

Src kinase inhibitor PP2 regulates the biological characteristics of A549 cells via the PI3K/Akt signaling pathway

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Abstract. Lung cancer is one of the most prevalent types of cancer worldwide, with a poor prognosis for patients and a concomitant financial burden on society. There are a number of different pathological subtypes, with non-small cell lung cancer (NSCLC) being the primary subtype. Although anticancer therapy has led to a marked improvement in the survival rate of patients in recent years, the survival rate remains poor. Potential reasons for this include a lack of early diagnosis and drug resistance, which is considered to be associated with mutations in components of signaling pathways, tumor suppressors and epidermal growth factor receptor, and certain other complex mechanisms to a certain extent. It is therefore imperative to develop novel therapies. In the present study, the pyrazolopyrimidine compound PP2 was used to inhibit Src family protein tyrosine kinases in A549 cells. It was demonstrated that PP2 was able to suppress cell viability, migration and invasion, and promote apoptosis via regulating the phosphoinositide 3-kinase/protein kinase B/B-cell

lymphoma 2/caspase-3 signaling pathway. PP2 may therefore be useful in anti-NSCLC therapy in the future.

Introduction

As the leading cause of cancer-associated mortality worldwide (1), lung cancer imposes an increasing burden on society and is a major challenge to clinicians. The Global Cancer Statistics reported that an estimated 1.8 million novel cases of lung cancer occurred in 2012 worldwide, accounting for ~13% of total cancer diagnoses (2). Non-small cell lung cancer (NSCLC) is the major pathological type, consisting of 85% of all types of lung cancer (3). In recent years, with the development of molecular biotechnology techniques, drugs for the treatment of NSCLC, particularly lung adenocarcinoma, targeting signal transduction and angiogenesis have achieved certain effects; however, patients with genetic disorders failed to benefit from these drugs and they are not available to those of limited means in certain countries (4).

Src family protein tyrosine kinases (SFKs), members of the receptor tyrosine protein kinase family, include the proto-oncogene 'Scr' was the first to be identified (5) and serves a key function in cellular signal transduction pathways, immune responses and inflammatory responses (6). Aberrant SFK activity is observed in a number of types of human cancer, and is associated with proliferation, invasion, apoptosis and migration of tumor cells. There is evidence that expression of SFKs in gastric cancer is associated with tumor invasion, and lymph node and distant metastases (6). Overexpression of SFKs was detected in ~80% of patients with colon cancer, and was demonstrated to accelerate tumor metastasis and lead to chemotherapeutic drug resistance via multiple downstream signaling pathways (7). Furthermore, it has been demonstrated that biological changes in breast cancer induced by cluster of differentiation 44 silencing may be mediated by cumulative downregulation of SFKs (8), and that knockdown of Lyn or other SFK members may decrease proliferation, migration and invasion of human pancreatic cancer cells (9).

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A number of studies have focused on the association between SFKs and NSCLC. One study identified sex-determining region Y box 2 as a novel target of epidermal growth factor receptor (EGFR)/Src/protein kinase B (Akt) signaling in NSCLC that modulates self-renewal and expansion of stem-like cells from NSCLC (10). Furthermore, targeting SFKs is a clinically applicable strategy to overcome resistance to insulin-like growth factor 1 receptor tyrosine kinase inhibitors (11). Lung cancer may be inhibited by silencing Lyn kinase expression using small interfering RNA, which decreased EGFR activation and cell viability (12). In addition, EGFR inhibition promotes innate drug resistance and is associated with limited primary responses (13). Furthermore, resistance of NSCLC to anticancer treatment is also prevented by mitochondrial changes and activation of caspases (14,15).

Pyrazolopyrimidine compound PP2 (Fig. 1A) is a selective inhibitor of SFKs. EGFR mutants of the H1299 cell line exhibited different sensitivity to PP2 (16). On this basis, we hypothesize that there may be overexpression of Lyn kinase in patients with lung cancer. This hypothesis was confirmed in our previous study (unpublished data). The aim of the present study was to determine whether PP2 is able to influence the biological characteristics of A549 cells.

