Paris polyphylla ethanol extract induces G2/M arrest and suppresses migration and invasion in bladder cancer

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Background: Paris polyphylla is a traditional Chinese medicinal herb with multiple antitumor activities, but the role of P. polyphylla in bladder cancer (BC) is under investigation. This study aims to examine the antitumor activities of P. polyphylla ethanol extract (PPE) on BC cells and elucidate the underlying mechanisms.

Methods: Viable cells were counted using the trypan blue exclusion assay. The cell cycle was analyzed using flow cytometry, and scratch wound-healing and transwell assays were used to evaluate cell migration and invasion abilities, respectively. The protein expression levels were determined by western blotting. A xenograft model was used to assess the in vivo inhibitory effect of PPE on BC tumor growth.

Results: Our results showed that PPE inhibited the growth of BC cells in vivo and in vitro. Mechanistically, PPE regulated the levels of cell cycle-associated proteins, with PPE-induced G2/M phase arrest occurring through cyclin-dependent kinase inhibitor 1 (CDKN1A) accumulation and cyclin B1 (CCNB1)/cyclin-dependent kinase 1 (CDK1) inhibition. BC tumor growth was also inhibited by PPE treatment. Moreover, the migration and invasion abilities of J82 cells were suppressed through modulating epithelial-mesenchymal transition (EMT) regulatory factors with upregulation of cadherin-1 (CDH1) and downregulation of cadherin-2 (CDH2), snail family transcriptional repressor 2 (SNAI2), and twist family bHLH transcription factor 1 (TWIST1).

Conclusions: PPE inhibited cell growth, induced G2/M arrest, and suppressed the migration and invasion of J82 cells. BC tumor growth in vivo was also inhibited by PPE. Our results lay the foundation for further studies on the antitumor mechanisms of PPE.

Keywords: Urinary bladder neoplasm; medicine, Chinese traditional; cell cycle; epithelial-mesenchymal transition (EMT)

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Introduction

Bladder cancer (BC) is a malignancy of the urinary system that is common in both China and western countries (1-3). The American Cancer Society has reported that BC is the fourth most common cancer in men with an estimated 62,100 new BC cases occurring in the United States in 2020 (4). Drug resistance is a major obstacle to BC chemotherapy, and more than fifty percent of superficial BC will recur in the future (5). Thus, BC patients need periodic urinary cytology and cystoscopy exams, which are costly and invasive. Because of this life-long recurrence monitoring, BC has become the most expensive disease among all cancers (6). Therefore, the identification of novel and effective drugs for BC treatment is urgently needed.

*Paris polyphylla*, a traditional Chinese medicinal herb distributed widely in Southwest China, might possess a broad spectrum of biological activities, including hemostatic, antimicrobial and antivenom activities, and it has been used for thousands of years (7-9). Clinical and pharmacological studies have shown that *P. polyphylla* has a hemostasis function via contraction of the uterus (10). The polyphyllins isolated from *P. polyphylla* possess antifungal activity against *Cladosporium cladosporioides* and *Candida species* (11). Polyphyllin I has been reported to possess anti-inflammatory functions by inhibiting the phosphorylation of IKKα/β and p65, and suppressing the nuclear localization of p65 in the peritoneal macrophage-mediated inflammatory response (12). The components of *P. polyphylla* are used in more than 70 proprietary medicines, such as Gongxuening capsules, Chonglou jieduding tablets, and Jidesheng sheyao tablets (9).

*P. polyphylla* has also been used as a traditional Chinese medicine to treat cancers, including lung cancer, osteosarcoma, brain tumors, and other cancers (13). Polyphyllins, phytosterols and ecdysones are bioactive components that have been identified and isolated from *P. polyphylla* (7). Some of these chemical constituents have been demonstrated to possess antitumor activity. For instance, polyphyllin I induces apoptosis in gefitinib-resistant non-small cell lung cancer via downregulating the MALAT1/STAT3 signaling pathway (14), caspase-3, -8 and -9 are activated and apoptosis is enhanced by polyphyllin G in human oral cancer cells (15). Additionally, invasion and epithelial-mesenchymal transition (EMT) are inhibited by polyphyllin I through the CIP2A/PPI2A/ERK signaling pathway in prostate cancer (16). Additional antitumor activities of polyphyllins have also been found in gastric cancer (17) and glioblastoma (18); however, less is known about the antitumor effects of *P. polyphylla* on BC.

