RESEARCH COMMUNICATION

Chemoprevention by *Prunella vulgaris* L. Extract of Non-Small Cell Lung Cancer Via Promoting Apoptosis and Regulating the Cell Cycle

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Abstract

Chemoprevention is one feasible approach to decreasing morbidity and mortality of non-small cell lung cancer (NSCLC). The present study aimed to explore the mechanisms of chemoprevention of NSCLC by *Prunella vulgaris* L. (PV) using a PV extract of 60% ethanol (P-60). In an A/J mouse model benzo[a]pyrene induction of lung tumors was significantly reduced difference by P-60 group. In addition, P-60 was found to have the ability to regulate cell cycle and induce apoptosis in SPC-A-1 cells. Therefore, we propose that P-60 has potential as a lung cancer chemopreventive agent.

Keywords: *Prunella vulgaris* L.- in vivo chemoprevention - NSCLC - apoptosis - cell cycle - SPC-A-1 cells

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Introduction

Lung cancer, including small cell lung cancer and non-small cell lung cancer, is the leading cause of cancer deaths in the world (Minna et al., 2002). Non-small cell lung cancer (NSCLC) is the main common type in all cases, accounting for about 80%. It can be caused by carcinogenic substances such as chemicals from tobacco smoke (Hecht, 2002), pollution such as ionizing radiation (Shin et al., 2002), and viral infection (Kountouri et al., 2010). Its four-year morbidity and mortality were found to be 95-100/100 000 and 87%, respectively (Parkin et al., 2001; Blanchon et al., 2006; Eilstein et al., 2008). Common treatments for lung cancer include surgery, radiotherapy and chemotherapy; however, these treatments often result in serious side effects such as bone marrow suppression, leukopenia, impaired immune function, nausea and vomiting (Nico, 2001). Therefore, early prevention and treatment need to be stressed.

Chemoprevention is one possibility (Han et al., 2009). It has been reported that p53 gene, mediating the cell apoptosis, is highly related to the occurrence and development of NSCLC. It is also known that cytoplasmic p53 may rapidly translocate to the mitochondria under pro-apoptotic stress (Robbins et al., 2010). Cell cycle regulation and apoptosis induction were recognized as the underlying apoptosis mechanisms of chemoprevention of NSCLC (Nakamura et al., 2009; Murugan et al., 2010).

Prunella vulgaris L., a Labiatae plant, is used commonly as dietary supplements in world, which is effective in preventing or treating diseases. It has been reported that it had immune modulatory effects through activating NF-kappaB and MAP kinase (Collins et al., 2009), antiestrogen receptor (Choi et al., 2010), and antitumor activity (Lee et al., 1988). An extract has inhibited mutagenicity and carcinogenicity of benzo[a] pyrene, 1,6-dinitropyrene and 3,9-dinitrofluoranthene (Horikawa et al., 1994; Vostálováet al., 2010). It also prevents UVB-induced DNA damage and oxidative stress in HaCaT keratinocytes (Cheung et al., 2008). PV is rich in phenolic acids, flavonoids, coumarins, triterpenes, volatile oil, polysaccharides (Feng et al., 2010; Moon et al., 2010), all demonstrating chemopreventive potential (Tanaka et al., 1993; Lin et al., 2008; Petronelli et al., 2009).

The purpose of this study was to investigate the chemoprevention effect of PV extract on non-small cell lung cancer both *in vitro* and *in vivo*. There are many lung cancer animal models (Malkinson et al., 1992) including carcinogen-induced (Das et al., 2007) and transgenic models (Liu et al., 2002). Here we used benzo[a]pyrene with A/J mice. In addition, the activity of cell cycle regulation and apoptosis induction were also studied to explore the possible chemoprevention mechanisms.

Materials and Methods

Plant material

4 kg dried spikes of PV was ordered from medicinal corporation of Bozhou, Anhui province, China. Herb was authenticated as *Prunella vulgaris* L. by Professor D.K. Wu, from Nanjing University of Chinese Medicine.

Chemicals

Cisplatin was ordered from Jiangsu Hengrui Medicine

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Co., Ltd., (Lianyungang, China, batch number: 08062524). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Sigma (USA) and benzo[a]pyrene (B[a]P) was purchased from Aladdin (Shanghai, China). V-FITC Apoptosis Detection Kit was ordered from Nanjing KeyGen Biotech. Co. Ltd (Nanjing, China).