Materials and methods

Cell culture. The human NSCLC cell line A549 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml) (both Beyotime Institute of Biotechnology, Haimen, China) at 37°C with 5% CO₂.

MTT assay. Cells were seeded at 5x10³ cells/well in a 96-well plate and incubated overnight at 37°C to reach 85% confluence. Cells were treated with different concentrations of PP2 (20, 40, 80, 160 and 320 µM; Abcam, Cambridge, MA, USA) which was dissolved in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), for 24, 36 and 48 h. The control group was treated with PBS. Following culture with PP2, 10 µl/well MTT solution (5 mg/ml) was added prior to incubation for a further 4 h. Subsequently, 100 µl DMSO was added to each well prior to incubation at 37°C for 3 h to solubilize the formazan crystals. The absorbance of plates was determined at 570 nm using a microplate reader (Tecan Group, Ltd., Männedorf, Switzerland).

Immunofluorescence microscopy. A549 cells treated with PP2 (0, 20, 40, 80, 160 and 320 µM) for 24 h were fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed A549 cells were washed with PBS three times, prior to being permeabilized with methanol (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Subsequently, 100 µl/well DAPI (Beyotime Institute of Biotechnology) was used for staining for 5 min in the dark at room temperature, prior to washing with PBS three times. Images of all specimens were captured using an SP5 Leica confocal microscope and analyzed with

Leica Application Suite software (Version number: 14.0.0162) (both Leica Microsystems GmbH, Wetzlar, Germany).

Colony formation assay. Cells were seeded in dishes of 60 mm diameter with 500 cells/dish and cultured overnight. Cells were exposed to PP2 at various concentrations (0, 20, 40, 80, 160 and 320 µM) for a total of 10 days when the colonies were visible to the naked eye, and medium was refreshed every 48 h. On the last day, the colonies were washed with PBS and fixed with 4% paraformaldehyde, then washed by PBS three times and stained with Wright-Giemsa stain (BaSO Biotech, Taipei, Taiwan) at room temperature for 11 min. The number of colonies formed in each group was determined, by counting with the naked eye.

Cell invasion assay. Matrigel (3 mg/ml) was added at 40 µl/well to the inner face of the membrane in the upper compartment of the Transwell insert and 40 µl/well fibronectin (125 µg/ml; Sigma-Aldrich; Merck KGaA) was added to the outer face of the membrane, prior to drying overnight. Cells were resuspended in FBS-free RPMI-1640 medium with 0.1% bovine serum albumin (BSA; Sangon Biotech Co., Ltd., Shanghai, China). Subsequently, 2x10⁵ cells were added into the upper compartment of the Transwell insert with 300 µl/well FBS-free medium containing 0.1% BSA. Cells were exposed to PP2 at various concentrations (0, 20, 40, 80, 160 and 320 µM). RPMI-1640 medium containing 10% FBS was added to the lower compartment at 1 ml/well. Cells were incubated at 37°C for 24 h. The insert was removed and cells on the outer face were fixed with 4% paraformaldehyde for 30 min and stained with Wright-Giemsa stain (BaSO Biotech, Taipei, Taiwan) at room temperature for 11 min. Five random fields were selected from each membrane with light microscope at x100 magnification, and the number of cells in each field was counted.

Immunofluorescence flow cytometry. Cells were seeded in 6-well plates and cultured in RPMI-1640 medium containing 10% FBS overnight. Cells were treated with PP2 at various concentrations (0, 20, 40, 80, 160 and 320 µM) for 24 h, and then collected for propidium iodide (PI) and fluorescein isothiocyanate/Annexin V (Annexin V-FITC) staining in the dark at room temperature, the FITC Annexin V Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's protocol. Apoptotic cells were analyzed using flow cytometry with a FACSaria II instrument (BD Biosciences) and data were analyzed by Cell Quest 5.1 (BD Biosciences).

