Pectolinarigenin inhibits non-small cell lung cancer progression by regulating the PTEN/PI3K/AKT signaling pathway

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Abstract. Lung cancer is the principal cause of cancer-associated mortality. Pectolinarigenin (Pec) reportedly has effective antitumor activity against certain cancer types. Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor and serves a vital role in cancer progression. However, the effect of Pec on non-small cell lung cancer (NSCLC) cell proliferation and metastasis, and the underlying mechanism, has not yet been elucidated. In the present study, it was demonstrated that Pec inhibited the proliferation of A549 and Calu-3 cells in dose- and time-dependent manners. The apoptosis rate significantly increased with increasing doses of Pec. Apoptosis-associated protein expression was additionally altered by Pec exposure. Pec was able to suppress the metastasis of NSCLC cells; it upregulated the mRNA and protein expression levels of E-cadherin, and downregulated the mRNA and protein expression levels of vimentin. Additionally, Pec was able to activate PTEN and subsequently downregulate the PI3K/protein kinase B (AKT) signaling pathway. In summary, Pec was able to inhibit cell proliferation, promote apoptosis and suppress metastasis in NSCLC cells through the PTEN/PI3K/AKT signaling pathway, indicating that Pec is a potential agent for NSCLC therapy.

Introduction

Lung cancer is one of the principal causes of cancer-associated mortality, and contributes to the economic burden worldwide.

In total, ~85% of confirmed lung cancer cases are classified as non-small cell lung cancer (NSCLC) (1-3). Traditionally, clinical methods, including surgery, chemotheraphy or a combination are the primary treatment approaches for lung cancer. Recently, molecular-targeted drugs (4), anti-angiogenic therapy (5) and cancer immunotherapy (6) have been widely applied in patients with lung cancer. Despite advances in early diagnosis and standard treatment, the overall 5-year survival rate for NSCLC is <15% (7), and there is an urgent requirement for the development of novel therapeutic approaches for NSCLC.

Phosphatase and tensin homolog (PTEN), located on chromosome 10q23, is well known as a tumor suppressor, and has lipid and protein phosphatase activities. It is involved in various cellular processes and signal transduction pathways in a complex network system (8-10). Emerging evidence has demonstrated that PTEN serves an important role in tumorigenesis and tumor suppression in multiple tumor types, including thyroid cancer (11), osteosarcoma (12), gastric cancer (13) and myeloma (14). The function of PTEN may be regulated by genetic mutations, transcriptional regulation and post-translational modifications (8,10). Loss of PTEN or a decrease in PTEN expression levels and activity has been associated with poorer overall survival among patients with NSCLC. Therefore, regulation of PTEN has emerged as a promising research topic for cancer therapy (15).

In recent years, natural agents have attracted increasing attention due to the richness of the resource, easy accessibility and cost effectiveness for clinical application. As previously demonstrated, treatment with Osthole and Morinda citrifolia edible leaf extract (16,17), regulated PTEN expression, inducing cancer growth and metastasis. Pectolinarigenin (Pec; C17H14O6; molecular weight: 314.28; melting point: 204-205˚C; storage conditions: 4˚C refrigerated, sealed and protected from light) is a flavonoid compound widely distributed in a number of medicinal plants, including Cirsium japonlcum, Eupatorium odoratum and Trollius chinensis. Under the appearance of a yellow crystal, it is soluble in dimethyl sulfoxide and hot methanol, and insoluble in petroleum ether, chlorine and other solvents. As documented in previous studies, Pec has demonstrated effective antitumor activities in vivo and in vitro. Cheng et al (18) observed that Pec may inhibit cell viability and migration of nasopharyngeal carcinoma cells, and induce...
mitochondrial-associated apoptosis through the accumulation of caspase-3 and caspase-9 in cells. Zhang et al (19) demonstrated that Pec may be able to disrupt signal transducer and activator of transcription 3 (STAT3) signaling and decrease STAT3 downstream proteins, including cyclin D1, B-cell lymphoma 2 (BCL-2) B-cell lymphoma extra-large (BCL-xL), Myeloid cell leukemia 1 (MCL-1), contributing to the suppression of cell proliferation and apoptosis in osteosarcoma cells. Additionally, Pec was able to inhibit cell migration and invasion, and preserved the epithelial-mesenchymal transition (EMT) phenotype. As uncontrolled cell proliferation and metastasis are considered hallmarks of malignant tumors, inhibition of associated signaling pathways is one important aspect of cancer treatment.

