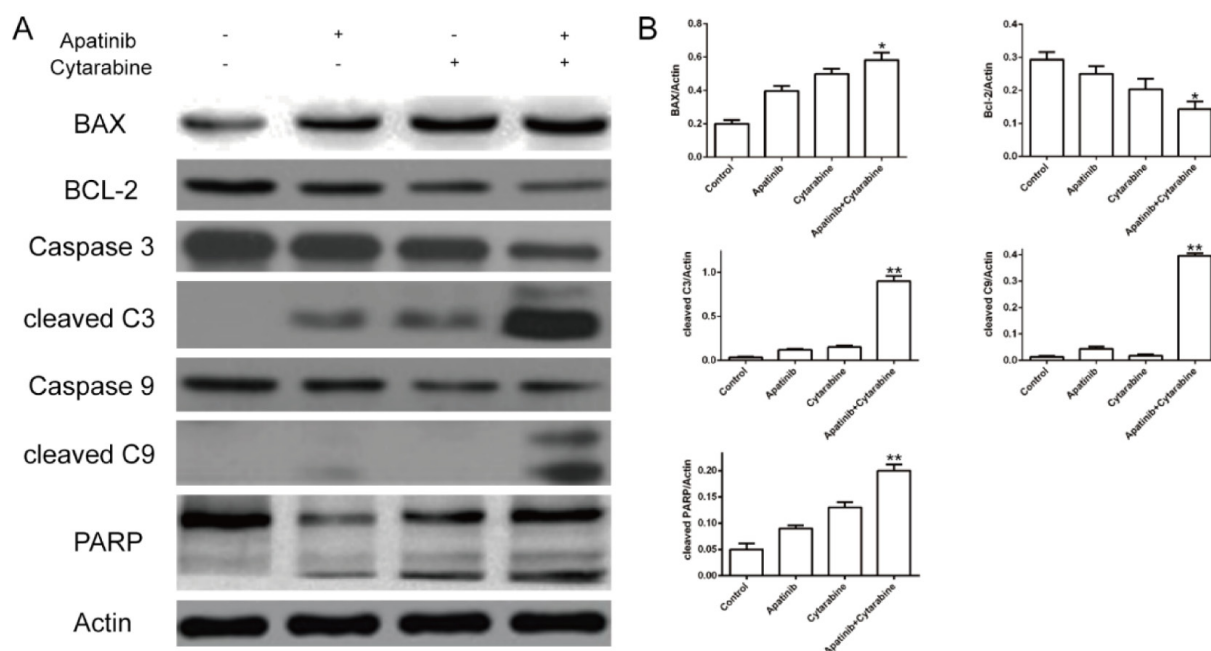
rate was 3.12% in the apatinib group, 10.8% in cytarabine group and up to 28.49% in apatinib + cytarabine group, indicating that the single application of 1  $\mu$ M apatinib has no significant inhibitory effect on apoptosis, and apatinib combined with cytarabine synergistically promotes the pro-apoptotic effect (Figure 3B).

#### *Apatinib and cytarabine synergistically destroyed the mitochondrial transmembrane potential of HL60 cells*

Apoptosis refers to a thorough, complete, active and ordered death process under the control of a variety of genes to maintain the homeostasis. There are two classical apoptotic pathways: intrinsic pathway and extrinsic pathway, also known as mitochondrial pathway and death receptor pathway. Mitochondria are closely related to apoptosis, and the loss of mitochondrial transmembrane potential and changes in mitochondrial membrane permeability are key events in the intrinsic apoptotic pathway. The effects of different treatments on the mitochondrial transmembrane potential of HL60 cells were detected using flow cytometry and DiOC6 (3) single staining to clarify whether apoptosis of HL60 cells was induced through the intrinsic pathway. The experimental results manifested that the proportion of cells with loss of mitochondrial membrane potential was obviously increased in the combination group (Figure 4), suggesting that apatinib and cytarabine synergistically destroy the



**Figure 4.** Effects of apatinib and cytarabine on mitochondrial membrane potential ( $\Delta\Psi_m$ ) in HL60 cells. **(A,B)** HL60 cells were treated with RPMI1640, 1  $\mu$ M apatinib, 60nM cytarabine or the combination of apatinib and cytarabine for 24 hrs. Evaluation of mitochondrial membrane potential ( $\Delta\Psi_m$ ) was done with Rhodamine 123 stain by flow cytometry. Data shown are representative of 3 independent experiments. Data are presented as means  $\pm$  SD (n=3). \*\*p<0.01.



**Figure 5.** Apatinib and cytarabine promoted the changes in the expression of apoptosis-related proteins in HL60 cells. **(A,B)** HL60 cells were treated with RPMI1640, 1  $\mu$ M apatinib, 60 nM cytarabine or the combination of apatinib and cytarabine for 24 hrs. Then, the expression level of target proteins (BAX, Bcl-2, procaspase-3, cleaved caspase-3, procaspase-9, cleaved caspase-9 and PARP) were measured by western blot. The results of western blot showed that in the combination group the expression of levels of cleaved caspase-3, while the Bcl-2 expression level was significantly downregulated compared with those in the single application groups. Data shown are representative of 3 independent experiments and presented as means $\pm$ SD (n=3). \* $p$ <0.05, \*\* $p$ <0.01.

mitochondrial transmembrane potential of HL60 cells and induce apoptosis of HL60 cells through the intrinsic pathway.

