Prevention and Immunomodulation Activity of *Prunella vulgaris* L. on Lung Cancer

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Abstract: The Chinese medicinal plant *Prunella vulgaris* L. (PV) has been successfully used for centuries in China for treatment of non small-cell lung cancer. However, there is seldom report about its function on lung cancer prevention. In the current study, two lung adenocarcinoma cell lines (A549 and SPC-A-1 cells) and heterogenic transplantation mouse cancer model(subcutaneous inoculated C57 BL/6J mouse with Lewis cancer cells) were used to investigate *Prunella vulgaris* L. effects on lung cancer prevention. We observed strong inhibitory effects of *Prunella vulgaris* L. on A549 and SPC-A-1 cells growth. The similar results were obtained in vivo study, inhibition rate of lung tumor deterioration by high dosage of PV was 63.56%±6.79% and low dosage was 33.45%±10.98%($P \leq 0.01$). Furthermore, we found *Prunella vulgaris* L. could enhance thymus index whereas no effect on spleen index in tumor-bearing mice. The content of TNF-α in serum was increased in *Prunella vulgaris* L. group($P \leq 0.05$) as well. Together, our results indicated that *Prunella vulgaris* L. possessed the prevention effects on lung cancer and might involved in immunomodulation during the cancer progression.

Keywords: Prunella vulgaris L.; TNF-α; prevention; Immunomodulation; Lung cancer

1 Introduction

In recent years, cancer has been considered as a disease with worldwide epidemic characteristics and major cause of hospitalization and death due to the environmental degradation and pollution worsening caused by global industrialization. Lung cancer is one of the most common malignant tumors worldwide [1-3]. Therefore, the development of new agents for prevention and therapy of lung cancer is important and urgent to reduce the mortality caused by this disease. To date, complementary and alternative approaches are considered effective way for prevention of lung cancer [4-8]. Traditional Chinese medicine (TCM) is an important part of alternative medicine. In China, TCM is the mainstream medicine system with thousands of year's clinical practices. There are many herbal extracts and combined prescriptions which are composed by hundreds of complex constituents [9] have been proved effective for cancer treatments clinical. In addition, new drugs development is getting more and more difficult considering cost, rate of success. By listed as a complementary and alternative medicine (CAM) category [4], now TCM has been regarded as an alternative health care system in Western Europe, as well as in North America as perceiving the concept of nature. Hence, research and development of effective drugs based on TCM might be promising and significant for prevention and treatment of lung cancer.

The genus *Prunella vulgaris* L. (PV) has been used in clinic effectively for prevention and therapy of non small-cell lung cancer for long history in Chinese folk medicine, showing powerful cancer supressive activity [10]. At the same time, it has been applied widely as well for clinical treatment of scrofula, gall tumor, mammary abscess swelling, thyromegaly, and glandular phthisis [11], etc. It is known PV contains some compounds associated with tumor inhibitory effects, such as terpenoids[12-13], flavonoids[14], phenolic acids[15], and polysaccharide[16]. It was reported that PV exerted its significant suppressive effect on lung cancer cells through antiproliferation of cancer cells, regulation of cell cycle, and promotion of

cell apoptosis [14]. Other cellular functions of PV such as anti-oxidation, anti-free radical[17], immune regulation[18], anti-mutagenic[18], stimulating macrophage phagocytic activity, nitric oxide (NO) production and cytostatic activity, and inducing production of macrophage-related cytokines such as TNF-a, IL-1b and IL-6[18], play roles on the effects of PV on lung cancer as well. However, there is seldom report about PV lung cancer prevention study in vitro and in vivo so far.

In the present study, we investigated the prevention activity of PV on lung cancer in vitro and in vivo and try to reveal the role of PV on the immune regulation. Our results indicated that *Prunella vulgaris* L. possessed the prevention effects on lung cancer and might involved in immunomodulation during the cancer progression. Our study would provoid new candidate for lung cancer therapy.