It has been demonstrated that Pec may inhibit growth and metastasis of nasopharyngeal carcinoma cells and osteosarcoma cells (18,19); however, the effect of Pec on NSCLC and its underlying mechanisms have not been reported. In the present study, the potential effects of Pec on human NSCLCs cells were investigated to clarify the possible underlying mechanisms. As a result, it was revealed that Pec may significantly inhibit cell proliferation, migration, invasion, EMT, and induce apoptosis in vitro by promoting the expression of PTEN.

Materials and methods

Cell lines and reagents. Human NSCLC cell lines A549 and Calu-3 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The two cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% CO2 incubator at 37°C. Pec reagent was purchased from Abmole Bioscience Inc. (Houston, TX, USA). Antibodies against Bax (cat. no. 2744), BCL-xL (cat. no. 2762), PTEN (cat. no. 9188), phospho-phosphoinositide 3-kinase (p-PI3K; cat. no. 4228), phospho-protein kinase B (p-AKT; cat. no. 4060), cellular tumor antigen p53 (p53; cat. no. 9282), Lamin B1 (cat. no. 13435) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Fluorescence-tagged secondary antibodies, IRDye® 680RD goat anti-rabbit IgG (cat. no. 925-68071) and IRDye® 680RD goat anti-mouse IgG (cat. no. 925-68070) were acquired from LI-COR Biosciences, Inc. (Lincoln, NE, USA). PTEN inhibitor SF1670 (cat. no. ab141303) and AKT activator SC79 (cat. no. ab146428) were purchased from Abcam (Cambridge, UK). SF1670 and/or SC79 (10 µM) were applied to the culture medium with 4% paraformaldehyde for 30 min at room temperature. The cells were washed twice with PBS and subsequently fixed in 10% neutral buffered formalin for 10 min. The fixed cells were blocked with 10% donkey serum and incubated with primary antibodies (cat. no. ab146428) for 1 h at room temperature. The membranes were washed three times with PBS and incubated with fluorescence-tagged secondary antibodies for 1 h. Images of the membranes were captured with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). GAPDH and Lamin B1 protein intensity was used as internal controls for cytoplasm and nucleus fractions, respectively.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of the NSCLC cells treated with or without Pec were collected and extracted with RNasy Mini RNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The concentrations of total RNA were determined spectrophotometrically at 260 nm with an automatic microplate analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell apoptosis assay. A cell apoptosis assay was conducted by flow cytometry with a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. After treatment with Pec for 48 h, the cells were harvested and resuspended in binding buffer at a concentration of 1x10⁶ cells/ml. Subsequently, 5 µl FITC Annexin V and 5 µl Propidium Iodide Staining Solution were added to a 200-µl cell suspension, and incubated for 30 min at room temperature in the dark. Cell apoptosis was detected using BD FACSVerse (BD Biosciences) and the apoptosis rate of cancer cells was analyzed using FlowJo software version 7.0 (FlowJo LLC, Ashland, OR, USA).

Colony formation assay. Cells were seeded in a 6-well plate at 400 cells/well. After 24 h, the cells were treated with Pec at various concentrations (0, 10 and 25 µM) and incubated at 37°C with 5% CO₂. The cells were consistently cultured for 10 days and the culture medium was changed every two days. The cells were washed twice with PBS and subsequently fixed with 4% paraformaldehyde for 30 min at room temperature. A crystal violet stain was used to observe colony formation.

Cell viability assay. Cells were grown in a 96-well plate overnight at a density of 4x10⁴ cells/well, and subsequently treated with different concentrations of Pec for 24, 48 and 72 h. Following incubation, 10 µl Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well for 1 h. The optical density of each sample was measured spectrophotometrically at 450 nm with an automatic microplate analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
GTT CAC GG-3’ and reverse, 5’-GGG TGT CGA GGG AAA AAT AGG-3’; vimentin forward, 5’-GAC GCC ATC AAC ACC GAG TT-3’ and reverse, 5’-CTT TGT CGT TGG TTA GCT GGT-3’; GAPDH forward, 5’-GGA GCG AGA TCC CTC CAA AAT-3’ and reverse, 5’-GGC TGT TGT CAT ACT TCT CAT GG-3’. The densitometry of relative mRNA expression was performed using the calculation of $2^{-\Delta \Delta Cq}$ (20) and GAPDH was used as an internal control.