#### *Apatinib and cytarabine synergistically promoted the changes in expressions of apoptosis-related proteins*

Apoptosis involves various processes, such as the activation, expression and regulation of a variety of proteins, in which caspase family proteins and Bcl-2 family proteins play important roles in the signal transduction in apoptosis. The effects of apatinib combined with cytarabine on the expression levels of caspase family proteins and Bcl-2 family proteins in HL60 cells were detected via Western blotting, so as to further clarify the mechanism of combined application of them in inducing apoptosis of HL60 cells. The results revealed that in the combination group, the expression levels of cleaved caspase-3, cleaved caspase-9 and Bax were significantly up-regulated, while the Bcl-2 expression level was significantly down-regulated compared with those in the single application groups (Figure 5). The activation of caspase-9 plays an important role in the intrinsic apoptotic pathway. Activated caspase-9 activates caspase-3, the latter of which hydrolyzes and activates various important proteins in cells (including the remaining caspase components), leading to complete

suicide cytolysis. PARP is a DNA repair enzyme and caspase-3 cleavage substrate first identified in apoptosis. Loss of DNA repair function leads to the DNA rupture, thus failing to maintain the genomic stability and promoting apoptosis. The above results suggest that apatinib combined with cytarabine induces apoptosis of HL60 cells through the intrinsic pathway.

## Discussion

AML is a malignant clonal proliferative disease of myeloid hematopoietic stem cells, which is highly heterogeneous [13,14]. AML is manifested as different degrees of differentiation and maturation disorders, abnormal proliferation and inhibited apoptosis of myeloid progenitor cells, and its clinical manifestations are hematopoietic dysfunction and accompanying complications, such as anemia, bleeding, infection and organ infiltration. Currently, chemotherapy is still one of the main treatment means for leukemia [15]. Cytarabine is a kind of classical therapeutic drug for AML and a pyrimidine antimetabolite mainly acting in the S phase, which interferes in the cell proliferation through inhibiting DNA synthesis in cells. However, its side effects, such as bone marrow suppression and gastrointestinal reactions, cannot be ignored, which

often limit the increase of its clinical dose [16,17]. Apatinib, as a novel small-molecule tyrosine kinase inhibitor, selectively inhibits the phosphorylation of vascular endothelial growth factor receptor and resists tumor angiogenesis, and its anti-tumor activity in various tumors has been demonstrated in many studies. Liang et al. [18] studied and showed that apatinib can inhibit the proliferation and promote the apoptosis of leukemia HL60 cell lines. Moreover, studies have revealed that apatinib combined with a variety of anti-tumor drugs can improve the curative effect [19,20]. Considering the above studies, the inhibitory effect of apatinib combined with cytarabine on AML cells and its related mechanism were explored in this study.

As it is well known, the cell cycle, also known as the cell division cycle, refers to the whole process of continuously dividing cells from the end of mitosis to the end of the next [21]. Cell cycle regulation is an extremely complex but fine regulatory process, and there is a check point in each cycle phase, which can induce the production of cycle inhibitors when errors are detected in the cell cycle, preventing the further development of cell cycle [22]. The occurrence of tumor involves the disorder of cell cycle regulation, leading to the infinite cell proliferation. Therefore, the effects of single application of apatinib and cytarabine and the combined application of them on the cell cycle were detected via flow cytometry, and it was found that their combined application could significantly arrest HL60 cells in the G0/G1 phase. The study on related mechanism revealed that the combined application could affect the expression level of cell cycle regulatory protein, up-regulate the expressions of CDK inhibitors p21 and p27, and down-regulate the expression of cyclin D1. P21 and p27 can inhibit the phosphorylation of pRb through inhibiting the formation of CDK-cyclin complex, and make free E2F bind to unphosphorylated pRb, so that E2F transcription-dependent genes cannot transcribe, and a variety of biochemical reactions including DNA synthesis are indirectly inhibited, thereby arresting the cell cycle progression [23]. However, the mechanism of combined application

of apatinib and cytarabine in regulating cyclin D1, p21 and p27 proteins remains unclear and needs further study.

Apoptosis is a spontaneous active death process occurring after various physiological or pathological stimuli in cells, which involves the activation, expression and regulation of a variety of genes, such as the oncogene c-myc and cancer suppressor gene p53, and a series of proteases, including caspase family proteins and Bcl-2 family proteins [24]. Studies have demonstrated that apatinib can exert an anti-tumor effect through promoting apoptosis of a variety of tumor cells. Interestingly, it was found in this study that apatinib combined with cytarabine could destroy the mitochondrial transmembrane potential, and activate the Bcl-2 family proteins and the key protein caspase-9 in the intrinsic apoptotic pathway, indicating that apatinib combined with cytarabine can induce apoptosis through the intrinsic apoptotic pathway.

## Conclusions

In conclusion, this study revealed that apatinib combined with cytarabine has a synergistic anti-AML effect, whose relevant mechanism is possibly related to inducing apoptosis through the intrinsic pathway and arresting cell cycle through the regulation of cyclin, laying a foundation for further investigation of the mechanism of the drug. This study provides a new perspective for the study on AML and a certain theoretical basis for the final selection of new clinical strategies.

## Acknowledgement

This study was supported by the Development Plan of Medical and Health Science and Technology in Shandong Province (2016NS0472) and Key Research and Development Project in Shandong Province (2018GSF118203).

## Conflict of interests

The authors declare no conflict of interests.

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