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Preliminary results indicate resveratrol affects proliferation and apoptosis of leukemia cells by regulating PTEN/PI3K/AKT pathway

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Abstract. – OBJECTIVE: PTEN-PI3K/AKT signaling pathway is widely involved in the regulation of cell proliferation, cell cycle, apoptosis, and invasion. Resveratrol (Resv) is a natural botanical ingredient involved in several biological activities. It is still unclear in terms of whether Resv may exert anti-leukemia effects by regulating the PTEN-PI3K/AKT pathway. This study investigated the effect of Resv on leukemia cell proliferation and apoptosis by regulating PTEN-PI3K/AKT pathway.

PATIENTS AND METHODS: Human [illegible]-mal peripheral blood PBMC cells, and hu[illegible] acute promyelocytic leukemia (APL) cell l[illegible] NB-4 and HL-60 cells were cultur[illegible] *vitro*. R[illegible]al-time quantitative reverse tr[illegible]n poly[illegible]merase chain reaction (qRT[illegible]R) wa[illegible]used to detect Phosphatase and t[illegible]n homo[illegible]g (PTEN) mRNA expression. Weste[illegible]t wa[illegible] test PTEN protein ex[illegible]ssio[illegible]0 and [illegible] cells were treated [illegible]0, 5, 10, [illegible]20 μM Resv, respectively. Cel[illegible]liferation w[illegible]nalyzed by cell counting [illegible]K-8) assay. [illegible]level of caspase-3 w[illegible]meas[illegible]d by Weste[illegible] blot. HL-60 cells w[illegible]re divided i[illegible]ontrol group, 20 μM Resv tr[illegible]ent group, an[illegible]sv+PTEN inhibitor SF167[illegible]roup. Cell apoptosi[illegible]as determined by flo[illegible]tomet[illegible]Cell proliferation was assessed b[illegible]I sta[illegible]g.

R[illegible] Compa[illegible]d with peripheral blood mono[illegible]ar cell ([illegible]C), PTEN mRNA and pro-[illegible]n leve[illegible]re[illegible]nificantly decreased in NB-4 [illegible] HL-60[illegible]Resv significantly inhibited the [illegible]liferation[illegible]ivity in HL-60 and NB-4 cells, and [illegible]d the activity of caspase-3. Resv treat-[illegible]egulated the expression of PTEN and reduced the expression of p-AKT protein in HL-60 cells. However, Resv treatment markedly sup-[illegible]essed the proliferation of HL-60 and induced [illegible]poptosis. SF1670 treatment in the presence of Resv significantly antagonized the down-regulation of p-AKT protein expression induced by Resv, resulting in decreased apoptosis and enhanced cell proliferation.

CONCLUS[illegible]S:[illegible]v can up-re[illegible]ate PTEN expression a[illegible]d inhib[illegible]e activity of PI3K/AKT pathway to play an anti[illegible]kemia effect through supp[illegible] cell prolif[illegible]n and inducing ap[illegible]sis.

Ke[illegible]Words:
[illegible] PTEN, PI3[illegible]KT, Leukemia, Apoptosis, Prolif-erat[illegible]

Introduction

Leukemia is a group of heterogeneous hematopoietic stem cell malignant clonal diseases caused by the differentiation blockage, apoptosis suppression, and malignant proliferation of hematopoietic stem/progenitor cell at different stages. Among them, acute myeloid leukemia ((AML) is a class of malignant leukemia that originates in myeloid hematopoietic cells[1,2].

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB, protein kinase B) signaling pathway is widely expressed in a variety of cells and participates in the regulation of biological processes, such as cell growth, survival, and apoptosis[3]. It plays a role in promoting a variety of activities related to tumors[4-6]. Abnormal PI3K/AKT signaling pathway plays a key regulatory role in the development, progression, and prognosis of leukemia, and has become a therapeutic target for multiple anti-leukemia drugs[7-9]. The phosphatase and tensin homologue deleted on chromosome ten (PTEN) is the only tumor suppressor gene with the dual activity of protein esterase and phosphatase to date, which can negatively regulate the activity of PI3K/AKT signaling pathway[10]. It plays a tumor suppressor role in the occurrence, progression, metastasis, and drug resistance of vari-

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ous tumors, such as breast cancer[11], lung cancer[12], esophageal cancer[13], and leukemia[14,15], etc.

