

RESEARCH COMMUNICATION

Paris polyphylla* Smith Extract Induces Apoptosis and Activates Cancer Suppressor Gene Connexin26 Expression*Fu-Rong Li^{1&}, Peng Jiao^{3&}, Shu-Tong Yao², Hui Sang², Shu-Cun Qin³, Wei Zhang¹, Ya-Bin Zhang², Lin-Lin Gao^{2*}****Abstract**

Background: The inhibition of tumor cell growth without toxicity to normal cells is an important target in cancer therapy. One possible way to increase the efficacy of anticancer drugs and to decrease toxicity or side effects is to develop traditional natural products, especially from medicinal plants. *Paris polyphylla* Smith has shown anti-tumour effects by inhibition of tumor promotion and inducement of tumor cell apoptosis, but mechanisms are still not well understood. The present study was to explore the effect of *Paris polyphylla* Smith extract (PPSE) on connexin26 and growth control in human esophageal cancer ECA109 cells. **Methods:** The effects of PPSE on Connexin26 were examined by RT-PCR, western blot and immunofluorescence; cell growth and proliferation were examined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. **Results:** PPSE inhibited the growth and proliferation on esophageal cancer ECA109 cells, while increasing the expression of connexin26 mRNA and protein; conversely, PPSE decreased Bcl-2 and increased Bad. **Conclusion:** This study firstly shows that PPSE can increase connexin26 expression at mRNA and protein level, exerting anti-tumour effects on esophageal cancer ECA109 cells via inhibiting cell proliferation and inducing cell apoptosis.

Keywords: Connexin - *Paris polyphylla* smith - esophageal cancer - apoptosis

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Introduction

Paris polyphylla Smith (Liliaceae) is distributed in many regions of the world, such as India, China, Vietnam, and Germany. As a traditional Chinese medicine, it grows widely throughout South China and has been used mainly as a folk remedy for treatment of abscesses, throat swelling and pain, thanatophidia bites, contused wounds and convulsions for centuries. It is also the major component medicine of the famous Chinese patent medicine yunnan baiyao powder and snake-bite therapeutics. It also has been used to treat liver cancer in China for many decades (Lee et al., 2005; Shoemaker et al., 2005).

The active components of *Paris polyphylla* smith are the saponin steroids polyphyllin D, dioscin, and balanitin 7. Among its three chemical constituents, polyphyllin D and dioscin have been previously reported (Deng et al., 1999; Li et al., 2001; Cheung et al., 2005; Gao et al., 2011) to circumvent drug resistance and elicit apoptosis in liver cancer HepG2, R-HepG2, cells (Deng et al., 1999; Li et al., 2001; Cheung, et al., 2005). However, as there has been no documentation of the use of the extract of *Paris polyphylla* smith (PPSE) in the treatment of cancer, its mechanisms in human esophageal cancer cells remain unknown. Therefore, the aim of the present study was to evaluate the effects of PPSE on human esophageal cancer

ECA109 cells and the signaling pathways involved in PPSE-induced apoptosis.

Gap junction (GJ) is specialized cell-cell junctions that directly link the cytoplasm of neighboring cells. They mediate the direct transfer of low-molecular-weight (<1000 Da) metabolites and ions, including second messengers such as cyclic AMP, inositol triphosphate and Ca²⁺, between adjacent cells (Oyamada et al., 2005). Each GJ channel is formed by two hemichannels (connexons), and each connexon is composed of six individual transmembrane proteins called connexins (Martin et al., 2004). To date, at least 21 members of connexins proteins have been identified in mammalian. Certain connexins have been reported to have tumor suppressing effect, including connexin43, connexin32 and connexin26 (Tanaka et al., 2004; Fujimoto et al., 2005; Decrock et al., 2009). Connexin 26 is one of the most frequently investigated Connexin proteins which shows growth inhibition and induction of apoptosis (Connexins) (Tanaka et al., 2004). Most of cancer cell express low level of connexin26 and restore the connexin26 show significantly inhibit the tumor cell growth and proliferation. Therefore connexin26 is a potential target for cancer therapy.

In the present study, we show that PPSE can increase connexin26 and inhibit the growth and proliferation partly by reduction of Bcl-2 and increased Bad in ECA109 cells.

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Materials and Methods

Chemicals and reagents

RPMI-1640 medium, fetal calf serum and trypsin were purchased from GIBCO (Canada). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, and trypsin were purchased from Amresco Chemical Co. Ltd. (USA). Propidium iodide (PI) was purchased from Sigma (USA). The Annexin V/PI-FITC apoptosis detection kit was purchased from BD Biosciences (USA). The primary antibodies for Bcl-2, Bad, β -actin and the secondary antibody were acquired from Cell Signaling Technology (USA), and all chemicals were of analytical grade and were obtained from Tianjin Chemical Reagents Co. Ltd. (Tianjin, China).