2 Experimental design and methods

2.1 Plant

Dried spikes of PV were purchased from Medicinal Corporation of Bozhou City, Anhui Province (batch number0711128) and authenticated by Professor D.K. Wu, college of pharmacy, Nanjing University of Chinese Medicine.

2.2 Preparation of the crude plant extract

The spikes (4 kg) were refluxed with 40 liters 95% ethanol for 2h for twice; the filter residue was refluxed with 40 l 60% ethanol, 30% ethanol and distilled water subsequently in the same way. The extractions were concentrated by rotary evaporator at 60°C and the resultant concentrated solutions were dried under reduced pressure at 50°C. The dark brown powders were shattered by pulverizer and ready for use. There are four powders of every crude plant, named XKC-95, XKC-60, XKC-30 and XKC-W, respectively. The Rest of crude plant and powders were deposited at Key Laboratory of New Drug Delivery System of Chinese Meteria Medica, Jiangsu Provincial Academy of Chinese Medicine.

2.3 Reagents and cell lines

Human lung cancer cell lines A549 and mice lung adenocarcinoma Lewis cell line was provided by Nanjing KeyGen Biotech Co., Human lung adenocarcinoma cell line SPC-A-1 was provided by Institute of biochemistry and cell biology, shanghai institute for biological sciences, chinese academy of sciences. TNF-α assay kit was purchased from Wuhan boster Biology Engineering Co., Ltd.(batch number:EK0527).

2.4 Animal model and prevention activity of PV on lung cancer in vivo

Male C57BL/6 mice, 6–8 weeks of age, weighing approximately 18-20 g, obtained from shanghai SLAC laboratory animal Co., Ltd. and maintained on a standard environmental condition were used. They were fed with standard diet and water ad libitum.

Primary lung tumors were generated by the subcutaneous (s.c.) injection of 10^6 Lewis cells/mouse in the right anterior limb of mouse. Afterwards, the mice were intragastric administration with 0.4ml mixture of XKC-95, XKC-60, XKC-30 and XKC-W (high dose: 10g crude plant/kg; low dose:5g crude plant /kg) every day. Mice were sacrificed for the following assay after 14 days treatment consecutively. Positive control mice were injected 0.2ml/d cyclophosphamide (20mg/kg). Mice received 0.4ml/d sodium chloride as blank control. 0.5ml blood was took from venous plexus in fossa orbitalis before sacrificed, centrifuged (3000rpm, 15min), separated serum, -20°C despoisted for TNF-α assay.

2.5 Cell culture and cytotoxicity assay

A549 and SPC-A-1 cells were grown in RPMI-1640 supplemented with FCS (10% v/v) , streptomycin (100 U/ml), penicillin (0.1 mg/ml) in a humidified atmosphere with CO_2 (5% v/v) at 37℃. For cytotoxicity assay, A549 and SPC-A-1 cells in exponential growth were placed down at a density 4×10^4 cells/cm^2 with 100μ1/well in 96 well plates respectively. After grown to confluence for 24h, the media was removed and cells were treated with 90 μ1 RPMI-1640 w/o FCS, then 10 μ1 reagent with different

concentration of crude extract of PV(16, 80, 400, 2000 μg crude plant /ml in A549 cell; 50, 100, 200, 400 μg crude plant /ml in SPC-A-1 cell)were added into wells. Final concentration of organic solvent (DMSO:ethanol=50:50, v/v) in medium was 0.48%. Cells were treated with 0.48% organic solvent as negative control and cells were treated with 10μ1 cisplatin as positive control. Afterwards, these cells were incubated in humidified atmosphere with CO_2 (5% v/v) at 37°C for 36 h.

For MTT assay, 10μ1 MTT (5mg/ml) were added into each well to generate formazan, then cells were incubated in humidified atmosphere with CO_2 (5% v/v) at 37°C for 4 h. After removing the supernatant, 100μ1 DMSO was added to dissolve the purple crystal with 10min vibration. The optical density of each well was measured at 550nm and 690nm by a microplate reader. The growth inhibitory ratio was calculated based on the following formula:

Growth inhibitory ratio of cell=(mean OD of control group － mean OD of treated group)/mean OD of control group ×100%

2.6 TNF-α assay

Serum TNF-α concentration was measured using TNF-α elisa kit according to the operation instructions.

