Puerarin suppresses cell growth and migration in HPV-positive cervical cancer cells by inhibiting the PI3K/mTOR signaling pathway

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Abstract. Puerarin is an effective component that is present in high concentrations in the *Pueraria lobata* plant and is extensively distributed throughout nature. Puerarin possesses a number of pharmacological effects and has strong pharmacological activity with few side effects and extensive clinical applications. The aim of the present study was to explore the effects of Puerarin on the apoptosis of human papillomavirus (HPV)-positive cervical cancer cells and the underlying molecular mechanisms. MTT assay, lactate dehydrogenase activity and Annexin V/fluorescein isothiocyanate/propidium iodide analysis were used to analyze cell growth of HPV-positive HeLa cervical cancer cells treated with Puerarin. Western blotting was performed to measure protein expression in the treated cells. Puerarin significantly reduced cell proliferation and induced apoptosis in HeLa cells. In addition, it was observed that Puerarin significantly enhanced caspase-3/9 activities and significantly increased B-cell lymphoma 2-asscoiate X protein expression in HeLa cells. Puerarin suppressed phosphatidylinositol-3 kinase (PI3K), phosphorylated (p)-protein kinase B (Akt) and p-mammalian target of rapamycin (mTOR) protein expression in HeLa cells. These results indicate that Puerarin induces apoptosis in HPV-positive HeLa cervical cancer cells via inhibiting PI3K/Akt/mTOR signaling.

Introduction

Cervical cancer is the second most common type of malignant tumor in females worldwide and the most common in a number of developing countries, representing a large socioeconomic burden (1). The International Agency for Research on Cancer reported that ~300,000 mortalities are attributed to cervical cancer annually in China (2,3).

Human papillomavirus (HPV)-18 is regarded as an important strain of HPV that causes precancerous lesions, which subsequently develop into cervical cancer and is associated with >90% of all cervical cancer cases (4). The prevalence of HPV-18 infection in women continues to increase globally; however, in the majority of cases infection does not progress to the disease stage (4). It is therefore important to determine factors aside from infection that lead to carcinogenesis and the progression of cervical cancer (5).

Recent studies have revealed that the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) signal transduction pathway (6) serves a key role in a number of cellular activities, including apoptosis, aging and proliferation (7). The PI3K/Akt signal transduction pathway responds to various intracellular and extracellular survival pressures to adaptively regulate these processes (7). A previous study demonstrated that ionizing radiation, ultraviolet rays and cytotoxic drugs may activate the PI3K/Akt signal transduction pathway (8).

Mammalian target of rapamycin (mTOR) is a type of serine/threonine protein kinase that is highly evolutionarily conserved and is observed in a wide variety of organisms (9). The mTOR signaling pathway serves an important role in the growth and proliferation of normal cells, however it is also closely associated with the growth, proliferation, differentiation, apoptosis and metabolism of a number of types of malignant tumor (10). mTOR expression has been reported to be upregulated in multiple tumors, including breast cancer, colon cancer and lymphoma (10).

Puerarin is an isoflavonoid monomer that may be isolated and extracted from the leguminous plant *Pueraria lobate* (11). Puerarin has strong pharmacological activity (12) and a wide range of pharmacological effects, including anti-arrhythmia, anti-myocardial infarction, anti-angiectasis, antitumor, microcirculation improvement, blood fat reduction, increased cerebral blood flow, protection against oxidation, regulation of bone metabolism and reduction of intraocular pressure (11,12). Huang *et al* (13) demonstrated that Puerarin induces cell

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apoptosis in human chondrosarcoma cells via inhibiting the PI3K/Akt signaling pathway. The aim of the present study was to explore the effects of Puerarin on apoptosis in HPV-positive cervical cancer cells and the molecular mechanisms responsible.

Materials and methods

Cell culture. A HeLa HPV-18-positive cervical cancer cell line was obtained from the American Type Culture Collection (cat. no. CRM-CCL-2; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mM/L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in a humidified atmosphere containing 5% CO_2.