Abnormal growth and metastasis are the main characteristics of cancer. Cell mitosis is a precisely regulated process, and the cyclin B1 (CCNB1)/cyclin-dependent kinase 1 (CDK1) complex is a key regulator in G2 phase to M phase transition. Metastasis leads to approximately 90% of tumor-related deaths, and EMT is an essential process during tumor progression and metastasis. The activities of *P. polyphylla* toward BC have rarely been investigated. In this report, we studied the effects of *P. polyphylla* ethanol extract (PPE) on the cell growth, cell cycle, migration, invasion, and tumorigenic potential of BC cells. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-1512).

Methods

Extraction of *P. polyphylla*

*P. polyphylla* was purchased from the Pharmacy Department, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, and it was identified by Professor Xueshun Zhang. Two kilograms of *P. polyphylla* were ground into coarse powder, and a six-fold volume of EtOH/H₂O (60/40 v/v) was added to reflux extraction two times, for 2 hours each. The extraction solution was combined and filtered, reducing the pressure of the filtrate to recover the ethanol and concentrate it to a relative density of approximately 1.10 (60 °C). Two-fold volume of water was added and mixed, followed by incubation at 4 °C for 12 hours, and centrifugation at 6000 rpm for 5 minutes, precipitation and drying under a reduced pressure (65–70 °C, −0.08 Mpa) to collect the ground extract. The powder was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg/mL.

Cell culture

The J82 BC-derived cell line (kindly provided by Associate Chief Technician Feng Kong, Shandong Provincial Hospital; RRID: CVCL_0359) was used in this present study. J82 cells were maintained in minimum essential medium (MEM; cat. no. CM10025; Macgene) supplemented with 10% (v/v) fetal bovine serum (FBS; cat. no. 04-001-1ACS; Biological Industries). Cells were incubated in humidified incubators at 37 °C under an
atmosphere with 5% CO₂.

**Trypan blue exclusion assay**

The test was performed using the Trypan Blue Staining Cell Viability Assay Kit (cat. no. KGY015; KeyGEN BioTECH) following the manufacturer’s instructions. Briefly, J82 cells were treated with various concentrations of PPE for 24 hours and digested with trypsin. Cells were centrifuged at 300 g for five minutes after adding complete medium. The resulting cell pellet was washed once with PBS and resuspended in 100 μL of PBS. The cell suspension (90 μL) was gently mixed with 10 μL of trypan blue solution, and allowed to stain for one minute, and the unstained cells were then counted using a blood cell count plate.

**CCK-8 assay**

The PPE concentrations used in the migration and invasion assay were analyzed using the Cell Counting Kit-8 (CCK-8; cat. no. CK04; Dojindo). J82 cells were seeded in 96-well plates and cultured at 37 °C with 5% CO₂ for 24 hours, and various concentrations of PPE were added for 48 hours. After removing the medium, the J82 cells were incubated with new culture medium supplemented with 10% CCK-8 for 2 hours. Absorption at 450 nm was detected using a microplate reader (Thermo Fisher Scientific).

**Colony formation assays**

J82 cells were seeded in 6-well plates (800 cells per well) and cultured with complete medium. The cells were then treated with various concentrations of PPE, and incubated at 37 °C for 10 days. The cells were then fixed with 4% paraformaldehyde for 15 minutes and stained with 0.2% crystal violet for 30 minutes. After washing with PBS for 5 minutes, colonies exceeding 50 cells were counted using Image-Pro Plus 6.0 (Media Cybernetics; RRID: SCR_007369).

**Scratch wound-healing assay**

J82 cells were seeded in 6-well culture plates and grown to confluence overnight. Monolayer cells were scratched with a 200 μL pipette tip, and the cells were washed at least three times with PBS to remove detached cells. Fresh medium with 1% FBS was added, and photographs were obtained at 0, 24, and 48 hours using a microscope (ZEISS). The cells were incubated in a humidified incubator at 37 °C with 5% CO₂.

**Transwell assay**

Transwell chambers (cat. no. 353097; Corning) were precoated with Matrigel (cat. no. 356234; BD) and incubated at 37 °C for 30 minutes. J82 cells were digested and resuspended in serum-free MEM. The cell suspension (2.5×10⁴ J82 cells in 500 μL of medium) was added to the upper wells, and the lower wells were filled with 600 μL of MEM supplemented with 10% FBS. Subsequently, the chambers were incubated at 37 °C for 48 hours, and the cells were then fixed with 4% paraformaldehyde for 15 minutes and stained with 0.1% crystal violet for 30 minutes. Cells in the upper chamber were gently wiped off with cotton swabs, and the invaded cells on the lower surface were counted under a microscope.