Preparation of ethanol extracts from *Paris polyphylla* Smith

The place of production of *Paris polyphylla* Smith was Yunnan, China and dried root parts were purchased from Jinan Jianlian herbal medicine drug shop, China. The identities of these herbs were confirmed by comparison with descriptions of characteristics and appropriate monograph in Chinese Pharmacopoeia. The lumpy roots (100g) were ground with a crushing machine to pass a 1 mm screen and were extracted with 95% ethyl alcohol (EtOH) for 3 days at room temperature and filtered through Whatman No.1 filter paper (Advantec, Tokyo, Japan). The EtOH solvent was then removed by evaporation in vacuo, and an auburn coloured dried extracts (14.8g) EtOH extracts were obtained. This EtOH extracts were named PPSE and stored in a refrigerator at 4°C until used.

Cell culture

ECA109 cells were obtained from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China), cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂; the medium was changed every other day. When the cultures were 80 to 90% confluent, the ECA109 cells were washed with phosphate-buffered saline (PBS, pH 7.4), detached with 0.25% trypsin, centrifuged and re-plated onto 96- or 24-well plates at an appropriate density according to each experimental scale.

MTT assay

The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsin and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μ l/well and incubated in an incubator with 5% CO₂ at 37°C. After 24 hours, 200 μ l of various concentrations (0 to 200 μ g/ml) of PPSE were added and incubated for 24, 48, 72 and 96 hours to evaluate their anti-proliferation effects on ECA109 cells. The cell proliferation in the microplate was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide) assay (Chang,

et al., 2008) after incubation. Twenty microliters of PBS solution containing 5 mg/ml MTT was added to each well. After incubation for 4 hours, the cells from each well were solubilized with 100 μ l DMSO for optical density determination at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control).

Reverse transcription PCR

Total RNA was isolated from cell lines using Trizol Reagent according to the manufacturer instruction. Synthesis of cDNA from mRNA transcript was performed with Reverse transcriptional kit (TAKARA, Shimon-Ku, Japan) The PCR reactions of Connexin26 (primer forward 5-GGAAG CTTCC TCCCG CGCAG -3, reverse 5-CTT CAC CAGACGCT GCAT-3) and β -actin (primer forward 5-'GATGCAGAA GGAGATCACTG-3, reverse 5-GGGTGT AACGCAACTAAGTC-3', consisted of a hot start (5 min at 94°C), 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 40 sec at 72°C, followed by final extension step at 72°C for 10 min.

Western blot

Cells were rinsed once with PBS and then scraped off with lysis buffer (Pierce, USA), added protease inhibitor cocktail tablets (Roche, Switzerland). Total protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, USA). Primary antibody Connexin26, Bcl-2, Bad and tubulin are pursued from (Santa Cruz, CA, USA). Western blot analysis was carried out as described previously (Liu et al., 2009).

Immunofluorescence staining

ECA109 cells were plated on glass slides in the 24-well plates. When cells reached 50% confluence, they were treated with 0.1% DMSO (V/V) vehicle control for 24 h. The Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100. Then blocked with 10% goat serum, the cells were reacted with anti-mouse monoclonal Connexin26 antibody followed by FITC-conjugated goat anti-mouse antibody (Sigma, USA) diluted in PBS-Evans blue. Fluorescence was visualized using fluorescent microscope.

Statistical analysis

All experiments were repeated at least three times. The values reported the mean of triplicates (\pm SD). Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS). A p-value of 0.05 (two-sided) was considered statistically significant.

Results

Cytotoxic activity of PPSE on ECA109 cells

MTT assays were performed to determine whether PPSE treatment suppressed the growth of ECA109 cells. As shown in Figure 1, PPSE significantly inhibited the viability of ECA109 cells and decreased the cell survival rate as compared to the vehicle control. Cells were observed that vehicle treated ECA109 cells (control) grew well with clear skeletons, whereas cells treated with PPSE

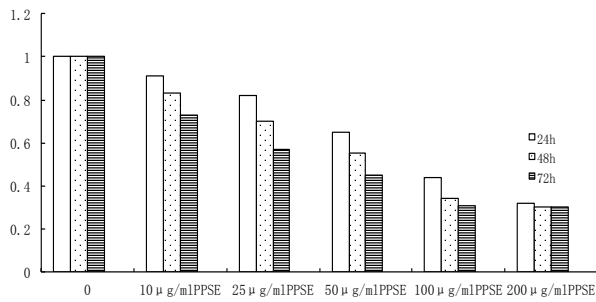


Figure 1. PPSE Inhibited the Growth and Proliferation on ECA109 Cells. (A) ECA109 cells were treated with vehicle control and PPSE of various concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) for 24, 48, and 72 h. After that, survival cell were assessed by MTT array