MTT assay and lactate dehydrogenase (LDH) activity. HeLa cells were seeded at a density of $5x10^3$ cells/well in 96-well plates and incubated overnight at 37˚C. The cells were treated with 0, 0.25, 0.50, 1.00 or 2.00 mM Puerarin (Sigma-Aldrich; Merck KGaA) for 24, 48 and 72 h at 37˚C as previously described (14). A total of 20 μl MTT solution (Sigma-Aldrich; Merck KGaA) was added to each well and the cells were incubated for a further 4 h. A total of 150 μl dimethyl sulfoxide was added to each well to dissolve the purple formazan and the absorption was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 492 nm.

HeLa cells were seeded in 6-well plates at a density of $5x10^6$ cells/well and were treated with 0.50, 1.00 or 2.00 mM Puerarin for 48 h at 37˚C. The LDH activity level was subsequently measured using an LDH activity kit (C0016; Beyotime Institute of Biotechnology, Haimen, China) and the absorption was measured with a microplate reader at 450 nm.

Annexin V/fluorescein isothiocyanate/propidium iodide (PI) analysis. HeLa cells were seeded in 6-well plates overnight ($5x10^6$ cells/well) at 37˚C. The cells were treated with 0.50, 1.00 or 2.00 mM Puerarin for 48 h at 37˚C, fixed with 4% paraformaldehyde for 15 min at room temperature, stained with Annexin V/phycoerythrin and PI (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min and analyzed using flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Migration assay. HeLa cells ($1x10^5$) were seeded in the upper chamber of 24-well plates with Transwell inserts (pore size, 8 μm; Millipore; Merck KGaA) containing DMEM. The lower chamber contained DMEM supplemented with 10% FBS. Following 48 h, cells from the lower surface of the inserts were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 1% crystal violet for 30 min at room temperature. Migrating cells were visualized using a light microscope (magnification, x100).

Measuring caspase-3/9 activity and a DAPI assay. Total protein was extracted from cells using a radioimmunoprecipitation buffer (Sigma-Aldrich; Merck KGaA). A total of 10 μg protein was incubated with caspase-3 and caspase-9 activity kits (C1115 and C1158; Beyotime Institute of Biotechnology) for 2 h at 37˚C, according to the manufacturer's instructions. The absorption was measured using a plate reader at 405 nm.

HeLa cells ($5x10^6$ cells/well) were seeded in 6-well plates and incubated overnight at 37˚C. The cells were then treated with 0.50, 1.00 or 2.00 mM Puerarin for 48 h at 37˚C, washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and stained with DAPI (5 mg/ml) for 30 min in darkness at room temperature. Cells were observed using a fluorescence microscope (magnification, x100).

Western blotting. Total protein was extracted from the cells as described above. The proteins (50 μg) were separated using 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBST for 1 h at 37˚C and incubated with primary antibodies against Bax (sc-6236; 1:10,000), PI3K (sc-293172; 1:2,000), phosphorylated (p)-Akt (sc-7985-R; 1:1,000), Akt (sc-135829; 1:1,000), p-mTOR (sc-293133; 1:1,000), mTOR (sc-1549; 1:1,000), and GADPH (sc-47724; 1:1,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. The membranes were subsequently incubated with goat anti-rabbit IgG-horseradish peroxidase secondary antibodies (sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h and developed using an enhanced chemiluminescent detection system (Beyotime Institute of Biotechnology). The protein bands were scanned using a Fujifilm LAS-3000 Imaging system (Fujifilm Corporation, Tokyo, Japan) and analyzed with Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Cell transfection. The PI3K plasmid was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). HeLa cells were transfected with 100 nM PI3K plasmid using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. At 4 h following transfection, medium was replaced and supplemented with 0.50, 1.00 or 2.00 mM Puerarin and cells were cultured for 44 h at 37˚C.

Statistical analysis. Data are expressed as the mean ± standard deviation and are representative of three replicates. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used in data analyses. Comparisons were made using one-way analysis of variance, with a Bonferroni post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Puerarin reduces cell proliferation in HeLa cells. It was investigated whether Puerarin (Fig. 1A) exerted any effects on the proliferation of HeLa cells. Puerarin inhibited the cell proliferation of HeLa cells in a dose- and time-dependent manner. Treatment with 0.5 mM Puerarin significantly inhibited the proliferation of HeLa cells compared with the control group at 72 h, however treatment with 1-4 mM Puerarin significantly inhibited the cell proliferation at all time points (Fig. 1B). In addition, treatment with 1 or 2 mM Puerarin significantly increased the LDH activity (Fig. 1C) and significantly reduced the migration rate of HeLa cells compared with the control group (Fig. 1D and E).