Resveratrol (Resv) is a natural botanical ingredient with many biological activities[16-19]. It was shown that Resv regulates the expression and function of PTEN through a variety of mechanisms. However, it is still unclear whether Resv may exert anti-leukemia effects by regulating PTEN-PI3K/AKT pathway[20-22]. This study investigated the effect of Resv on leukemia cell proliferation and apoptosis.

Materials and Methods

Main Reagents and Materials

Human normal peripheral blood mononuclear cells (PBMCs) were purchased from Beijing Beina biological Co. (Beijing, China). Leukemia HL-60 cells were purchased from Nanjing Kezhen biological Co. (Nanjing, China). Leukemia NB-4 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640), Opti-MEM, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). RNA extraction reagent TRIzol was purchased from Invitrogen (Beijing, China). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen Sciences (Germantown, MD, USA). Rabbit anti-human polyclonal PTEN and β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-human p-AKT monoclonal antibody was purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Goat anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). CCK-8, Annexin V-FITC/PI apoptosis kit, and BCA protein quantification kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). EdU Flow Cytometry Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Resv was purchased from Tocris Cookson Chemicals (Houston, TX, United States).

This study has been approved by the Ethical Committee of Affiliated Hospital of Weifang Medical College (Shandong, China). All participants have acknowledged this research and have signed informed consents.

Cell Culture

PBMC, HL-60, and NB-4 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) at 5% CO_2 and 37°C. The cells in the log phase were subjected to experiments.

CCK-8 Assay

HL-60 and NB-4 cells were seeded in 96-well plate at 10000/well and treated by different concentrations (0, 5, 10, 20 μM) of Resv for 48 h. Next, the cells were added with Cell Counting Kit-8 (CCK-8) to measure the absorbance (A450). Relative viability = (A450 in drug group – A450 in blank group)/(A450 in control – A450 in blank group) ×100%.

Flow Cytometry Detection of HL-60 Cell Proliferation

Cell proliferation was tested by EdU Flow Cytometry Kit. The cells were resuspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS). After been incubated in 10 μM EdU at 37°C for 2 h, the cells were seeded in 6-well plate and divided into three groups, including control group without special treatment, 20 μM Resv group that was treated by 20 μM Resv, and Resv + PTEN inhibitor SF1670 group that was treated by 20 μM Resv and 1 μM SF1670. Post incubation for 48 h, the cells were fixed in paraformaldehyde. The cells were then incubated in 100 μL TritonX-100 at room temperature and in 500 μL reaction fluid at room temperature under dark for 30 min. At last, the cell was tested on FC500MCL flow cytometry.

Cell Apoptosis Detection

The cells were digested by enzyme and collected followed by being resuspended in 100 μl binding buffer. After that, 5 μl Annexin V-FITC kit and 5 μl PI were added into cells and incubated at room temperature under dark for 15 min. Then the cells were tested on flow cytometry.

qRT-PCR

Total RNA was extracted using TRIzol. QuantiTect SYBR Green RT-PCR Kit was used for qRT-PCR. The PCR reaction system was composed of 10.0 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL forward primer (0.5 μmol/L), 1.0 μL reverse primer (0.5 μmol/L), 2.0 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O. The reaction was performed on Bio-Rad CFX96 at 45°C for 5 min and 94°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The primer sequences were: PTEN-F-5'-TG-

GGCCCTGTACCATCCCAAGT-3'; PTEN-R-5'-TGTGGCAACCACAGCCATC-GT-3'; GAPDH-F-5'-CATGAGAAGTATGA-CAACAGCCT-3'; GAPDH-R-5'-AGTCCTTC-CACGATACCAAAG-3'.

Western Blot

Total protein was extracted from the cells by radioimmunoprecipitation assay buffer (RIPA buffer). After quantified by bicinchoninic acid assay (BCA assay), a total of 40 μg protein was separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 250 mA for 100 min. After blocked by 5% skim milk at room temperature, the membrane was incubated in primary antibody at 4°C overnight (PTEN, p-AKT, and β-actin at 1:1500, 1:800, and 1:10000, respectively). The membrane was washed by Phosphate-Buffered Saline with Tween 20 (PBST), and was then incubated in horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for 60 min (1:10000). At last, the membrane was treated by ECL (enhanced chemiluminescence) reagent developed.