2.7 Statistical analysis

The results were expressed as mean±SD. All data were analysized with the spss10.0 software.

3. Results and discussion

3.1 PV supressed growth of A549 and SPC-A-1 lung cancer cell lines

We found PV showed strong cytotoxic effect on A549 cells in a dose dependent manner, especially at the dosage 2000μg crude plant /ml(Fig1). When the cells were observed under a light microscope, they looked dead and floating, with strongly shrunken cytoplasm in this study. In blank control group, cells was uniform and transparent cytoplasm, good refractive index. However, vision in a decrease in the number of normal cells in PV group with changes in cell morphology, cell body shrinkage, poor refractive index and increase in intracellular granules (Fig.2). The inhibition rate of different concertration groups were 20.06±1.98%, 32.25±5.49%, 49.79±13.41%, 97.45±5.94%, respectively. A sharp increase of the inhibition of proliferation could be observed between the concentrations 16 and 2000 μg crude plant /ml for A549 lung cancer cell(Fig. 1 A).

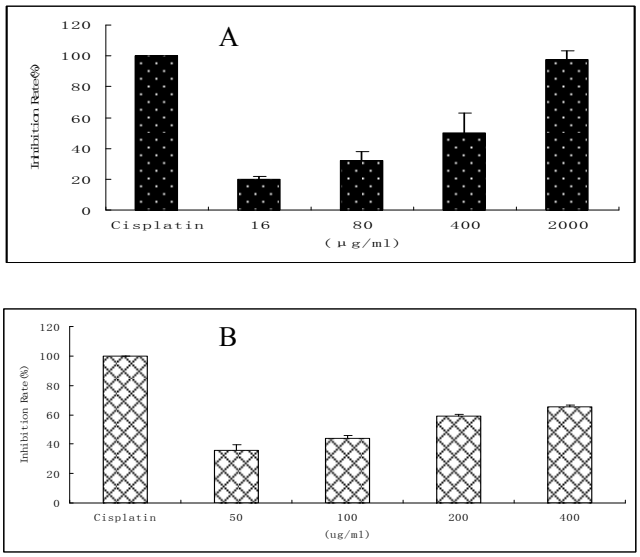

Fig.1 Cytotoxic activity on A549 lung cancer cell (A)and SPC-A-1 lung cancer cell (B). Concentration dependent inhibition of combretum fragrans methanolic leaf extract on proliferation of A549 lung cancer cell. In A549 cytotoxicity detection, drug concentrations of 16-2000 crude plant μg/ml（converted to concentration of extract 2.45，12.25，61.23，306.16μg/ml）was choiced; in SPC-A-1 cytotoxicity test, drug concentrations of 50-400 crude plant μg/ml was choiced（converted to concentration of extract 7.66,15.31， 30.62， 61.23μg/ml）. Results are expressed as mean % inhibition of control ± standard error of mean. The results were done in duplicate and repeated two times.

In test of PV against SPC-A-1 human lung cancer cell, we found the extract of PV showed the strong cytotoxic activity in this study. At 50, 100, 200, 400 μg crude plant /ml, the inhibition rate were 35.83±4.00%, 43.77±1.98%, 58.89±1.16%, 65.32±0.97%, respectively.(Fig. 1B). the inhibition rate also showed a increase with the concertration of PV. The results of PV against A549 and SPC-A-1 cancer lung cell showed that PV possessed cytotoxic activity in vitro.