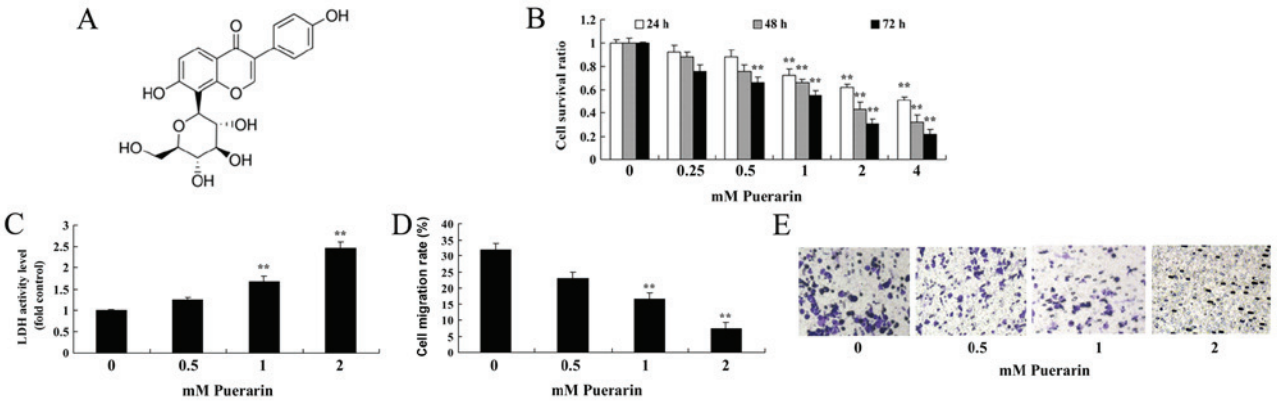

Figure 1. Puerarin reduces cell proliferation in HeLa cells. (A) Chemical structure of Puerarin. (B) Cell survival, (C) LDH activity and (D) migration rate in HeLa cells. (E) HeLa cells following treatment with 0, 0.5 1 or 2 mM Puerarin for 48 h (magnification, x100). **P<0.01 vs. control group. LDH, lactate dehydrogenase.