Wound scratch assay. Cancer cell migration was assessed using a cell wound scratch assay. NSCLC cells were seeded in a 6-well plate and incubated overnight to form a confluent cell monolayer, and a scratch was made with a 100-µl sterile pipette tip. The cancer cells were subsequently treated with or without Pec for 24 h and images of the scratches were obtained with an inverted microscope (Olympus Corp., Tokyo, Japan).

Transwell assay. For the invasion assay, the upper chambers were pre-coated with Matrigel (Invitrogen; Thermo Fisher Scientific, Inc.), the NSCLC cancer cells (1x10^5 cells/well) were plated on the upper chamber with Pec (25 µM) or alone; whereas, the lower chamber contained RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin. After 24 h of incubation, the upper chambers were swabbed out, and the lower chambers were fixed with paraformaldehyde and stained with crystal violet. Images of the invaded cells were captured with a Leica fluorescence microscope (magnification, x100; Olympus Corp.).

Statistical analysis. All data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and comparisons between groups were determined by Student’s t-test. The results are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times independently.

Results

Pec inhibits cell proliferation in A549 and Calu-3 cells. It was reported that Pec exhibited an inhibitory effect on nasopharynx carcinoma (18); thus, in order to investigate whether Pec had a similar effect on NSCLC cells, a cell proliferation assay was conducted using a CCK-8 assay. The results revealed that the cell viability of A549 and Calu-3 cells was decreased following administration of Pec. The CCK-8 assay demonstrated that Pec was able to significantly inhibit the cell proliferation in dose- and time-dependent manners, with half maximal inhibitory concentration values of 21.49 and 22.63 µM, respectively. In addition, the colony formation assay demonstrated that the growth rate was markedly decreased in the two cell lines treated with Pec. The CCK-8 assay revealed that the cell viability of A549 and Calu-3 cells was decreased following administration of Pec. The CCK-8 assay demonstrated that Pec was able to significantly inhibit the cell proliferation in dose- and time-dependent manners, with half maximal inhibitory concentration values of 21.49 and 22.63 µM, respectively. In addition, the colony formation assay demonstrated that the growth rate was markedly decreased in the two cell lines treated with Pec (Fig. 1D). These results indicated that Pec exhibited an inhibitory effect on the proliferation of NSCLC cells.

Pec induces the apoptosis of human NSCLC cells. To determine the effect of Pec on NSCLC cell apoptosis, flow
cytometry was conducted to detect the apoptosis of cells treated with Pec. As presented in Fig. 2, the apoptosis rate of the A549 and Calu-3 cell lines increased gradually with the administration of different concentrations of Pec (0, 5, 10, 25, 50 and 100 µM) for 48 h, particularly the A549 cell line.

Furthermore, western blot analysis demonstrated that the pro-apoptotic Bax protein expression level was upregulated; whereas, the anti-apoptotic BCL-XL protein expression level was downregulated in the A549 cell line (Fig. 3A), and similar results were additionally observed in the Calu-3 cell line (Fig. 3B). Therefore, it was hypothesized that Pec exhibited an antitumor effect by inducing NSCLC cancer cell apoptosis and apoptosis-associated protein expression levels.

Pec decreases the migration, invasion and EMT of NSCLC cells. To examine whether Pec was able to inhibit the migration, invasion and EMT of NSCLC cells, a cell wound scratch assay was performed and the two cell lines were treated without or with Pec (25 µM). After 24 h of incubation, Pec significantly inhibited the migration of NSCLC cells compared with the control groups (Fig. 4A). A Transwell assay was conducted to detect the invasion of NSCLC cells; the results demonstrated that Pec suppressed the invasion of A549 and Calu-3 cells (Fig. 4B). Furthermore, the expression of epithelial marker E-cadherin and mesenchymal marker vimentin was detected by RT-qPCR and western blotting. The EMT process is considered the initial step for cancer metastasis; Pec markedly upregulated the expression of E-cadherin and downregulated the vimentin expression at the mRNA (Fig. 4C) and protein expression levels (Fig. 4D). Collectively, treatment with Pec suppressed the metastasis and EMT of NSCLC cells.