Western blot analysis. A549 cells were seeded in 6-well plates and cultured overnight, prior to treatment with PP2 at various concentrations (0, 20, 40, 80, 160 and 320 µM) for 24 h. Following incubation, cells were lysed in radioimmunoprecipitation assay lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40 and 1% sodium deoxycholate) containing proteinase (phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF and Na₃VO₄) and maintained on ice for 30 min. The lysate was centrifuged at 11,113 x g for 10 min at 4°C on an Allegra X-22R centrifuge (Beckman Coulter, Inc., Brea, CA, USA). The protein concentration of each specimen was determined quantitatively using

a bicinchoninic acid protein concentration assay kit (Beyotime Institute of Biotechnology). The suspension was transferred to a new tube and kept on ice, then mixed with 5X SDS-PAGE sample loading buffer (Beyotime Institute of Biotechnology), and boiled at 100°C for 10 min. A 50 µg amount of each protein sample was loaded per lane of an SDS-PAGE gel (10% acrylamide) with two lanes of 2 µl protein molecular mass marker. The gel was electrophoresed for 30 min at 80 V for stacking and 100 V for separation, then the protein was electrotransferred onto a polyvinylidene fluoride membrane for 2.5 h at 300 mA. Non-specific binding was blocked with 5% dried skimmed milk diluted in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h and washed with TBST three times. The membranes were incubated with primary mouse monoclonal anti-human phosphoinositide 3-kinase (PI3K; 1:1,000; cat. no. ab86714), rabbit polyclonal anti-human phospho-PI3K (1:1,000; cat. no. ab182651), rabbit monoclonal anti-human Akt (1:1,000; cat. no. ab32505), rabbit monoclonal anti-human phospho-Akt (1:1,000; cat. no. ab81283), mouse monoclonal anti-human B-cell lymphoma 2 (Bcl-2; 1:500; cat. no. ab692), rabbit polyclonal anti-human caspase-3 (1:1,000; cat. no. ab2302), and mouse monoclonal anti-human GAPDH (1:1,000; cat. no. ab8245) antibodies (all obtained from Abcam) at 4°C overnight. Following removal of unbound antibodies and washing three times with TBST, the membranes were incubated with secondary antibodies (goat anti-rabbit polyclonal; 1:5,000; cat. no. ab6721) and goat anti-mouse polyclonal (1:1,000; cat. no. ab6789) (both obtained from Abcam) for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Statistical analysis. All data are expressed as the mean ± standard error of the mean. The statistical significant differences were analyzed using one-way analysis of variance followed by Bonferroni's correction for comparison tests, using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PP2 has a cytotoxic effect on A549 cells. The effect of PP2 on lung cancer remains unclear. In order to elucidate this function, an MTT assay was used to determine the effect of PP2 on the viability of A549 cells. Cells were treated with various concentrations of PP2 (0, 20, 40, 80, 160 and 320 µM) at three different times (24, 36 and 48 h). The results indicated that PP2 was cytotoxic towards A549 cells (Fig. 1B), with the survival rate decreasing with increasing concentrations of PP2 and the extension of the incubation time. Similarly, the morphological features of A549 cells treated with PP2 were also altered (Fig. 1C). The cell nuclei were irregular and rather ambiguous at increased concentrations of PP2. These results suggested that PP2 is able to decrease the viability of A549 cells and alter the morphology of the cell nucleus.

PP2 suppresses the viability of A549 cells and decreases colony formation. In order to further verify the negative effect of PP2 on the viability of A549 cells, a colony formation assay was

used to determine the effect of PP2 on cell viability. Following treatment with PP2, the number of colonies formed decreased with increasing concentrations of PP2 (Fig. 1D). Compared with the untreated control group, following administration of PP2 at 320 µM, the number of A549 cell colonies formed decreased significantly (Fig. 1E). These results suggested that PP2 decreased the viability and colony formation ability of A549 cells effectively.

PP2 inhibits A549 cell invasion. A tumor invasion assay was used to detect the effect of PP2 on the invasive ability of A549 cells *in vitro*. Following treatment with PP2 for 24 h, the number of transmembrane cells at different concentrations were compared (Fig. 2A). With increasing concentration of PP2, the number of transmembrane cells decreased (Fig. 2B). Compared with the control group, there was a decrease of >50% in the number of transmembrane cells treated with 80 µM PP2.