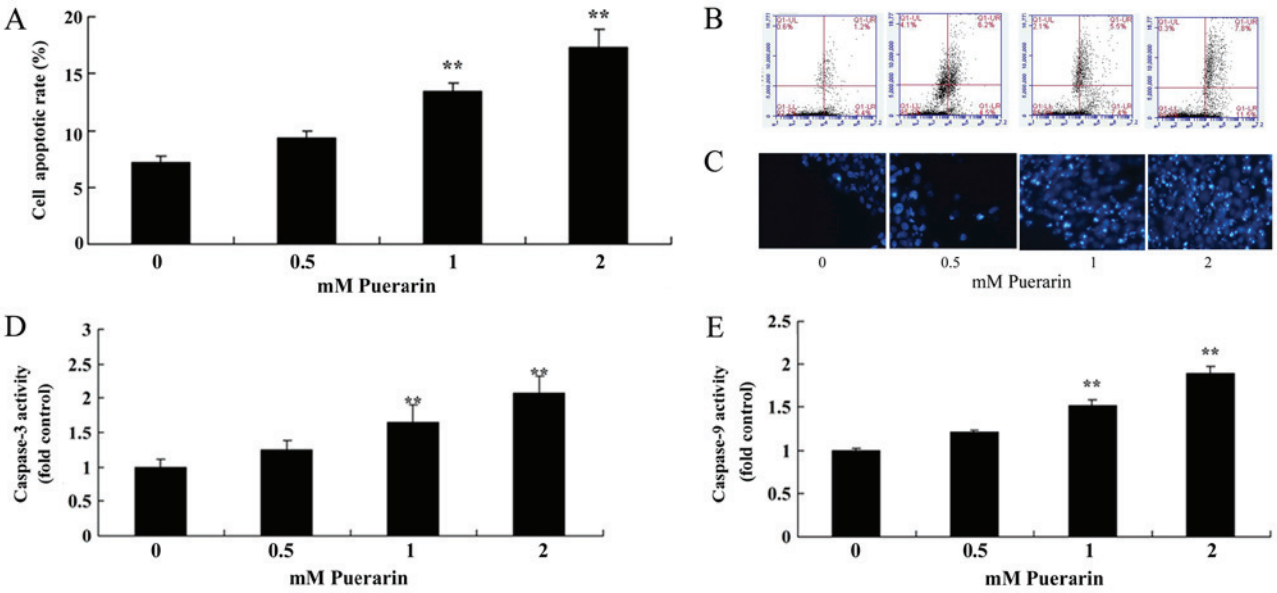

Figure 2. Puerarin induces apoptosis in HeLa cells. (A) Apoptosis rate was determined by (B) flow cytometry. (C) DAPI assay performed on HeLa cells following treatment with 0, 0.5 1 or 2 mM for 48 h (magnification, x100). (D) Caspase-3 and (E) Caspase-9 activity. **P<0.01 vs. the control group.

Puerarin induces apoptosis in HeLa cells. Flow cytometry analysis was performed to determine whether Puerarin affects the apoptosis of HeLa cells. The apoptosis rate in cells treated with Puerarin (1-2 mM) was significantly increased compared with the control group (Fig. 2A). In addition, Puerarin (1-2 mM) significantly increased Ccaspase-3 and -9 activity in HeLa cells compared with the control group (Fig. 2D and E).

Puerarin suppresses PI3K, p-Akt and p-mTOR protein expression in HeLa cells. The results of western blotting demonstrated that treatment with Puerarin (1-2 mM) significantly reduced PI3K (Fig. 3A), p-Akt (Fig. 3B) and p-mTOR (Fig. 3C) protein expression and significantly increased Bax (Fig. 3D) protein expression in HPV-18 positive HeLa cells compared with the control group (Fig. 3E).

An increase in PI3K reduces the anticancer effect of Puerarin in HeLa cells. The role of PI3K/p-Akt/p-mTOR signaling in the anticancer effects of Puerarin was further investigated. Transfection with the PI3K plasmid significantly increased the protein expression of PI3K (Fig. 4A), p-Akt (Fig. 4B) and p-mTOR (Fig. 4C), while Bax (Fig. 4D) expression was significantly reduced compared with the Puerarin treated group (Fig. 4E). It was also revealed that transfection with PI3K plasmids significantly ameliorated the effects of Puerarin on cell viability (Fig. 5A) and also significantly decreased the LDH activity compared with the Puerarin group (Fig. 5B). PI3K plasmid transfection significantly decreased the migration rate of HeLa cells compared with the Puerarin group (Fig. 5C and D). It was also demonstrated that PI3K transfection significantly decreased the anticancer effect of Puerarin on the rate of apoptosis and caspase-3/9 activities in HeLa cells compared with the Puerarin group (Fig. 6).

Discussion

Cervical cancer associated with HPV-infection is a potentially preventable malignancy, as a vaccination for HPV is currently available and undergoing further development. In addition, if identified at an early stage cervical cancer is readily