**Effects of Pec on the PTEN/PI3K/AKT pathway in A549 and Calu-3 cells.** A previous study suggested that the PTEN/PI3K/AKT pathway is involved in cancer progression (21-25), thus, the effect of Pec on the PTEN/PI3K/AKT pathway in A549 and Calu-3 cells was evaluated by western blotting to examine the underlying mechanism of Pec-mediated inhibition of cell proliferation and metastasis. In the present study, the results of western blotting demonstrated that the expression of phospho-PI3K and phospho-AKT was significantly decreased; whereas, the PTEN protein expression
Figure 2. Continued. Pec induces apoptosis in lung adenocarcinoma cells. (C and D) Calu-3 cells were treated with Pec at different concentrations (0, 5, 10, 25, 50 and 100 µM) for 48 h. Flow cytometric analysis demonstrated that Pec induced increased apoptosis with increased doses of Pec. Each experiment was performed three times. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. Pec, pectolinarigenin.

Figure 3. Pec-mediated expression of apoptosis-associated proteins is altered. (A and B) Western blot analysis demonstrated that Pec downregulated the expression of anti-apoptosis protein BCL-xL and upregulated pro-apoptosis protein Bax with increasing concentrations in A549 and Calu-3 cells. Each experiment was performed three times. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. Pec, pectolinarigenin; BCL-xL, B-cell lymphoma-extra large; Bax, apoptosis regulator BAX.
Figure 4. Pec inhibits the migration, invasion and EMT of NSCLC cells. (A) A cell wound scratch assay was performed to assess NSCLC cell migration; cells were treated without or with Pec (25 µM) for 24 h. (B) Transwell invasion assays were performed for NSCLC (A549, upper images; Calu-3, lower images) cells. (C) Quantitative analysis of E-cadherin and vimentin mRNA expression. NSCLC cells were not treated or treated with Pec (25 µM) and RNA was subjected to reverse transcription-quantitative polymerase chain reaction. (D) NSCLC A549 and Calu-3 cells were treated without or with Pec (25 µM), and the cellular extract was subjected to western blotting for analysis of E-cadherin and vimentin protein expression. Each experiment was performed three times. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. Pec, pectolinarigenin; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.
level was increased with increasing concentrations of Pec (Fig. 5). Previous studies suggested a complicated association and interaction between PTEN and p53 (26,27). To confirm whether p53 is involved in the pathway mediated by PTEN, cells were pre-treated with Pec and/or an inhibitor of PTEN (SF1670), and the protein expression level of PTEN and p53 was detected. The present results indicated that the altered expression of PTEN was associated with p53 expression. Treatment with SF1670 alone inhibited PTEN and p53 expression; whereas, combined treatment of Pec and SF1670 in NSCLC cells abolished the SF1670-induced effect on PTEN and p53 (Fig. 6A). Furthermore, activation of AKT by SC79, a novel AKT activator, decreased the expression of p53, which indicated that p53 was negatively regulated by PTEN/PI3K/AKT (Fig 6B). Generally, the present results revealed that Pec exerted antitumor activity through the PTEN/PI3K/AKT signaling pathway and p53 served an important role in the signaling pathway mediated by PTEN. The specific schematic diagram of PTEN/PI3K/AKT and the crosstalk between p53 and PTEN is presented in Fig. 7. Additionally, it was identified that Pec was able to promote the translocation of PTEN from the cytoplasm to the nucleus to exert antitumor activity (data not shown), which is consistent with previous studies (28).

**Discussion**

Lung cancer, particularly NSCLC, is responsible for the majority of cancer-associated mortalities; there were 1.69 million mortalities of patients with lung cancer reported in 2015 worldwide (3,7). Although the development of novel therapies has greatly ameliorated the conditions of patients with lung cancer, the 5-year survival rate has not improved much primarily due to resistance to antitumor agents and toxic side-effects (29). In clinical treatments, drug resistance remains one of the principal issues in developing a successful treatment for cancer. It is important to develop more effective drugs against lung cancer with fewer side-effects.