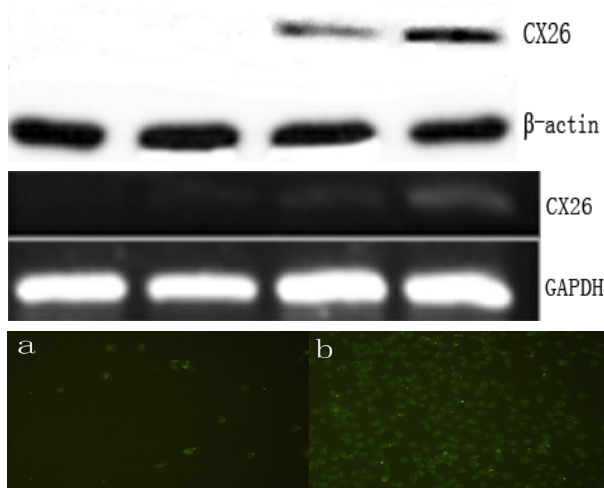


Figure 2. PPSE Induced Connexin26 mRNA and Protein Expression on ECA109 Cells. (A) Total RNA was isolated from the cells treat with different concentration of PPSE and the gene expression was assessed by RT-PCR. Results of the Connexin26 mRNA expression of the cells treated with different concentrations of PPSE for 24 h. (B) Lysate from the cells with different treatments and the protein expression was assessed by Western blot. Results of the Connexin26 protein expression of the cells treated with different concentrations of PPSE for 24 h. (C) Connexin26 expression and localization following 25 µg/ml PPSE treatment for 24 h, assessed by using immunofluorescence staining analysis. The images were visualized by fluorescent microscope

exhibited cytoplasmic shrinkage and either detached from each other, floated in the medium, or became distorted and blurry under a phase contrast microscope. The number of sloughed cells increased with increasing drug concentrations.

PPSE induce Connexin26 mRNA and protein expression

As we showed previously, PPSE inhibited the esophageal cancer ECA109 cell growth and proliferation, however the mechanism of the PPSE anti-cancerous effect was not clear. Most of the esophageal cancer cell lines have low level of Connexin26. ECA109 cells are highly metastatic human esophageal cancer cell line, which express barely detects level of Connexin26 and do not form functional gap junction. We used it as a model to test whether PPSE induced the Connexin26 expression and displayed the anticancer effect. According the MTT assay results, we treated the ECA109 cells with three different

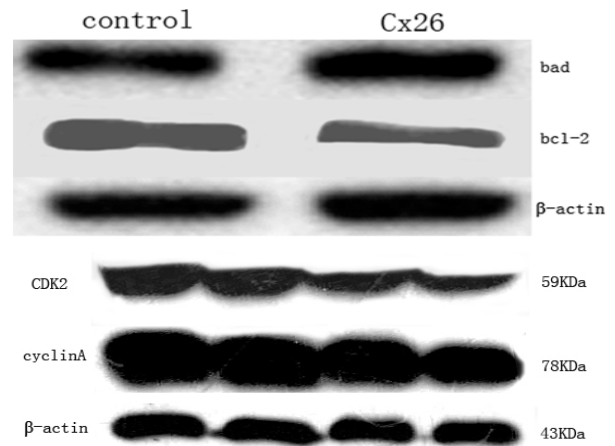


Figure 3. WB Detected the Connexin26 Protein Overexpression in the ECA109 Cells

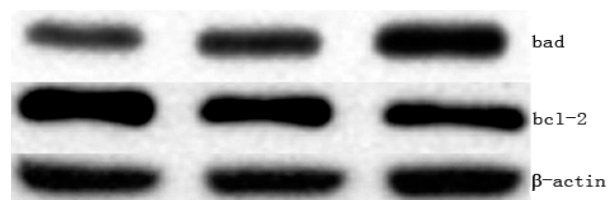


Figure 4. PPSE Treatment Down-regulated Bcl-2 and Upregulated Bad Expression. Cell treated with two different concentrations of PPSE (50 µg/ml and 100 µg/ml), Bcl-2 and Bad expression was assessed by Western blot analysis, β-actin as loaded control

concentrations of PPSE for 24 h. Semi-quantitative RT-PCR (Figure 2A), Western blot analysis (Figure 2B) and immunofluorescence (Figure 2C) revealed an increase in endogenous Connexin26 mRNA and protein expression following by PPSE treatment, especially on 25 µg/ml PPSE. However higher concentration of PPSE, such as 100 µg/ml, did not induce higher level of Connexin26 (data not show).

