Effect of Shanxian Granule on Immunity in Lewis Lung Cancer Mice

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Abstract Objective: To observe the effect of Shanxian Granule on the proliferation of Lewis lung cancer cells and anti-tumor immunity of Lewis lung cancer bearing mice, and explore the molecular mechanism of Shanxian Granule in inhibiting tumors and improving the anti-tumor immunity of the body, so as to provide a profound theoretical basis for its clinical application.

Methods: Lewis lung cancer cells were implanted into subaxillary skin to establish tumor bearing mice model, the mice were divided into control group, model group, chemotherapy group and Shanxian granule group, calculating the inhibition rate by weighing the tumor of Lewis lung cancer bearing mice, calculating the spleen index by weighing the spleen, immunohistochemical method was used to detected the expression of lymphocyte CD4+ and CD8+ in spleen.

Results: 1. The inhibition rate of Shanxian granule group on Lewis lung cancer was 45.99%, significantly higher than that of chemotherapy group (P < 0.05); 2. Spleen index, ratio of CD4+ T cell and CD8+ T cell in model group and chemotherapy group were significantly lower than those in the control group and Shanxian granule group (P < 0.05); while there was no significant difference between the control group and Shanxian granule group (P > 0.05). Conclusion: Shanxian Granule could obviously inhibit the tumor growth in Lewis lung cancer bearing mice, increase spleen index, enhance T lymphocyte activity and restore immune homeostasis of mice, indicating that the anti-tumor effect of Shanxian Granule may be related to the improvement of immunity and the enhancement of immune surveillance function.

1. Experimental Materials

1.1. Laboratory Animals and Tumor Cell Lines
48 healthy SPF Kunming mice, half male and half female, aged 4-5 weeks, weighing 18-22g (provided by Experimental Animal Center of Xi'an Jiaotong University, SCXK-2015-005). Mouse Lewis lung cancer cells line (LLC) was purchased from Saiqi (Shanghai) Bioengineering Co., Ltd.

1.2. drugs and reagents
Main drugs: Shanxian granule (SXG), preparation center of Affiliated Hospital of Shaanxi university of Chinese Medicine (batch number 150328), cisplatin injection; main reagents: Hyclone DMEM high glucose culture fluid, CD4+, CD8+, rabbit anti-mouse polyclonal antibody (Wuhan Doctor De Bioengineering Co., Ltd.).

1.3. main instruments
Carbon dioxide incubator (Germany BindCD-150), ultra-clean workbench (Suzhou purification),
inverted microscope (Auter BDS-200), ordinary paraffin slicing machine (A0-800, Shanghai Medical Device Factory), etc.

2. Experimental methods and project testing

2.1. Preparation of LLC suspension
Lewis lung cancer cells were cultured and amplified in vitro, tumor cells in logarithmic growth phase were taken to prepare 1×10⁸/ml cell suspensions.

2.2. Establishment and Grouping of Tumor-bearing Mouse Model
48 healthy Balb/c mice, half male and half female, aged 4-5 weeks, weighing 18-22g, were selected. After a week of adaptive feeding, sex in half, 12 mice were randomly selected as control group according to BMI, 0.4 ml saline was injected subcutaneously into the armpit of the right forelimb of the mice, and the remaining 36 mice were inoculated with 0.2 ml LLC (about 2 x 10⁷ tumor cells) suspension into the same part as experimental group, the tumorigenesis was examined and measured after three days. When tumors diameter was about 5mm (about day5), sex in half, the mice were divided into model group, chemotherapy group and Shanxian granule group according to the tumor size index, 12 in each group.

2.3. preparation of medicine
Based on the conversion algorithm of human and rat weights, Shanxian Granule Suspension (0.05g/ml) was prepared by Shanxian Granule (g): Normal Saline (mL) = 1:20 and stored in refrigerator at 4 C for reserve.

2.4. medication
The medication was started the next day after grouping. The control group, model group and chemotherapy group were given 0.4mL of normal saline respectively, and Shanxian granule group was given 0.4ml (1g/kg/d) Shanxian granule suspension by gavage for 14 consecutive days at 9 a.m. and 3 p.m. each day. On the 1st, 3rd and 5th day, the control group, model group and Shanxian granule group were intraperitoneally injected with 1 ml saline respectively, the chemotherapy group was injected Cisplatin (DDP) saline solution 1ml (containing DDP 0.1 mg) intraperitoneally. That is, control group and model group were given 0.4ml saline intragastric administration and 1ml saline peritoneal injection, chemotherapy group was given 0.4ml saline and 1ml DDP saline, Shanxian granule group was given 0.4ml Shanxian granule suspension and 1ml saline).