In recent years, there have been an increasing number of previous studies investigating the antitumor activity of medicinal plant extracts, which have successfully demonstrated their potential use in treatments. Previous modern pharmacological studies demonstrated the excellent antitumor effects of various medicinal plant extracts, including oxymatrine (30) and deguelin (31). Pec is a natural extract from a diverse range of herbal medicine and was previously demonstrated to have an antitumor effect on nasopharyngeal carcinoma (18) and osteosarcoma (19). However, there has been no study, to the best of our knowledge, on the biological activity of Pec in human NSCLC cells. With regard to the identification of the anticancer effects of Pec on NSCLC cells, in the present study, experiments were conducted to demonstrate the inhibitory effect of Pec on NSCLC cell proliferation and metastasis.

In the present study, it was observed that Pec was able to significantly decrease the cell viabilities of A549 and Calu-3 cancer cells in dose- and time-dependent manners with the administration of Pec. Pec-mediated cell apoptosis was
Figure 6. p53 is involved in the PTEN-mediated PI3K/AKT signaling pathway. (A) A549 and Calu-3 cells were treated with Pec (25 µM; 24 h) and/or PTEN inhibitor (SF1670; 10 µM; 1 h). Expression of the indicated proteins was detected by western blotting and subsequent statistical analysis was performed. (B) Following activation of AKT with SC79 (10 µM; 1 h), PTEN and p53 protein expression levels were additionally quantified by western blot analysis. Each experiment was performed three times. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. p53, cellular tumor antigen p53; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; PKT, protein kinase B; Pec, pectolinarigenin.
augmented with increasing Pec concentrations, demonstrating a dose-dependent effect. Furthermore, it was observed that Pec significantly inhibited the migration and invasion of cancer cells, and upregulated E-cadherin expression; whereas it downregulated vimentin expression. Collectively, these results indicated that Pec exerted an effective anticancer activity against lung cancer.

Furthermore, an aim of the present study was to elucidate the underlying mechanisms involved in Pec-induced antitumor progression. It was previously observed that PTEN, a tumor suppressor phosphatase, may negatively regulate the activation of the PI3K/AKT signaling pathway (22). The PI3K/AKT pathway is essential for maintaining cell growth, survival, death and metabolism, and is commonly activated in cancer initiation and progression (32). Activation of the PTEN/PI3K/AKT pathway may be a therapeutic molecular target for lung cancer. (26). In the past decade, it was demonstrated that various natural compounds positively regulated PTEN gene expression, consequently exerting an inhibitory effect against tumors. Similarly, in the present study, it was demonstrated that Pec activated PTEN and downregulated the activity of PI3K/AKT, which indicated that the PTEN/PI3K/AKT signaling pathway was involved in the Pec-induced antitumor effect. It was additionally revealed that Pec was able to promote the nuclear translocation of PTEN. Furthermore, p53 is a well-documented tumor suppressor protein and is involved in apoptosis, autophagy, cell cycle regulation, and DNA replication and repair. Previous studies have indicated that PTEN could regulate p53 protein stability by antagonizing the PI3K/AKT-mediated MDM2 (murine double minute 2)-p53 pathway or by interacting with p53 directly. The present results indicated that p53, as a complementary signal, was involved in the PTEN-mediated PI3K/AKT pathway. However, cancer cell growth and metastasis is a complicated process in which multiple signaling pathways are involved (33-35). Therefore, whether other signaling pathways are involved in the Pec-induced anticancer effect and the PTEN-mediated p53 signaling pathway through a phosphatase-dependent manner or phosphatase-independent manner requires further investigation.

In conclusion, the present study demonstrated that Pec exhibited an antitumor effect on human NSCLC cancer cell lines, which was able to inhibit NSCLC cell proliferation, metastasis and EMT, and promote apoptosis through the
PTEN/PI3K/AKT signaling pathway. Pec has potential for further development and may provide additional strategies for mono-therapy or combination treatments for NSCLC.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

FX and HP designed and conceived the study. FX and XG performed the experiments, analyzed the data and wrote the manuscript. HP reviewed and edited the manuscript, as well as provided funds. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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