Statistical Analysis

SPSS 18.0 software (SPSS in[illegible]icago, IL, USA) was applied for data anal[illegible]. Measurement data was presented as mean ± standard deviation and compared by the Student *t*-test [illegible] way ANOVA with Bonferroni as [illegible] analysis. $P < 0.05$ was depicted as significant difference.

Results

Decreased PTEN Expression in Leukemia HL-60 and NB-4 Cells

qRT-PCR showed that the expression of PTEN mRNA in leukemia HL-60 and NB-4 cells was significantly lower than that in healthy human PBMC (Figure 1A). Western blot demonstrated that the expression of PTEN protein in HL-60 and NB-4 cells was obviously lower than that of PBMC (Figure 1B).

Resv Suppressed HL-60 and NB-4 Cell Proliferation

CCK-8 exhibited that compared with the control group, the proliferation activity of HL-60 (Figure 2A) and NB-4 cells (Figure 2B) in Resv treatment group was markedly reduced in a dose-dependent manner. As revealed that compared with the control group, the activity of caspase-3 in HL-60 (Figure 2C) and NB-4 cells (Figure 2D) of Resv treatment group was significantly enhanced in a dose-dependent manner.

Resv Upregulated PTEN Expression in HL-60 Cells

qRT-PCR showed that the expression of PTEN mRNA in HL-60 cells of Resv treatment group was significantly higher than that of the control group in a dose-dependent manner (Figure 3A). Western blot observed that compared with the control group, the expression of PTEN protein in HL-60 cells was significantly upregulated, while the expression of p-AKT protein was signifi-

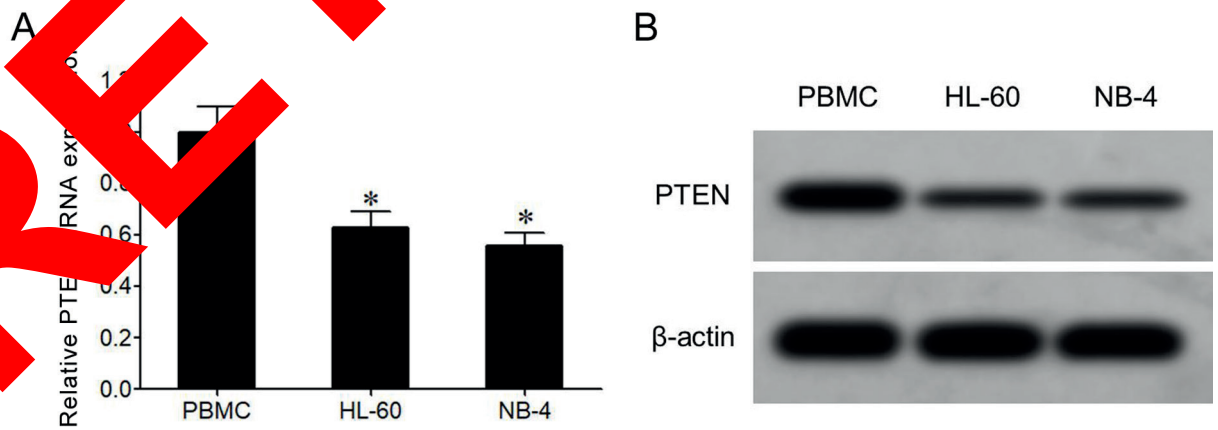

Figure 1. PTEN expression decreased in leukemia HL-60 and NB-4 cells. ***(A)*** qRT-PCR detection of PTEN mRNA expression; ***(B)*** Western blot detection of PTEN protein expression. *$p < 0.05$, compared with PBMC.