**Western blotting**

Whole cell protein lysates were obtained using RIPA buffer (cat. no. P0013E; Beyotime) supplemented with 1% phenylmethanesulfonyl fluoride (PMSF; cat. no. ST506; Beyotime), and protein concentrations were quantified using the BCA Protein Assay Kit (cat. no. P0012; Beyotime). Proteins (40 μg) were separated on SDS-PAGE gels (cat. no. P0012A; Beyotime) and then transferred onto polyvinylidene fluoride (PVDF) membranes (cat. no. IPVH00010; Millipore). Membranes were blocked with 5% nonfat milk and then incubated with the following primary antibodies: CCNB1 (cat. no. #12231; RRID: AB_2783553) and CDK1 (cat. no. #28439; RRID: AB_2798959) (purchased from Cell Signaling Technology); cyclin-dependent kinase inhibitor 1 (CDKN1A; cat. no. 10355-1-AP; RRID: AB_2077682 and cadherin-1 (CDH1; cat. no. 20874-1-AP; RRID: AB_10697811) (purchased from Proteintech Group Inc.); and cadherin-2 (CDH2; cat. no. ab98952; RRID: AB_883292) and twist family bHLH transcription factor 1 (TWIST1; cat. no. ab50581; RRID: AB_10696943), snail family transcriptional repressor 2 (SNAI2; cat. no. ab27568; RRID: AB_777968) and twist family bHLH transcription factor 1 (TWIST1; cat. no. ab50581; RRID: AB_883292) (purchased from Abcam); all diluted 1:1,000. After incubation with secondary antibodies, a chemiluminescent horseradish peroxidase (HRP) substrate (cat. no. P90719; Millipore) was added, and the blots were imaged.
Flow cytometry

After starvation overnight and treatment with various concentrations of PPE for 24 hours, J82 cells were collected and stained using a flow cytometry analysis kit (cat. no. GMS10021.1; GenMed Scientifics Inc.). The cell cycle was analyzed by flow cytometry (BD), and ModFit LT (Verity Software House; RRID: SCR_016106) was used to analyze the results.

Xenograft studies

All animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong University (No.: 2019-005). Six-week-old male BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China) and housed in separate cages in rooms with a controlled temperature (22±2 °C), strict light-dark cycle (12:12) and airflow regulation, with free access to food and water throughout the study.

J82 cells (5×10⁶) were injected subcutaneously into the right flanks of the mice. When the tumors were palpable (2×2 mm), the mice were pair-matched by tumor size and divided into the following three groups: control group (0.9% sodium chloride, n=3), PPE (100 mg/kg) treatment group (n=3), and 5-FU (5 mg/kg) treatment group (positive control, n=3). Intragastric administration (control and PPE treatment groups) and intraperitoneal injections (5-FU treatment group) were performed daily, and the body weights and tumor sizes of the nude mice were measured every three days. After 21 days of treatment, the mice were sacrificed by cervical vertebra dislocation, and tumor tissues were collected for further analysis.

Statistical analyses

Data comparisons were conducted with one-way ANOVA, and the results are presented as means ± SD. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.; RRID: SCR_002798). Differences were considered to be significant when P<0.05.

Results

PPE inhibits the growth of J82 cells

To evaluate the effect of PPE on BC, J82 cells were treated with various concentrations of PPE (0, 1, 3, 9, 27, and 81 μg/mL) for 24 hours. Cisplatin was used as a positive control, and viable cells were counted using the trypsin blue exclusion assay. The results showed a dose-dependent decrease in the number of J82 cells after exposure to PPE. The IC₅₀ value of PPE for J82 cells was 5.06±0.89 μg/mL, which is comparable to that of cisplatin (IC₅₀ =3.10±0.77 μg/mL) (Figure 1A). J82 cells were seeded into 6-well plates, and PPE was added as indicated. The imaging results showed that the morphology of cells in the PPE treatment and control groups was not obviously different. However, 3.2 μg/mL PPE notably inhibited J82 cell numbers after treatment for 24 hours (Figure 1B). Furthermore, the effect of PPE on the colony formation ability of J82 cells was assessed. The colony formation assay results showed that PPE significantly inhibited colony formation after treatment with PPE at 0.6, 1.2, and 1.8 μg/mL for 10 days (Figure 1C,D). These results indicated that PPE suppressed the growth of J82 cells in a dose-dependent manner. Furthermore, 3.2 μg/mL PPE was used as the highest concentration assayed in subsequent experiments.