PP2 induces apoptosis in A549 cells. To further elucidate the underlying molecular mechanism by which PP2 induced the inhibition of cell viability, it was investigated whether PP2 leads to induction of apoptosis in A549 cells. A549 cells were treated with various concentrations of PP2 for 24 h and analyzed using flow cytometry. The results indicated that the percentage of apoptosis cells increased with increasing concentrations of PP2 (Fig. 2C), the apoptosis rate increased with increasing concentrations of PP2 (Fig. 2D). These results indicate that PP2 was able to induce apoptosis in A549 cells in a dose-dependent manner.

Effects of PP2 on PI3K/Akt/Bcl-2/caspase-3 signaling pathway in A549 cells. The results of the present study indicated that PP2 is able to inhibit viability and induce apoptosis in A549 cells; however, the underlying molecular mechanism remains unclear. To elucidate the underlying mechanism of the effect of PP2 on A549 cells, the expression of associated proteins of the PI3K/Akt/Bcl-2/caspase-3 signaling pathway was determined following treatment with PP2. The results of the western blot assay are presented in Fig. 2E. PP2 decreased phospho-PI3K and phospho-Akt with increasing concentration of PP2. These results suggest that PP2 was able to inhibit the protein phosphorylation and activity of this signaling pathway, which are consistent with the result of the present study.

Furthermore, the expression level of apoptotic regulatory proteins was also determined. Compared with the control group, the expression level of Bcl-2 was markedly down-regulated, whereas that of caspase-3 was increased following treatment with PP2. These western blot results were consistent with those of the flow cytometric analysis. Therefore, PP2 was identified to be able to inhibit the viability and invasion of A549 cells, and also induce apoptosis; effects achieved by regulating the activation of the PI3K/Akt/Bcl-2/caspase-3 signaling pathway.

Discussion

Lung cancer is the leading cause of mortality from cancer among males worldwide, with an increase in lung cancer associated with increasing populations, aging populations