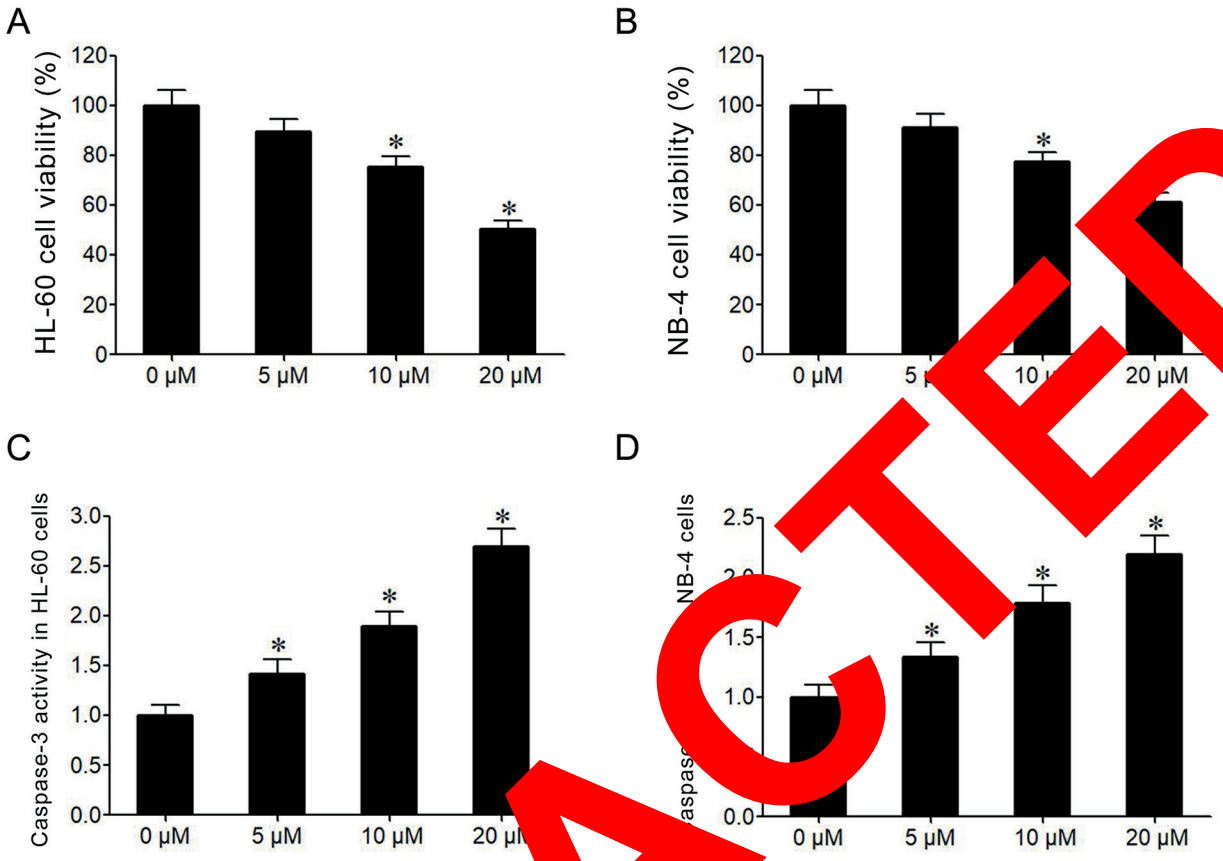

Figure 2. Resv suppressed HL-60 and NB-4 cell proli... ***(B)*** CCK-8 detection of HL-60 cell proliferation; ***(B)*** CCK-8 detection of NB-4 cell proliferation; ***(C)*** ...-3 activi... HL-60 cells; ***(D)*** caspase-3 activity in NB-4 cells. *$p < 0.05$, compared with 0 μM.

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Resv Inhibited HL-... Cell Proliferation and Induced Apopto... via Regulating PTEN/PI3K/AKT Signali... Pathway

... PCR f... d that the expression of PTEN m... A in H... 60 cells was significantly elevated i... μM Res... eatment group compared with th... ntrol g... p. SF1670 treatment did not ...ect the ... on of PTEN mRNA (Figure ...). Wester... t showed that compared with the ...trol group, the expression of PTEN protein w... ...icantly upregulated, whereas the expression of p-AKT protein was markedly declined in HL-60 cells of Resv treatment group. SF1670 ... not affect PTEN protein expression but significantly enhanced p-AKT protein expression (Figure 4B). Flow cytometry exhibited that 20 μM Resv significantly induced HL-60 cell apoptosis, while SF1670 apparently antagonized the apoptosis-inducing effect of Resv on HL-60 cells (Figure 4C). EdU staining observed that HL-60 cell proliferation in the 20 μM Resv treatment group was significantly weakened compared with the control group, and SF1670 significantly antagonized the inhibitory effect of Resv on HL-60 cell proliferation (Figure 4D).