Preparation of PV extracts

4 kg spike of PV was weighted and refluxed successively in vacuum reflux accumulator (20 L) with 10-fold amount of 60% ethanol (v/v), 30% ethanol (v/v) and distilled water for 2 h/time (2 times). Extracts were merged and concentrated by rotary evaporation at 60°C and further dried in a vacuum oven at 60°C. The final crude yields were obtained after volatilizing the solvent, and the obtained extracts were 180 g, 157 g and 157g, respectively.

Cell culture

SPC-A-1 cells were routinely maintained in RPMI-1640 medium, supplemented with 10% fetal calf serum, 100 U/mL of penicillin and 100 U/mL of streptomycin in a humidified incubator at 5% CO_2 and 37°C. The medium was renewed every 2 days. The cells were digested by 0.25% trypsin-0.01% EDTA and used for seeding into 96 or 24-well plates.

Cell proliferation assay

Cell proliferation inhibition of different concentration P-60 on SPC-A-1cell was determined by MTT assay. Briefly, cells were seeded in 96-well plates (4×10^4 cells/mL) and treated with the crude extract (16, 80, 400 and 2000μg/mL) for 48 h. Each well was added with 100 μL of MTT (5 mg/mL) and incubated at 37°C for 4 h. The MTT solution was discarded and the wells were added with 100 μL of DMSO to dissolve the formed formazan. The samples were examined on a SPECTRAmax 190 microplate spectrophotometer (Molecular Devices, USA) at 580 nm.

Chemoprevention activity in A/J mice

Female A/J mice were obtained from Nanjing Model Animal Research Center. The mices were maintained under standard conditions at 25°C ± 2°C and 50% ± 10% relative humidity, and fed with a standard diet and water at random. A/J mice received B[a]P in corn oil (100 mg/kg) by intraperitoneal injection. One week after injection, mices of the control group were given orally 0.4 mL saline solution (0.9% NaCl) everyday for 24 weeks, while mices of the treatment group were given orally 0.4 mL P-60 (10.0 g/kg) everyday for 24 weeks. After 24 weeks, the mices were sacrificed with CO_2. Lungs were removed and fixed in Tellyesniczky's solution (70% ethanol, methanol and acetic acid in a ratio of 20:5:3) for at least 24 h, and then stored in 70% ethanol (v/v). The number of tumors was calculated to determine the tumor inhibition ratio. Photographs of the samples were taken using a (SZX7, OLYMPUS, Cannon camera) with auto-focus.

Apoptosis detection

The cells were seeded in 25cm^2 flask (Gibco, Invitrogen, USA) for apoptosis analysis. After being cultured for 2 days, the cells were treated with the P-60 in 125, 250 and 500 μg crude drug/mL and then maintained at 5% CO_2 and 37°C for 48 h. These cells were detached with 0.25% trypsin-0.01% EDTA solution and centrifuged at 2000 × g for 5 min. After removing supernatant, the cells were washed twice with phosphate buffered solution (PBS, pH =7.4) and centrifuged at 2000 × g for 5 min to collect 5×10^5 cells. Cells were stained with 5μL annexin V-FITC and 5μL propidium iodide according to the manufacturer's instructions of V-FITC apoptosis detection kit. Then the cells samples were detected by using a flow cytometer (Beeton-Diekinson, USA) with fluorescence excitation wavelength at 488 nm and emission wavelength at 530 nm.

Cell cycle analysis

SPC-A-1 cells (4×10^4 cells/mL) were seeded in 25cm^2 flask for cell cycle distribution analysis. The cells were treated with various concentrations of P-60 (125, 250 and 500 μg crude drug /mL) for 48 h and then detached by using 0.25% trypsin-0.01% EDTA solution. Cell suspension was fixed with 70% ethanol (v/v) for 2 h and washed in PBS, then added with 100 μL RNase A (1 mg/mL) and heated in a warm bath at 37°C for 30 min. The cells were then stained with 400 μL propidium iodide (50 μg/mL) and incubated in the dark at room temperature for 30 min. The samples were detected by flow cytometry with fluorescence excitation wavelength at 488 nm and emission wavelength at 530 nm. Data from 10,000 cells were collected for each data file.

Statistical analysis

All data were expressed as means ± standard deviation (SD), and analyzed by one-way ANOVA with SPSS 16.0 software. The level of significance was set at $p < 0.05$.