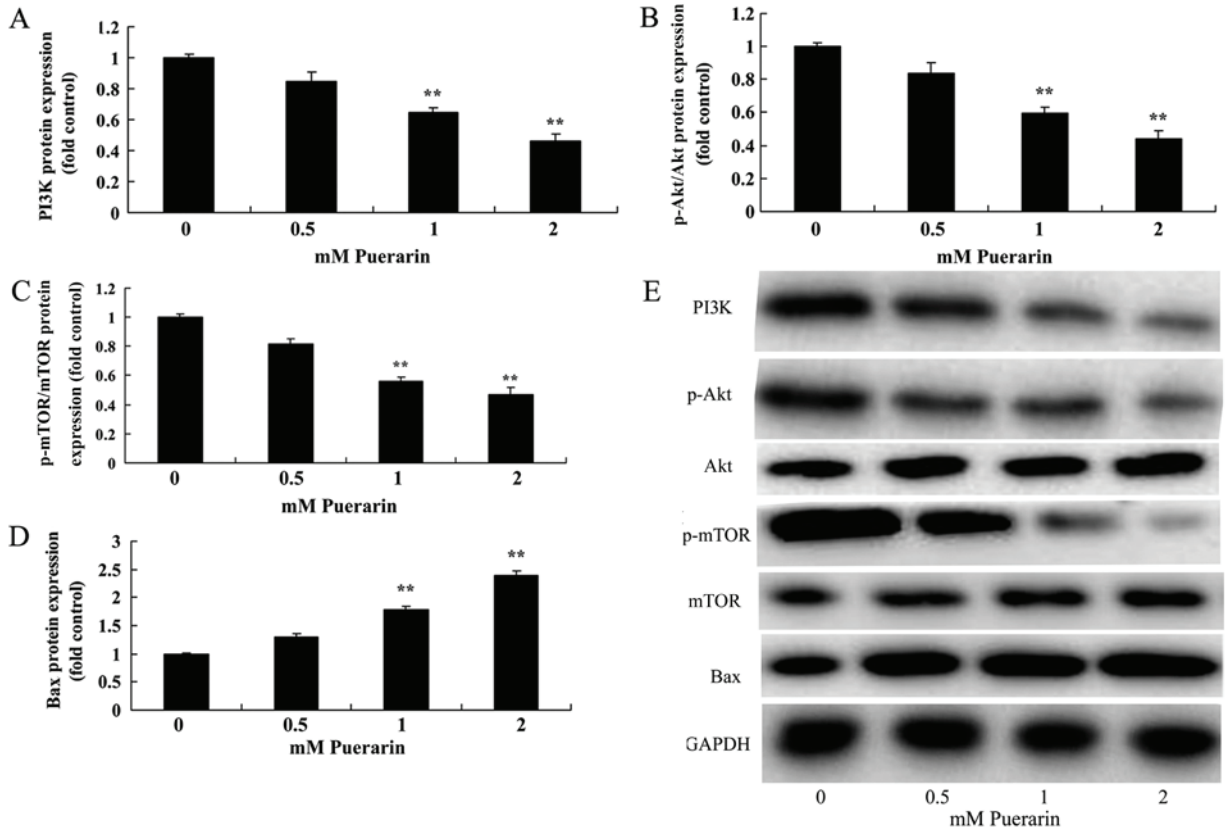

Figure 3. Puerarin suppresses PI3K, p-Akt and p-mTOR protein expression in HeLa cells. (A) PI3K, (B) p-Akt, (C) p-mTOR and (D) Bax protein expression was determined using (E) western blotting in HeLa cells. **P<0.01 vs. control group. PI3K, phosphatidylinositol-3 kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; p, phosphorylated.