PPE induces G2/M phase arrest in J82 cells

To assess the effect of PPE on cell cycle arrest in J82 cells, flow cytometry was used to analyze the cell cycle distribution. The results revealed an accumulation of cells in G2/M phase after exposure to different concentrations of PPE, which gradually increased in a dose-dependent manner from 14.66% in the control group to 32.46% in the 3.2 μg/mL PPE treatment group (Figure 2A). Statistical analysis showed that the distribution of cells in G2/M phase arrest significantly increased after treatment with PPE at 1.6 and 3.2 μg/mL (Figure 2B). Taken together, these results suggested that PPE induced G2/M phase arrest in J82 cells.

PPE inhibits xenograft tumor growth in vivo

To investigate the effect of PPE on BC cells in vivo, a BC xenograft model was established as described in the Methods section. After 21 days of PPE treatment, mice were sacrificed, and tumor tissues obtained. Compared with the control group (n=3), the tumors from the 5-FU (n=3) and PPE (n=3) treatment groups were relatively small (Figure 3A). Furthermore, statistical analysis showed that both 5-FU and PPE significantly limited tumor weights (Figure 3B). The tumor growth curves for the control, PPE treatment, and 5-FU treatment groups are shown in
Figure 1 PPE inhibits J82 cell growth. (A) J82 cells were treated with PPE or cisplatin for 24 hours. Subsequently, viable cells were enumerated using the trypan blue exclusion assay. (B) Different concentrations of PPE were used to treat J82 cells for 24 hours. Cell morphologies and cell growth were recorded, and representative images are shown. Scale bar =100 μm. (C) J82 cells were treated with PPE as indicated, and representative images of colony formation are shown. (D) The number of colonies was calculated and presented as the means ± SD of three independent experiments (* indicates P<0.05; *** indicates P<0.001). PPE, *P. polyphylla* ethanol extract.
Figure 2 PPE induces G2/M phase arrest in J82 cells. J82 cells were seeded into 6-well plates and starved for 12 hours. Different concentrations of PPE (as indicated) were used to treat J82 cells for 24 hours, and cell cycle analyses were then performed. Cell cycle distributions are shown in (A and B). G2/M cell cycle arrest was statistically analyzed, and the results are presented as the means ± SD of three independent experiments (NS represents not significant; * P<0.05; *** P<0.001). PPE, *P. polyphylla* ethanol extract.

Figure 3 PPE inhibits xenograft tumor growth *in vivo*. (A) After 21 days of PPE treatment, the mice were sacrificed, and tumor tissues were obtained (scale bar =1 cm). (B) Tumor weights were calculated, and the results are presented as the means ± SD. The tumor sizes and weight curves of the three groups are shown in (C and D), respectively (NS represents not significant; * indicates P<0.05). PPE, *P. polyphylla* ethanol extract.
Figure 3C. Tumor volumes were significantly lower in the PPE and 5-FU treatment groups than the control group on days 21 (P<0.05). Moreover, the inhibitory activity of PPE with respect to tumor volume was comparable to that of 5-FU. The weights of the mice in the three groups are shown in Figure 3D. These results demonstrated that PPE inhibited xenograft tumor growth in vivo.

**PPE suppresses migration and invasion of J82 cells**

Migration and invasion are crucial processes in tumor cell metastasis. The CCK-8 assay was used to select PPE concentrations to be used in the migration and invasion assays, and this assay showed that cell viability was not affected by PPE at 0.8 and 1.6 μg/mL (Figure 4A). The effects of PPE on migration and invasion of J82 cells were determined by scratch wound-healing and transwell assays, respectively. According to the images and statistics, the cells treated with PPE (0.8 and 1.6 μg/mL) for 48 hours showed significantly inhibited cell migration rates (Figure 4B,C) and numbers of invading cells (Figure 5A,B) compared with the control group (0 μg/mL). In addition, the inhibitory effect of PPE on migration and invasion was dose dependent.

**PPE modulates the levels of cell cycle-associated proteins and EMT-associated biomarkers in J82 cells**

To elucidate the molecular mechanisms underlying the growth inhibitory effects of PPE, J82 cells were starved overnight, and the levels of G2/M phase regulation-associated cytokines, including CDKN1A, CCNB1, and CDK1, were measured in J82 cells. The results revealed
that PPE treatment increased the level of CDKN1A, whereas it decreased those of CCNB1 and CDK1 (Figure 6A). These results indicated that PPE-induced G2/M phase arrest may occur via CDKN1A accumulation and inhibition of CCNB1/CDK1 expression.