Results

As can be seen from Figure 1, P-60 showed the strongest antiproliferative activity on SPC-A-1 cells compared with P-30 and P-w ($p\leq0.01$). The IC50 were 0.65 ± 0.15, 1.63 ± 0.25 and 4.84 ± 0.32 mg crude drug/mL, respectively. Based on determination result of this experiment, P-60 fraction was chosen for the further study. As can be seen in Figure 2, P-60 showed anti-lung cancer activity against SPC-A-1 cells in dose-dependent. With the increase of dose, the inhibition rates of 16, 80, 400 and 2000 μg crude drug/mL were 20.1 ± 1.98%, 32.3 ± 5.48%, 49.8 ± 13.4%, 97.5 ± 5.95%, respectively. The results showed that the optimal dose was selected between 16 and 2000 μg crude drug /mL for further study.

As shown in Figure 3, after being treated with 10 mg crude drug/mL P-60 for 24 weeks, the number of tumors in back side and front side was lower than untreated group (31.2 ±5.66 vs. 3.0 ± 2.16, $p\leq0.01$). The figures and data showed that treatment with P-60 decreased the tumor multiplicity by 90.3%.

Apoptosis plays a crucial role for it is an important mechanism of chemoprevention (Nakamura, 2009; Murugan et al., 2010; Robbins et al., 2010). In the apoptosis study, SPC-A-1 cells were stained with annexin

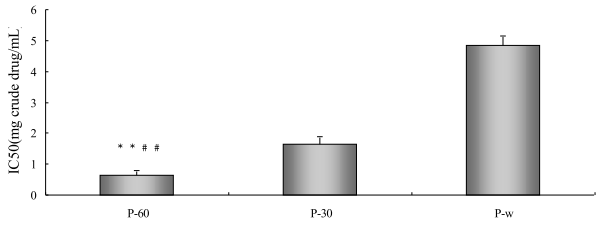

Figure 1. Cell Proliferation Inhibition of P-60, P-30 and P-w on SPC-A-1 Cells. ** p ≤ 0.01, P-60 Group vs. P-30 Group; ## p ≤ 0.01, P-60 Group vs. P-w Group

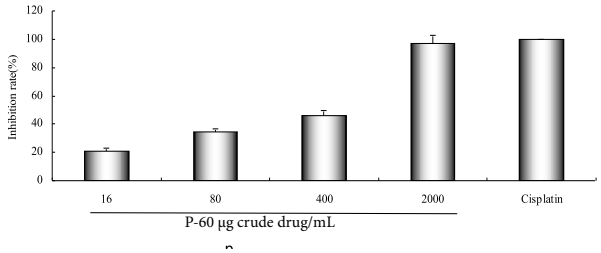

Figure 2. Cell Proliferation Inhibition of Different dose P-60 on SPC-A-1 Cells. Cisplatin was Used as Positive Control. The Data are Expressed as Mean ± SD from Three Triplicate Experiments (n=12)

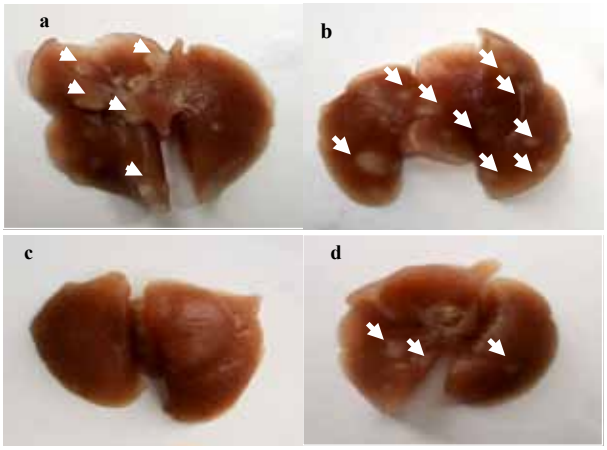

Figure 3. Efficacy of P-60 Against B[a]P-induced Lung Tumorigenesis in A/J Mice. a,b untreated P-60 Group, c,d Treated P-60 Group, Tumors are indicated by the white arrows