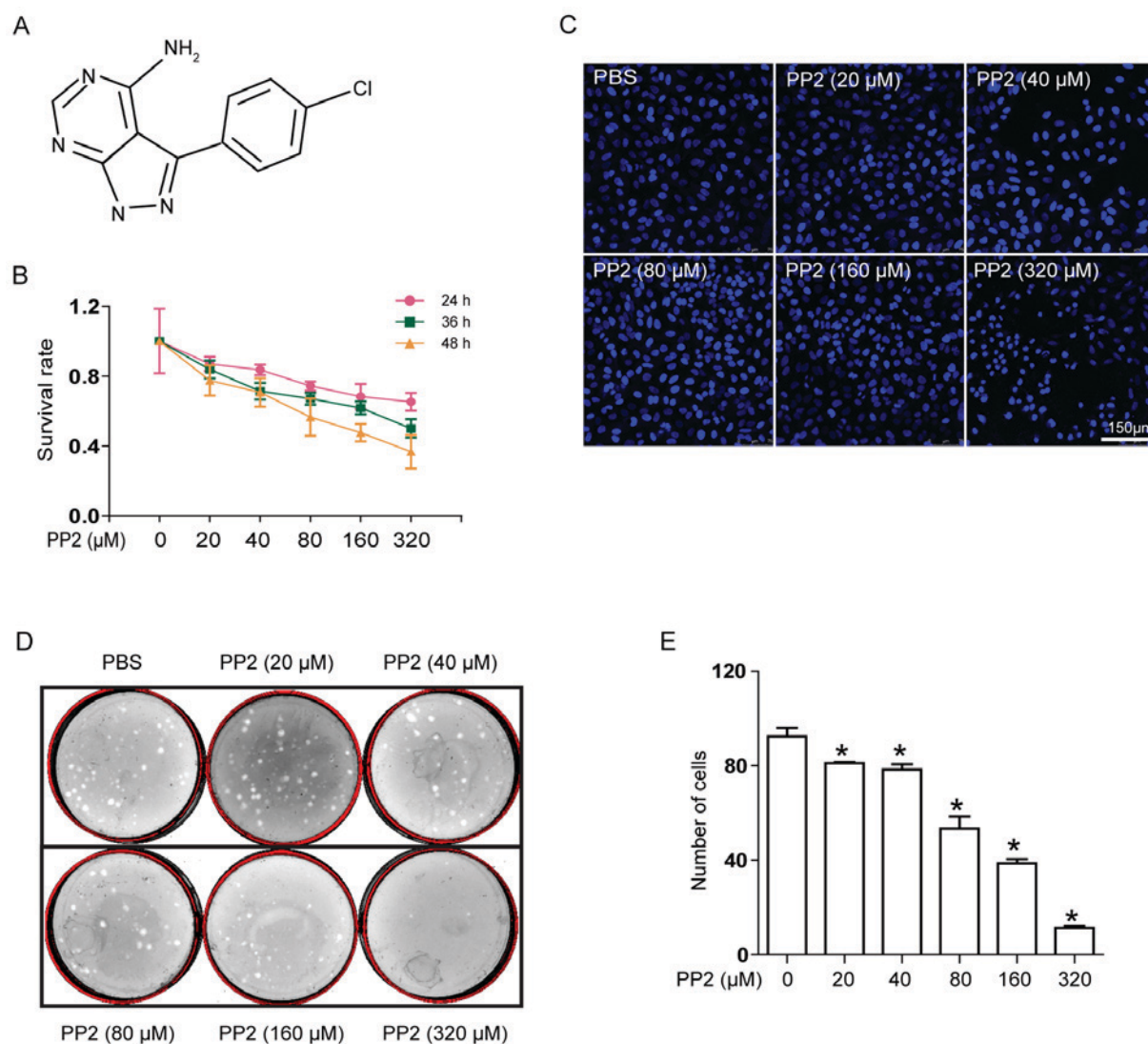


Figure 1. PP2 has a cytotoxic effect on A549 cells. (A) Chemical structure of PP2. (B) Survival rates of A549 cells treated with PP2 at different concentrations and times. (C) Morphological features of cell nuclei treated with various concentrations of PP2. (D) Colony growth of A549 cells treated with various concentrations of PP2. (E) Quantification of colony formation assay results. * $P < 0.05$ vs. control group (PBS/0 μM PP2).

and air pollution (2). Although anticancer therapies, including chemotherapy, radiation therapy and molecular targeted therapy, are currently commonly used in the clinic, a marked proportion of patients failed to benefit from these treatments. In 2015 the five-year survival rate of patients with lung cancer is only 16% (17). Furthermore, the effects of drug resistance and genetic mutations on lung cancer are becoming common problems for anticancer therapies. In previous studies, SFKs have been recognized as having a vital function in cancer cell proliferation, migration and invasion (18,19). As a member of the SFK family, Lyn was identified to be markedly expressed in lung tissue of patients with lung cancer in our previous study (unpublished data). PP2 is a selective inhibitor of SFKs, therefore PP2 was used to treat A549 cells in order to investigate its influence on biological characteristics of cells and elucidate the underlying molecular mechanisms.

Our previous study (unpublished data) identified that PP2 was able to inhibit the viability of A549 cells and decrease colony growth of cells, the morphological changes of nucleus were the main characteristics in A549 cells, and the effect

was markedly dose- and time-dependent. Furthermore, PP2 was able to markedly decrease the invasiveness, while promoting apoptosis, of A549 cells. The underlying molecular mechanism was modulation of the PI3K/Akt/Bcl-2/caspase-3 signaling pathway. These results suggested that molecular targeted agents against SFKs may have potential for anticancer therapies.