2.5. sample collection
The mice in each group were weighted and recorded after the last intragastric administration for 24 hours, then anesthetized with 2% chloral hydrate, removed tumors, free the spleen and weighed on the superclean workbench disinfected by 75% alcohol, the tissues were fixed in formalin for reserve.

2.6. Index detection
2.6.1. Calculating Tumor Suppression Rate
The average weight of tumor tissue in each group was calculated according to the previous recording, calculating the inhibitory rate according to (model group tumor weight − treatment group tumor weight)/model group average tumor weight *100%".

2.6.2. Calculating Splenic Index of Mice
According to recorded weight of spleen and mice, the spleen index was calculated based on the formula of "spleen index (mg/g) = spleen weight/body weight * 100%".
2.6.3. Immunohistochemical staining to detect the expression of CD4⁺, CD8⁺ in T lymphocyte of mice spleen
The spleen tissues were prepared into paraffin sections, and routinely hydration—washing—sealing—washing—antigen repair—adding CD4⁺ (or CD8⁺) rabbit anti-mouse polyclonal antibody (1:150)—washing—adding biotinylated sheep anti-rabbit second antibody (1:200)—washing—sealing—chromogenic—hematoxylin lining—dehydration, transparent—sealing. 10 high power microscopic fields (×400) was randomly selected from each specimen to calculate the percentage of positive cells.

2.7. Statistical Analysis of Data
In this experiment, the data was analyzed by SPSS19.0 statistical analysis software and expressed by $x \pm s$, one-way ANOVA was used for comparison among groups.

3. Results

3.1. The effect of SXG on solid tumors growth in Lewis lung cancer-bearing mice (Table 1).
Table 1. Inhibitory rate of SXG and chemotherapeutic drugs on solid tumor in mice bearing tumor ($x \pm s$, n=12)

| Group         | Mouse weight before treatment (g) | Mouse weight after treatment (g) | Tumor weight (g) | Tumor inhibition rate (%) |
|---------------|----------------------------------|----------------------------------|-----------------|--------------------------|
| Model group   | 19.53±0.66                       | 25.13±1.62                       | 3.25±0.36       | —                        |
| Chemotherapy  | 19.60±0.67                       | 24.23±2.27□                    | 2.58±0.42       | 20.02±4.57               |
| SXG Group     | 19.55±0.72                       | 27.13±1.21☆▽                   | 1.76±0.32       | 45.99±6.29☆▽             |

Note: Compared with the model group, ☆P < 0.01, □P > 0.05, Compared with the chemotherapy group, ▽P < 0.01.

3.2. The effect of SXG on spleen index in LLC-bearing mice (Table 2).
Table 2. The spleen index of mice in each group ($x \pm s$)

| Group         | n   | Spleen index (mg/g)  |
|---------------|-----|---------------------|
| Control group | 12  | 8.03±0.89           |
| Model group   | 12  | 7.36±0.60□          |
| Chemotherapy  | 12  | 7.14±0.49☆          |
| SXG Group     | 12  | 7.96±0.70△▽         |

Note: Compared with the control group, □P < 0.05, △P > 0.05; compared with the model group, ☆P > 0.05, ○P < 0.05; compared with the chemotherapy group, ▽P < 0.01.

3.3. Immunohistochemistry was used to detect the effect of SXG on the expression of CD4 (Fig. 1, Table 3) and CD8 (Fig. 2, Table 3) in spleen T lymphocytes of LLC bearing mice.
Table 3. Expression rates of CD4⁺, CD8⁺ and CD4⁺/CD8⁺ in spleen T lymphocyte of mice ($x \pm s$)

| Group          | n   | CD4⁺ positive expression rate (%) | CD8⁺ positive expression rate (%) | CD4⁺/CD8⁺ |
|----------------|-----|----------------------------------|----------------------------------|-----------|
| Control group  | 12  | 45.93±1.97                       | 24.04±1.49                       | 1.92±0.16 |
| Model group    | 12  | 33.36±1.53□                      | 19.40±2.63                       | 1.75±0.14 |
| Chemotherapy   | 12  | 25.02±2.28☆                      | 15.26±1.82                       | 1.64±0.12☆|
| SXG Group      | 12  | 44.56±1.98△▽                     | 23.61±1.35                       | 1.89±0.17△▽|