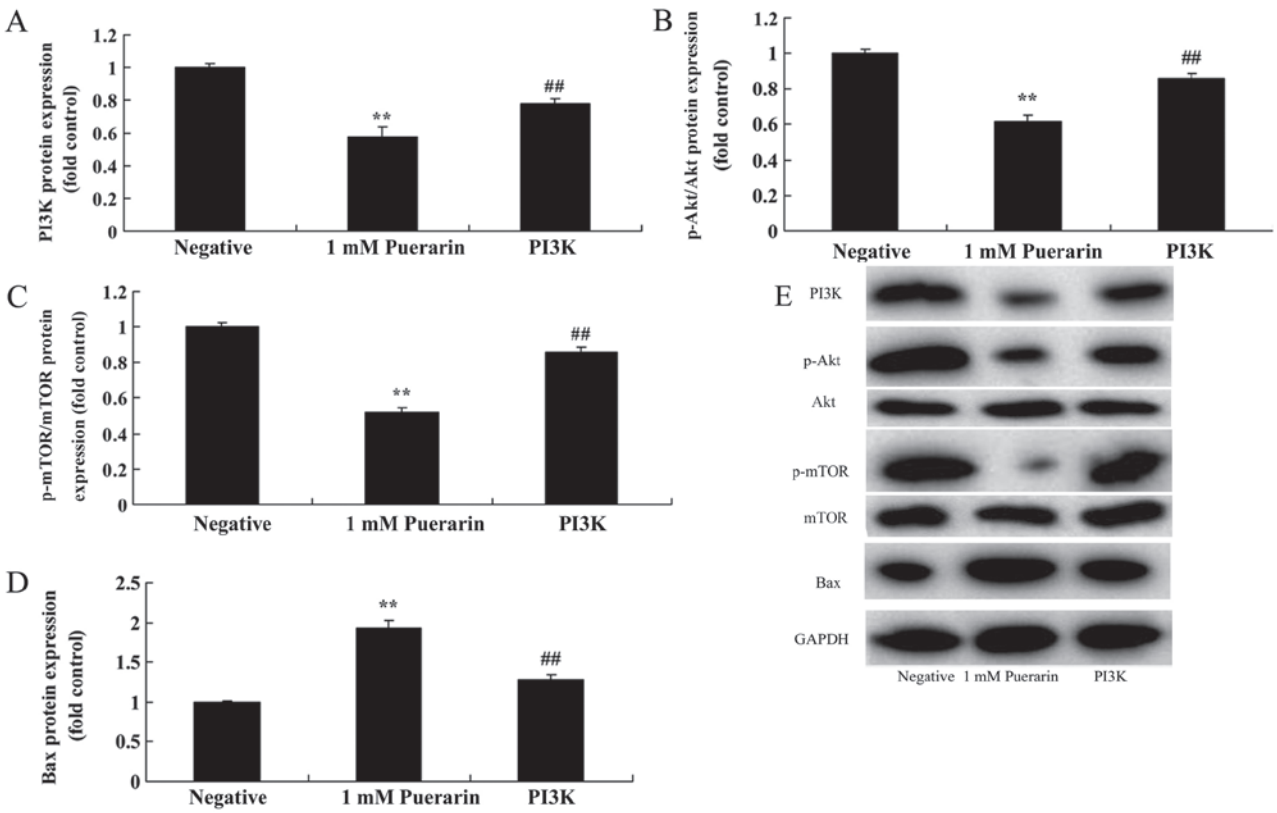

Figure 4. Transfection with PI3K plasmids reduces the anticancer effect of Puerarin in HeLa cells. (A) PI3K, (B) p-Akt, (C) p-mTOR and (D) Bax protein expression was determined using (E) western blotting in HeLa cells. **P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. 1 nM Puerarin group. PI3K, phosphatidylinositol-3 kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; p, phosphorylated.