Discussion

Under the stimulation of growth factors, mitogens, and other factors, PI3K can be activated after conformational changes. Once PI3K/AKT signaling pathway is activated, PI3K can catalyze its substrate phosphatidylinositol 4,5-diphosphate (phosphatidylinositol). 4,5-trisphosphate, (PIP2) phosphorylation to produces phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits Ser/Thr protein kinase AKT from the cytoplasm to the membrane and phosphorylates AKT at Ser473 and

Thr308 under the action of phosphoinositide-dependent protein kinase (PDK). Phosphorylated AKT continues to transmit signals downstream to regulate the expression and function of proteins that play a key role in cell proliferation, cell cycle, and apoptosis. Over-activation of PI3K/AKT signaling pathway promotes cell proliferation, migration, invasion, and apoptosis, which is closely related to the development of ovarian cancer[4], gastric cancer[5], breast cancer[6], and other tumors.

Resv is a natural botanical ingredient with many biological activities. It was found that Resv has a variety of biological activities and pharmacological effects in regulating lipid metabolism, inhibiting platelet aggregation, anti-inflammation, anti-tumor, and anti-leukemia[16-19]. Moreover, several studies have shown that Resv can regulate the expression and function of PTEN through a variety of mechanisms. However, there have been very few studies[20-22] about whether Resv plays a role in anti-leukemia by regulating PTEN-PI3K/AKT pathway. This study investigated the effect of Resv on leukemia cell proliferation and apoptosis as well as on PTEN-PI3K/AKT pathway.

This study showed that compared with h normal PBMC cells, PTEN mRNA and p levels in leukemia HL-60 and NB-4 cells v significantly decreased, indicating that the duced expression of the tumor sor ge PTEN may play a role in prom genesis of leukemia. Yao et al [23] fou hat PT expression was downregulated i kemi lls. Wang et al [24] observed t tu PTEN expression signific reduced in leukemia HL-60 In this stud decrease in the expres EN was ass ted with leukemia, which was similar to the results of Yao et al[23] and Wang et al[24]. CCK-8 assay revealed that different concentrations of Resv significantly inhibited the proliferation and enhanced caspase-3 activity of HL-60 and NB-4 cells dose dependence. Fan et al[25] reported th esv obviously induced leukemia NB-4 cel optosis and inhibited cell proliferation. Su et a mon-strated that Resv treatment mark ly sup ed the proliferation activity of H 0 cells. F al[27] reported that Resv pro ed leu mia H 60 cell autophagy throu KB1-A K-mTOR pathway and induced cell a ia upreg at-ing Bax/Bcl-2 ratio activ caspase nd caspase-3. Cakir al[28] exhibit at v ap-parently restr -60 cell pr tion and promoted ce popt ith dose dependence. In this study, Resv signi ly inhibited leukemia cell p ion, induced ptosis, and showed ant kemia effects, which was consistent with pr ous studies conducted by Su et al [26], Fan et al nd Cakir et .

resent, nu rous studies found that anti-le ia dr can play their roles by regulating ao et al[23] observed that PTEN pression level was significantly reduced in leu-B-4 cells. The administration of EGCG d ATRA could inhibit the activity of AKT by upregulating PTEN expression to promote cell myeloid differentiation and attenuate malignancy. Wang et al[24] showed that the decrease of PTEN expression was related to the malignant biological characteristics of leukemia HL-60 cells. The administration of carnosic acid (CA) significantly upregulated PTEN expression, inhibited AKT and ERK2 phosphorylation activities, promoted