EMT-associated biomarkers play essential roles in the process of metastasis. We analyzed whether PPE could repress BC cell invasion and migration through regulating the biomarkers. J82 cells were treated with PPE at concentrations of 0, 0.4, 0.8, 1.6, and 3.2 μg/mL for 48 hours, and EMT-related proteins were detected by western blot analysis. The results showed that the expression of CDH1 was increased after PPE treatment, but CDH2 expression was decreased. Moreover, the transcription inhibitors of CDH1 were also evaluated by western blot analysis, and the results showed that the expression levels of SNAI2 and TWIST1 were decreased (Figure 6B). These results indicated that PPE-induced inhibition of migration and invasion might occur through modulating EMT-associated biomarkers.

**Discussion**

BC is a common malignancy, and the cost of BC examination and treatment is high. Although resection, radical cystectomy, chemotherapy, and immunotherapy have been used to treat BC (19), drug resistance and recurrence remain unsolved issues. Therefore, the development of
more effective drugs has been widely awaited. Antipyretic-
detoxicate herbs are often used for cancer treatment in
traditional Chinese medicine (20,21). As a heat-clearing and
detoxifying herb, \textit{P. polyphylla} shows potential antitumor
properties (22,23). Nevertheless, reports on the use of \textit{P.
polyphylla} to treat BC are rare. The results of the present
study demonstrated that PPE has multiple antitumor
functions in BC, including the inhibition of cell growth,
induction of G2/M arrest and suppression of cell migration
and invasion. BC cell growth \textit{in vivo} is also inhibited by
PPE.

The results of our previous study demonstrated that
PPE could decrease the expression of mutant TP53 protein
in BC cells (8). The results of the current study provide
further insight into the mechanism of PPE activity against
BC, and we found that CCNB1 and CDK1 are downstream
effectors of PPE-induced G2/M phase arrest. We further
showed that PPE not only induces G2/M phase arrest but
also suppresses the migration and invasion of BC cells.
Moreover, we confirmed that PPE inhibits tumor cell
growth \textit{in vivo}.

Because sustained and proliferative signaling are
hallmarks of cancer (24), targeting the aberrant expression
of cell cycle regulators is a common cancer treatment
strategy. CCNB1 and CDK1 are involved in regulating G2/
M phase arrest, and CDKN1A is located upstream of these
two regulators and inhibits their activity (25,26). CDKN1A
expression is promoted by TP53 accumulation, but TP53
is mutated in J82 cells (27). Although PPE may reduce
mutant TP53 protein levels and increase CDKN1A levels,
CDKN1A is also regulated by factors independent of TP53,
including ERs, STATs, and E2Fs (28). Thus, identification
of the target sites of PPE and determination of whether it
acts in a TP53-independent manner are important points
that remain to be further investigated.

Tumor metastasis is the main reason cause of cancer-
related death (29), and it is an incurable disease. Therefore,
the screening of anti-metastasis agents has become a
priority. EMT is involved in tumor metastasis, and the
vital characteristics of EMT are the upregulation of
mesenchymal markers, such as CDH2, and downregulation
of epithelial markers, including CDH1 (30). Traditional
Chinese medicine provides a material and structural basis
for drug screening. \textit{P. polyphylla} possesses multiple biological
activities, and PPE is an ethanol extract of \textit{P. polyphylla}. Our
results showed that PPE inhibits BC cell migration and
invasion. We further showed that PPE upregulates CDH1
but downregulates CDH2, SNAI2, and TWIST1 protein
expression levels. These findings indicated that PPE may be
a potential candidate for anti-metastasis agents and merits
further study.

Although natural and traditional herbs have some
limitations, such as heterogeneity, methodological flaws
and a limited regulatory framework, natural products have
been an essential source of new drugs in the past nearly
40 years (31), and traditional Chinese medicine has been
applied in anti-tumor treatments in the clinic (32-34).
With quality control optimization and detailed mechanism
of action elucidation, recognition of traditional Chinese
medicine will be further improved.

Although our results showed that PPE could induce
G2/M arrest and inhibit the migration and invasion of BC
cells, there is still a long way to go before its application
in clinical treatment, including elucidation of the detailed
molecular mechanism underlying the anti-tumor effect
of PPE, whether the main polyphyllins possess the anti-
tumor effect, and which polyphyllin exerts the most
prominent antitumor activity against BC. Furthermore,
oral or intravesical administration also merits further study.
Nevertheless, we provide a potential candidate drug for
further development in the treatment of BC.

Conclusions

The results of this study demonstrated that PPE has
multiple antitumor functions toward BC, including the
inhibition of cell growth, induction of G2/M arrest, and
suppression of migration and invasion. BC cell growth
\textit{in vivo} was also inhibited by PPE. Thus, our results lay the
foundation for further studies of the antitumor mechanisms
of PPE.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No.: 2019-005) granted by the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong University, in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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