It has been identified that SFKs are translocated to the sites of cell adhesion (18). Owing to its particular localization, the catalytic activity of Src initiates the intracellular signal transduction pathways that influence cell proliferation and adhesive strength, the latter contributing to regulation of cell migration. In addition, the migration of cancer cells may be suppressed by PP2, because of its function of activating the epithelial cadherin-mediated cell adhesion system (19). In addition, overexpression of c-Src and EGFR in fibroblast cells causes synergistic increases in DNA synthesis, colony growth and tumor formation in nude mice, whereas knockdown of Src may decrease human pancreatic cancer cell proliferation, migration and invasion (9,20). These results suggested that Src is

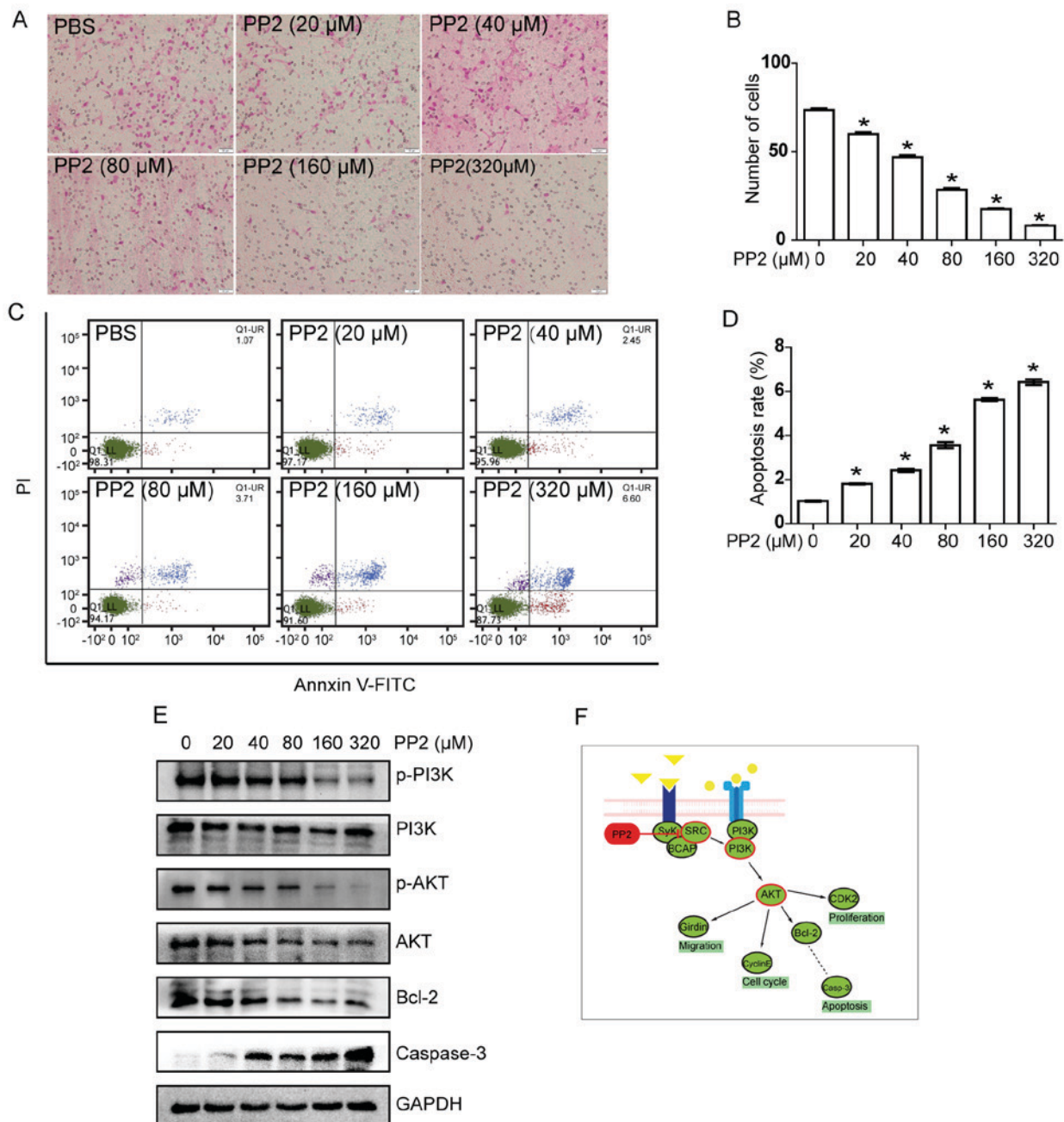


Figure 2. PP2 inhibits invasiveness and induces apoptosis of A549 cells in a dose-dependent manner by regulating activation of the PI3K/Akt/Bcl-2/caspase-3 signaling pathway. (A) Cell invasion assay. Representative images of cells treated with various concentrations of PP2 (magnification, x100). (B) Quantification of invasion assay. (C) Flow cytometric analysis of apoptotic cells treated with various concentrations of PP2. (D) Quantification of apoptotic cells. (E) Protein expression of the PI3K/Akt/Bcl-2/caspase-3 signaling pathway members in A549 cells treated with various concentrations of PP2. (F) Schematic diagram of the mechanism of action of PP2 on A549 cells. *P<0.05 vs. control group (PBS/0 μM PP2). PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; Bcl-2, B-cell lymphoma 2; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Q, quadrant; UR, upper right; LL, lower left; p-, phospho-; BCAP, B-cell adaptor for PI3K; CDK2, cyclin-dependent kinase 2; Casp-3, caspase-3.

associated with proliferation, metastasis and invasion of cells, in agreement with the results of the present study. Accordingly, it was identified that the expression of PI3K/Akt and phosphorylated PI3K/Akt was decreased. The PI3K/Akt/mammalian target of rapamycin signaling pathway is an important intracellular signal transduction pathway with an important function in cell viability and survival, inhibition of apoptosis, angiogenesis, metastasis and resistance to chemotherapy-radiotherapy (21,22). In a previous study, activated Akt was traced in primary NSCLC tumors and was suggested to be a poor

prognostic factor for patients with early-stage NSCLC (23). Overexpression of the downstream kinase Akt may also result in activation of the PI3K signaling pathway (21). These results indicated that PP2 is an effective inhibitor to inhibit SFKs and suppress the downstream PI3K/Akt signaling pathway, to inhibit cellular viability, migration and invasion.