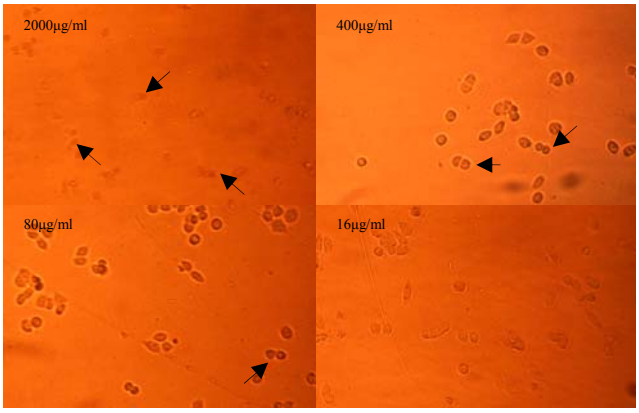

Fig.2 A549 cell morphology after diagram after administering 24h under inverted microscope （10×10）

3.2 PV prevented mouse primary lung tumor formation in vivo

As can be seen from Fig.3, compared with the saline group, cyclophosphamide group, high-dose group, low dose group has a very significant difference （ p≤0.01 ）， Inhibition rate(%)of positive control was 62.93%±11.05%, high dosage was 63.56%±6.79%, and low dosage was 33.45%±10.98%. This result show PV possessed powerful prevention activity on tumor.

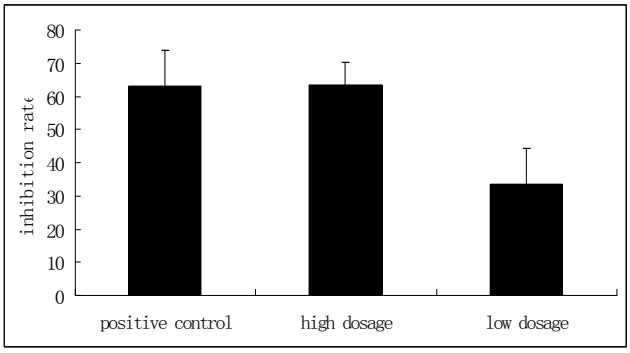

Fig.3 Inhibition rate of PV in tumor-bearing mice. Inhibition rate(%)of positive control was 62.93%±11.05%, high dosage was 63.56%±6.79%, low dosage was 33.45%±10.98%(P≤0.01).The observations were repeated in triplicate in three separate experiments(n=24). Values were expressed as mean±SD. Blank control: sodium chloride. positive control: cyclophosphamide.

3.3 PV increased immune organs index of tumor-bearing mice

From the Fig.4, we learned that high-dose group, low dose group enhanced obviously the thymus weight of tumor-bearing mice compared with the blank control group: thymus index(mg/g)of blank control was 1.20±0.14, high dosage was 2.06±0.50, low dosage was 1.39±0.36.

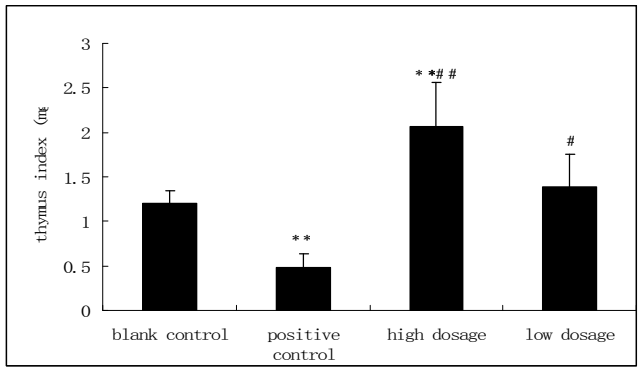

Fig.4 Thymus index in tumor-bearing mice. Thymus index(mg/g)of blank control was 1.20±0.14, positive control was 0.49±0.16; high dosage was2.06±0.50, low dosage was 1.39±0.36. The observations were repeated in triplicate in three separate experiments(n=24). Values were expressed as mean±SD. *comprasion with blank control(*P≤0.05, ** P≤0.01), # comprasion with positive control(# P≤0.05, # # P≤0.01), Student's t-test.

Unexpectedly, in Fig.5, there was no significant difference between spleen index of PV group and the blank control group, whether high-dose group or the low dose group. This showed that the medicinal herbs PV could enhance immune function of tumor-bearing mice though regulation of thymus while not spleen. PV induced immune function through the regulation organism for the prevention and treatment of lung cancer. The thymus index and spleen index in cyclophosphamide was significantly lower than in saline group and PV group. Results indicated that suppression of the immune function of cyclophosphamide.