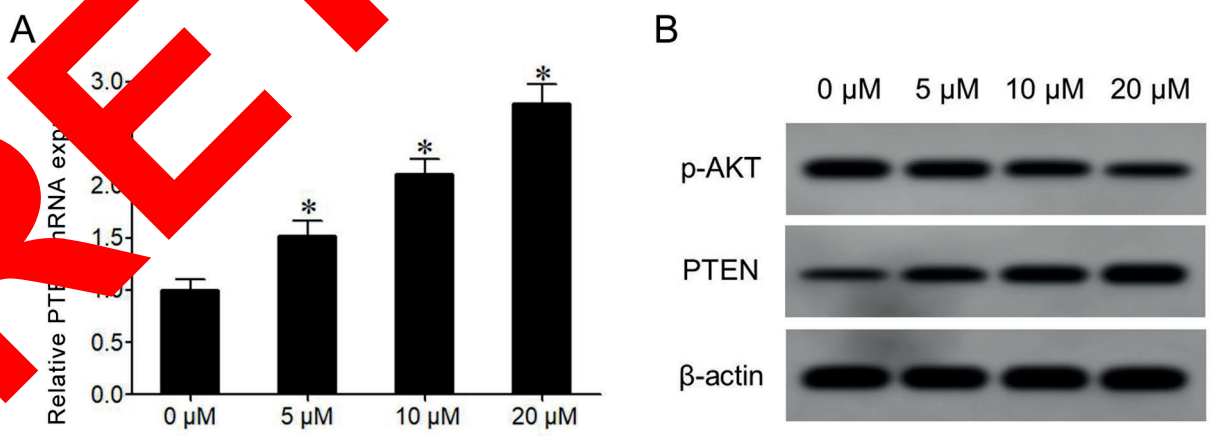
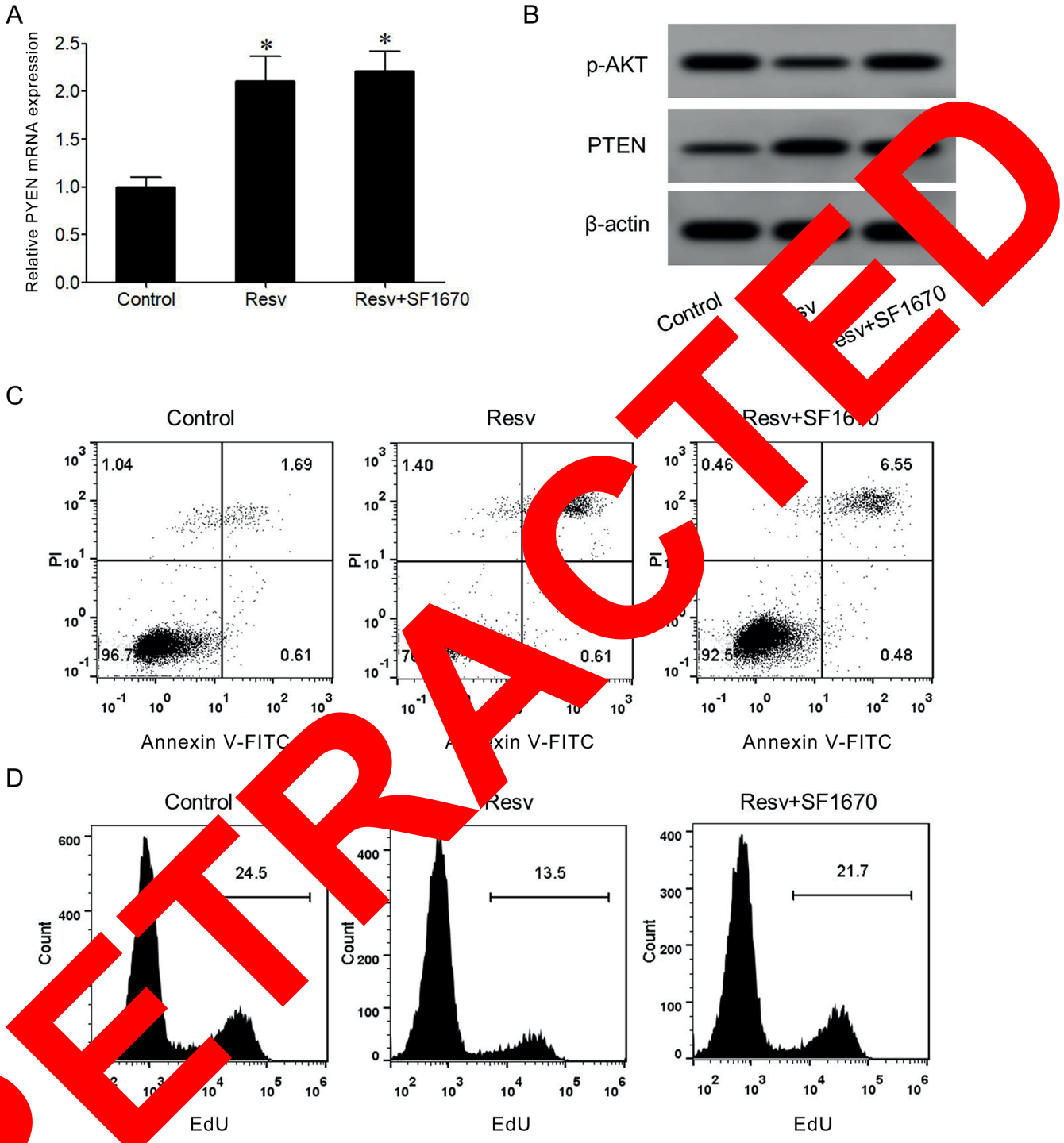