Additionally, from the flow cytometry data in the present study, we hypothesize that PP2 is able to promote apoptosis of A549 cells; the underlying molecular mechanism may be associated with suppressing the expression of Bcl-2 and upregulating

caspase-3 in the downstream pathway, as observed in the western blot analysis of the present study. The Bcl-2 family of pro- and anti-apoptotic proteins has been recognized as the important components of regulating the mitochondrial pathway of apoptosis, with the major anti-apoptotic proteins being Bcl-2, B-cell lymphoma extra-large and myeloid cell leukemia-1. These proteins promote cellular survival by sequestering pro-apoptotic proteins including Bcl-2-interacting mediator of cell death and Bcl-2-associated death promoter, which function as apoptotic 'sensitizers' or 'effectors' like Bcl-2-associated X protein or Bcl-2 homologous antagonist killer (24). Therefore, we hypothesize that Bcl-2 is a key protein in the apoptotic pathway which is suppressed by PP2.

Joseph *et al* (15) reported that caspase-3 serves a vital function in regulating nuclear changes during apoptosis. It was identified that caspase-3 is associated with loss of the integrity of the nuclear membrane, decreased synthesis of poly(ADP-ribose) and DNA fragmentation (25). There is evidence that effector caspases are responsible for initiating the hallmarks of the degradation phase of apoptosis, including DNA fragmentation, cell shrinkage and membrane blebbing (14), and poly(ADP-ribose) is critical for DNA repair, regulation of chromosome structure, transcriptional regulation, mitosis and apoptosis (26). Treatment with caspase-3 initiates DNase activity and causes DNA fragmentation in nuclei (26,27). Nam *et al* (19) identified that PP2 is able to induce morphological changes in cancer cells. In the present study, it was identified that the morphology of the nucleus under light microscopy was irregular and ambiguous following treatment with PP2. The alterations in nuclear characteristics demonstrated that PP2 promotes the apoptosis pathway by upregulating caspase-3. These results demonstrated the inhibition of PP2 on A549 cell viability, and that the PP2-promoted apoptosis in A549 cells occurred downstream of mitochondrial changes and caspase activation, and upstream of nuclear events.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GPL and XQY conceived and designed the study. XD, LJW, JW and YXS performed the experiments. XD and LJW wrote 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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