Note: Compared with control group, □P < 0.01, △P > 0.05, Compared with the model group, ☆P < 0.01; compared with the chemotherapy group, ▽P < 0.01
4. Discussion

Lung cancer is one of the most common malignant tumors, especially with the aggravation of environmental pollution, in recent years, the incidence of lung cancer rank first and mortality second among malignant tumors, which have increased significantly. At present, the treatment of lung cancer has not achieved satisfactory effect, survival rate in 5 years is only 10%-15%[2]. It was found that the immune of patients with malignant tumors was disorder and decrease[3], which was closely related to the occurrence and development of tumors[4]. In 2013, cancer immunotherapy was ranked in 《Science》 as the most important scientific breakthrough, and was considered that "immunotherapy can stand the test completely". Tumor immunotherapy is to control and kill tumor cells by stimulating or mobilizing the body's immune system and enhancing the anti-tumor immunity.

T lymphocyte-mediated cellular immunity is the main anti-tumor immunity (figure), mature T lymphocytes can be divided into CD4+ T cell (Th) and CD8+ T cell (Tc) according to whether CD4 and CD8 are expressed on the cell surface. CTL as mainly activated CD8+ T cells is the main effector cell of anti-tumor immunity, [4]. Tumor antigens are presented to CD8+ T cells by antigen presenting cell uptake, which directly activates proliferation and differentiation into CTL. Tumor antigens are presented to CD4+ T cells and induce Th0 cells to differentiate into Th1 and Th2. Th1 cells secrete IL-2 and IFN-gamma, which can co-stimulate CTL proliferation and differentiation[4]. CTL kills target cells through Fas-Fasl and TNF-TNFR pathways specifically, mediating necrosis or apoptosis of tumor cells[4]. The ratio of CD4+ and CD8+ T lymphocyte subsets in normal physiological state keep a dynamic balance to maintain the stability of cellular immune function. Studies have shown that patients with malignant tumors have immune imbalance, mostly manifested as cellular immune dysfunction and the changes in T lymphocyte subsets, specifically manifested in the lower content of CD4+ T lymphocyte than normal people, and the ratio of CD4+/CD8+ decrease[5-7]. Therefore, we can observe the anti-tumor immunity by detecting the changes of CD4+ T cell content and the ratio of CD4+/CD8+.

Shanxian Granule is an anti-cancer Chinese medicine compound preparation developed by Oncology Department of the First Affiliated Hospital of Shaanxi University of Chinese Medicine. Based on the basic theory of traditional Chinese medicine, combined with many years of clinical experience and modern pharmacological research, according to the basic treatment principle of "strengthening the body, promoting blood circulation and removing blood stasis". It is composed of gen-seng, turtle version, turtle shell, zedoary, crane grass, hawthorn, etc, which have been shown that they can inhibit tumor growth and promote apoptosis of tumor cells in modern pharmacological studies[8-12]. In addition, clinical studies have shown that Shanxian Granule can improve the patients' anti-tumor immunity and clinical symptoms, inhibit metastasis and recurrence of the tumor.[13, 14] The results suggested that Shanxian Granule could significantly inhibit the growth of tumor tissue in Lewis lung cancer bearing mice. The detection of CD4+ T cells and CD4+/CD8+ ratio in spleen of
mice showed that the model group was lower than the control group, the chemotherapy group was lower than the model group, the SXG group was higher than the model group, and there was no significant difference between the SXG group and the control group. We can found that the immune function of Lewis lung cancer bearing mice was disorder, and chemotherapy could further destroy it, but Shanxian Granule can restore the immune system of Lewis lung cancer bearing mice to homeostasis. It is suggested that Shanxian Granule can significantly improve the immune function and enhance the anti-tumor immunity of Lewis lung cancer bearing mice. Combined with previous research, the study suggested that anti-tumor effect of Shanxian Granule may restore the homeostasis of immune function, improve the anti-tumor immunity and strengthen the immune surveillance function of cancer patients.

5. Conclusions
Shanxian Granule could obviously inhibit the tumor growth in Lewis lung cancer bearing mice, increase spleen index, enhance T lymphocyte activity and restore immune homeostasis of mice, indicating that the anti-tumor effect of Shanxian Granule may be related to the improvement of immunity and the enhancement of immune surveillance function.

Acknowledgment
Source of fund: Special scientific research and education project of Shaanxi Education Department (15JK1190); General Projects in the Field of Social Development of Shaanxi Science and Technology Department (2017SF-306)

Platform: Project of Innovation Team Shaanxi University of Chinese Medicine

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