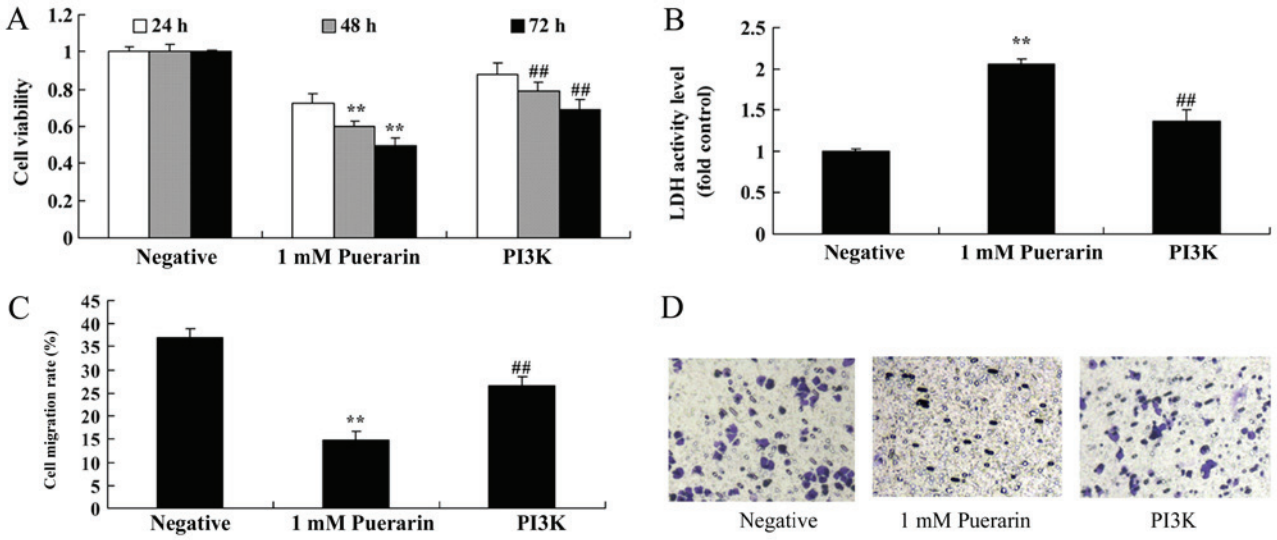

Figure 5. Transfection with PI3K plasmids reduces the effect of Puerarin on HeLa cell growth. (A) Cell viability, (B) LDH activity and (C) migration rate were assessed; (D) HeLa cells were assessed using DAPI by microscopy (magnification, x100). **P<0.01 vs. control group. ##P<0.01 vs. 1 nM Puerarin group. PI3K, phosphatidylinositol-3 kinase; LDH, lactate dehydrogenase.

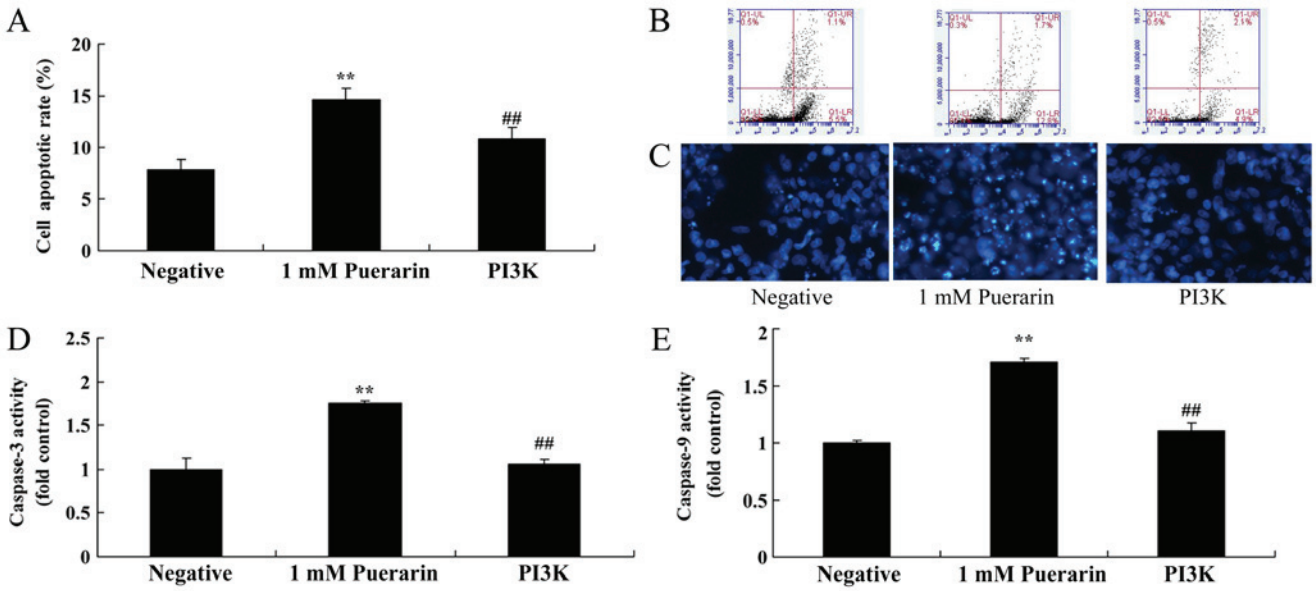

Figure 6. Transfection with PI3K plasmids reduces the effect of Puerarin on the apoptosis rate in HeLa cells. (A) Apoptosis rate was determined by (B) flow cytometry. (C) A DAPI assay was performed (magnification, x100). (D) Caspase-3 and (E) caspase-9 activity were assessed. **P<0.01 vs. the control group. ##P<0.01 vs. the 1 nM Puerarin group. PI3K, phosphatidylinositol-3 kinase.

curable (15). In the present study, it was revealed that Puerarin inhibited cell proliferation, increased the LDH activity, reduced the migration rate and increased apoptosis rate in HeLa cells in a dose- and time-dependent manner. A MTT assay, an LDH activity assay and Annexin V-FITC/PI analysis were utilized to analyze cell growth and apoptosis. Hu *et al* (16) reported that Puerarin inhibits non-small cell lung cancer cell growth via apoptosis induction. However, in future studies it is recommended that the number of experimental methods be expanded to include Ki-67 and Cyclin D analysis. As the current study was only performed with HeLa cells further investigations are required to validate the reported findings.