3.4 PV increase TNF-α production

As can be seen from Fig.6, cyclophosphamide group, high-dose group and low-dose group was significant difference(p≤0.05)compared with the saline group, this reveals the medicinal herbs PV can enhance TNF-α content in plasme of tumor-bearing mice and strengthen immune function of organism. Cyclophosphamide significantly reduced the body content of TNF-α. This might be why it suppressed immune organs.

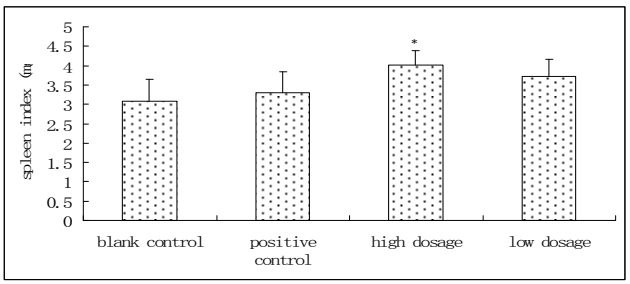

Fig.5 Spleen index in tumor-bearing mice. Spleen index(mg/g)of blank control was 1.20±0.14, positive control was 0.49±0.16; high dosage was2.06±0.50, low dosage was 1.39±0.36. The observations were repeated in triplicate in three separate experiments(n=24). Values were expressed as mean±SD. *comprasion with positive control(*P≤0.05), student's t-test.

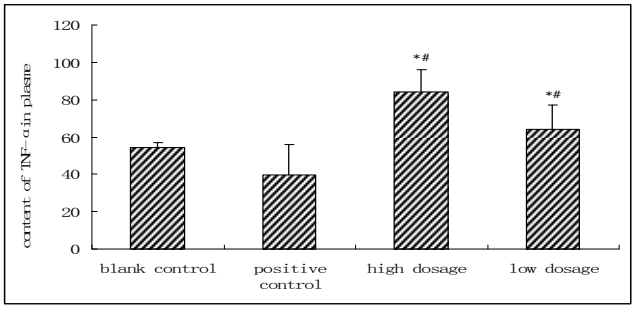

Fig.6 TNF-α content in sereum Prunella vulgaris L. Values were expressed as mean±SD. Blank control: sodium chloride. positive control: cyclophosphamide. * comprasion with blank control(P≤0.05), # comprasion with positive control(P≤0.05), Student's t-test.

Thymus was the main parts of T cell developing gradually to maturation, so it was referred to as the central immune organs. After T cells from embryonic liver cell or bone cell marrow into the thymus, it was induced to differentiation and development under thymic micro-environment. In the process of its differentiation and maturation, expression of a variety of cell differentiation antigens had happened and the formation of T cell bank emerged finally after positive-negative selection process. After that, mature T cells migrated from the thymus and settled in peripheral lymphoid organs, involved in lymphocyte recirculation and distributed in the body organizes via a series of complex processes.

Tumor necrosis factor is a molecular family, a 17 kDa protein consisting of 157 amino acids, including TNF-α and TNF-β. TNF-a is mainly produced by activated T lymphocytes, macrophages, and natural killer (NK) cells. In the course of disease, different T cell clones in T cell bank happened change, activated Th cells and activated Th cells secrete cytokines TNF-α and other cytokines. In this study, we found PV could enhance the induced thymus index and production of T cell -related cytokines TNF-a.

4. Conclusion

In this paper, we investigated the prevention activity and immunomodulation effect of PV in vitro and in vivo. PV could inhibit growth of the cancer cell lines in vitro and interruptted tumor generation of tumor-bearing mice in vivo. These results showed that PV possessed the prevention activity on lung tumor. From the perspective of the immunomodulation, TNF-α play a important role in prevention on cancer in vivo. PV was able to increased content of TNF-α to regulate the thymus for inhibiting lung cancer and had a certain immuneregulation function in vivo.

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