PPSE down-regulate Bcl-2 and up-regulate Bad expression

Now PPSE upregulated the Connexin26 mRNA and decreased the cell survival on ECA109 cells. We next detected the anti-apoptotic and pro-apoptotic family member Bcl-2 and Bad, to figure out whether it was involved in apoptotic pathway. We examined the Bcl-2 and Bad protein following the PPSE treatment on ECA109 cells by Western blotting. The result showed that PPSE treatment decrease the Bcl-2 level, especially on 100 µg/ml, and stable express of Connexin26 gene on ECA109 cells showed the similar result (Figure 3); We also found that apoptotic protein Bad exhibited the inverted result (Figure 4), which showed that Bcl-2 and Bad palyed an important role on the PPSE involved anticancer effect in esophageal cancer ECA109 cells.

Discussion

So far, the underlying mechanisms of the pharmacological effect of *Paris polyphylla* Smith in cancer therapy have been unclear, and this study examined the effect of PPSE and its underlying mechanisms on

inhibition of tumor cell proliferation. In the present study, PPSE also has been shown to inhibit the growth on ECA109 cells. However, the antitumor effects of PPSE on esophageal cancer cells through gap junctional mechanism have not been investigated. This study provides the first evidence that PPSE induce the Connexin26 mRNA and protein expression and exhibit its growth inhibition on ECA109 cells.

Most of the esophageal cancer cell is lost or impaired of Connexins comparing with the normal esophageal epithelial cell, which suggest that the Connexins is related to the esophageal carcinogenesis. Some studies also showed that Connexins is involved in the esophageal cancer progression (Jadranka et al., 2003). The raise of Connexins can inhibit the growth of esophageal cancer cells and have the synthesis effects of chemical treatments (Singal et al., 2000). A number of studies showed that Connexins can exert its anti-tumor effects by forming gap junctional intercellular communication (GJIC), therefore increase Connexins and improved GJIC may establish a new, effective therapy for esophageal cancer. However, transfection of Connexin26 gene into esophageal cancer ECA109 cells reversed the transformed phenotype without enhancing the activity of GJIC, which suggest that independent function of Connexins also play an important role on cell growth, tumorigenicity and differentiation (Tanaka et al., 2001).

Esophageal cancer ECA109 cells expressed low levels of Connexin26 which were used as the model to examine the effects of PPSE on Connexin26 by RT-PCR, western blot and immunofluorescence. The data showed that PPSE induced the expression either Connexin26 mRNA or protein, and both stable expression also had growth suppressive effect on ECA109 cells. Thus Connexin26 played an important role on PPSE induced growth control. It has been reported (Inose et al., 2009) that clinicopathologic outcome of abnormal expression of Connexin26 showed prognostic significance in human esophageal squamous cell carcinoma, In accord with this study which ECA109 cell displayed a upregulation of Connexin26 by the treatment of PPSE. The results showed that PPSE induced the expression of Connexin26 on ECA109 cell, and Connexin26 is contributed to the anti-tumor effect of PPSE.

To examine whether the raise of Connexin26 by PPSE would lead to the activation of GJIC, the localization of Connexin26 protein was detected by immunofluorescence. The results showed that followed the PPSE treatment, the Connexin26 staining was enhanced, suggesting increase in the number and size of gap junction plaques, and it also has been confirmed by the exogenous expression of Connexin26 in ECA109 cells. However the mechanism which PPSE elicits Connexin26 upregulation is still unclear, and need further research.

Apoptosis plays a critical role in embryogenesis, carcinogenesis and virally infected cell death. Prior to this study, our research group founded that the anti-tumor effects of Dioscin from traditional Chinese anti-snake venom medicine *Paris chinensis* (PCD) and correlated mechanisms regarding apoptosis in human gastric cancer SGC-7901 cells (Gao et al., 2011) and human ovarian

cancer SKOV-3 cells (Gao et al., 2011). And in this study PPSE also displayed the growth and proliferation inhibition on ECA109 cells. We next tested that Bcl-2 family member Bcl-2 and Bad were involved in the PPSE inducing ECA109 cell growth inhibition and apoptosis. Interestingly, previous studies showed that Connexin26 served as an anti-tumor gene, one of the mechanism was that it could reduce the Bcl-2 (Singal et al., 2000). The data showed that PPSE treatment not only decreased the Bcl-2, increased Bad, but also had growth inhibition by exogenous expression of Connexin26 in ECA109 cell.

Therefore, our results suggest that PPSE can increase the Connexin26 gene expression and the raise of Connexin26 can inhibit the growth of ECA109 cell partly by the reduction of Bcl-2 and increase of Bad.

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