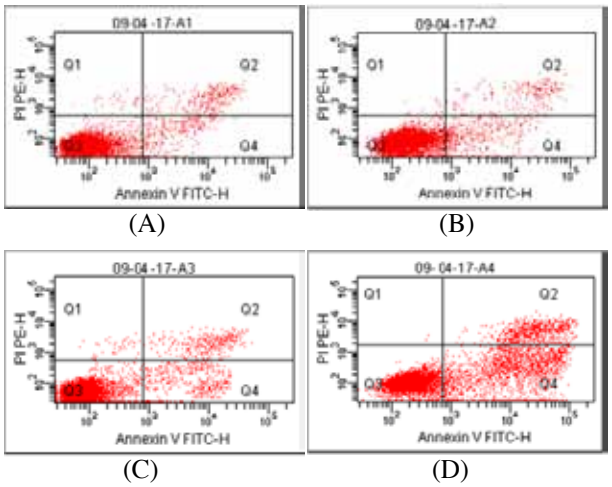

Figure 4. Apoptosis Effect of P-60 on SPC-A-1 Cells After Annexin V-FITC/propidium Iodide Staining. (A) Blank control group; (B) 125 μg crude drug/mL P-60 group; (C) 250 μg crude drug/mL P-60 group; (D) 500 μg crude drug/mL P-60 group. The percentage of apoptotic cells was expressed as Q2+ Q4

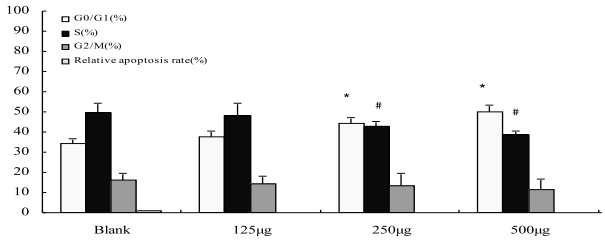

Figure 5. Effects of Different Concentration P-60 on Cell Cycle Progression in SPC-A-1 Cells. Percentage of cell cycle phases of SPC-A-1 Cells. *p ≤ 0.05, 250 & 500 μg crude drug/mL Group vs. Blank Group; #p ≤ 0.05, 250 & 500 μg Crude drug/mL Group vs. Blank Group. Data are mean ± SD from two independent experiments

V/PI and then analysed with flow cytometry. The cells in Q3 quadrant were viable and were negative for both PI/annexin V; the cells in Q4, early apoptotic cells, were positive for annexin V and negative for PI; Q2, were positive for annexin V and PI. It can be clearly seen in Figure 4, the cells were significantly increased in Q2+Q4 quadrant with the increase of dose (5.7% in blank group to 37.0% in 500 μg crude drug/mL group). Moreover, the percentage of apoptotic cells induced by 500 μg crude drug/mL P-60 was increased remarkably after 48 h treatment compared with the blank group (4.2%).

As shown in Figure 5, the cells were arrested in G0/G1 phase after being treated with P-60. After being treated with P-60 for 48 h, the percentage of G0/G1 phase increased from 34.3 ± 2.3% in normal group to 49.9 ± 3.1% in 500 μg/mL P-60 group ($p \leq 0.01$). The percentage of G2/M phases decreased from 16.1 ± 3.3% to 11.5 ± 2.3% with 500 μg/mL P-60 ($p \leq 0.01$). Moreover, P-60 regulated dose-dependently cell cycle.

Discussion

Chemoprevention is the potential and optimal strategy for reversing, delaying and preventing the occurrence and development of NSCLC (Johnson et al., 2008). Natural dietary agents have drawn a great deal of attention for NSCLC prevention due to its potential activity. The different compounds in natural dietary agents can protect normal cells against carcinogenesis (van Breda et al., 2008). From the results of our experiments, we can find that the P-60 possessed chemopreventive activity on NSCLC in vitro and in vivo. The mechanism studies on the chemoprevention has been shown that apoptosis is an important pathway for chemoprevention of NSCLC (Takeshi et al., 2000; Adhami et al., 2009; Das et al., 2009; Amin et al., 2010). The cell cycle process is regulated to lead the cancer cell to death. Taken together, our results, for the first time, suggest that the P-60 extract has inhibitory effects in SPC-A-1 cell line and promoting the cell apoptosis via regulating cell cycle phase.

From the above results, our investigation, for the first time, showed that chemoprevention ability and efficacy of PV extract against NSCLC in SPC-A-1 cells, and A/J mice in vivo and in vitro. Moreover, its apoptosis effect on SPC-A-1 cells was evaluated. The results showed that PV extract could induce apoptosis of SPC-A-1 cells via

regulating cell cycle from G0/G1 phase to S phase. The all results from this study indicate that PV extract can potentially be used as a lung cancer chemopreventive agent.

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