Figure 3. Resv upregulated PTEN expression in HL-60 cells. ***(A)*** qRT-PCR detection of PTEN mRNA expression in HL-60 cells; ***(B)*** Western blot detection of PTEN and p-AKT protein expressions in HL-60 cells. *$p < 0.05$, compared with 0 μM.



Resv inhibited HL-60 cell proliferation and induced apoptosis via regulating PTEN-PI3K/AKT signaling pathway. ***(A)*** qPCR detection of PTEN mRNA expression in HL-60 cells; ***(B)*** Western blot detection of PTEN and p-AKT protein expressions in HL-60 cells; ***(C)*** flow cytometry detection of cell apoptosis; ***(D)*** flow cytometry detection of cell proliferation. $^{*}p < 0.05$, compared with Control.

cell apoptosis, inhibited cell proliferation, and arrested cell cycle. Cellai et al[29] reported that the decrease of PTEN expression was related to the malignant characteristics of leukemia NB-4 cells. Treatment with WEB-2170 significantly up-regulated the expression of PTEN, inhibited the phos-

phorylation activity of AKT and ERK2, inhibited cell proliferation, and induced cell apoptosis. This study focused on investigating whether Resv may exert anti-leukemia effects by regulating PTEN. It was found that Resv clearly upregulated PTEN mRNA and protein levels, and suppressed p-AKT protein expression in HL-60 cells, indicating that Resv inhibited PI3K/AKT pathway activity by enhancing PTEN to exert an anti-leukemia effect. Furthermore, it was showed that treatment with PTEN inhibitor SF1670 on the basis of Resv can antagonize the down-regulation of p-AKT protein, enhance cell proliferation and decrease apoptosis induced by Resv, thus attenuating the anti-leukemia effect of Resv. It was suggested that the anti-leukemia effect of Resv is exerted through the PTEN-PI3K/AKT pathway. Jing et al[20] found that Resv can significantly upregulate the expression and function of PTEN, inhibit the activity of PI3K/AKT pathway, arrest cell cycle arrest, restrained cell proliferation, and attenuated cell malignancy of gastric cancer MGC803 cells. Dhar et al [21] revealed that Resv exerts anti-prostate cancer function by increasing PTEN expression, inhibiting AKT activity, reducing prostate c cell proliferation, and suppressing tumorige in animals. This study revealed that Resv in ited cell proliferation and promoted apoptosis regulating the activity of the P I3K/AK pathway. However, whether R pla role i regulating PTEN-PI3K/AK pathwa activity and anti-leukemia effect still un- clear and requires furt in

usions

We re ed that Resv up-regulate PTEN expre and inhibit the a ty of PI3K/AKT path y to pl an anti-leukemia effect by sup- p g ce oliferation and inducing apoptosis.

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authors de no conflicts of interest.

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