It has been demonstrated that the risk of developing cervical cancer is positively correlated with the number of sexual partners an individual has, as this increases their exposure to the HPV virus. Smoking, economic status, race and geographical location are all additional risk factors for the development of cervical cancer (17). Gan *and* Yin (14) demonstrated that Puerarin induced apoptosis in mantle cell lymphoma; based on this, the authors of the present study hypothesized that Puerarin may increase the apoptosis rate of HPV-18 positive cervical cancer cells, which was confirmed in the present study.

The function of Bcl-2 regulates programmed cell death (18). Members of the Bcl-2 family are inhibitors of apoptosis (19). Initiator and executor caspases are key molecules associated with regulation of the apoptosis signaling cascade (19). Apoptosis may occur through

either the intrinsic or extrinsic signaling pathway (18). The extrinsic pathway is activated by signals from other cells, while the intrinsic pathway may be activated by a number of internal cell signals associated with cell stress, caused by radioactive rays, cytotoxic drugs, the elimination of growth factors and proteins released by mitochondrial membranes, including cytochrome C (20). The intrinsic pathway is often referred to as the mitochondrial apoptosis-signaling pathway. Cytochrome C combines with apoptosis protease activating factor 1 and inactivated caspase-9 to form a protein complex called the apoptosome (21). The formation of apoptosomes activates Caspase-9, which in turn activates of a series of caspase proteins (caspase-3, caspase-6 and caspase-7) to trigger changes in cell morphology and biochemistry associated with apoptosis (22,23). The results of the present study indicate that Puerarin effectively induced Bax protein expression and promoted apoptosis in HPV-18 positive cervical cancer cells. Liu *et al* (24) revealed that Puerarin also suppressed lipopolysaccharide-induced breast cancer cell migration, migration and adhesion.

Activation of the PI3K/mTOR signaling pathway may promote cell cycle progression, reduce apoptosis and promote the migration of cancer cells (23), which are factors associated with the occurrence of multiple tumors (10). Activated PI3K activates downstream Akt (23), which infers an increased tolerance against apoptosis in cancer cells, as well as inducing cell growth and abnormal metabolism (24). The excessive activation of Akt activates downstream mTOR, which may cause the rapid proliferation of cancer cells, increase oncoprotein secretion, accelerate cell cycle progression and shorten the G1 time interval, which increases the occurrence and development of tumors (24). *In vivo*, mTOR realizes its physiological effects by phosphorylating multiple substrate proteins (24). The translation products include translation elements, such as ribosomal proteins and elongation factors (24). Previous research indicates that mTOR phosphorylates the 412th threonine residue of P70S6 kinase, which increases its activity 100-fold and promotes the biosynthesis of proteins (25). The results of the present study indicate that Puerarin significantly suppressed PI3K, p-Akt and p-mTOR protein expression in HeLa HPV-18 positive cervical cancer cells, thereby reducing their proliferative and migration abilities; however, PI3K upregulation reduced the anticancer effect of Puerarin on HeLa cells. Huang *et al* (13) revealed that Puerarin induces cell apoptosis in human chondrosarcoma cells via inhibition of the PI3K/Akt signaling pathway, which supports the results of the present study.

In conclusion, the present study demonstrated that Puerarin effectively inhibits cell proliferation, increases apoptosis and promotes caspase-3/9 and Bax protein expression in HeLa cells, in part by inhibiting the PI3K/Akt/mTOR signaling pathway. However, further studies are required to provide additional evidence for the anticancer effects and underlying mechanisms of Puerarin in HPV-18 positive cervical cancer cells. Puerarin has potential as a novel drug for the treatment of cervical cancer in future clinical practice.

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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

PL designed the experiments. LJ, YH and GY performed the experiments and analyzed the data. PL wrote the manuscript. All authors read and approved the final version of the 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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