Effect of Shanxian Granule on IFN-γ, TNF-β and IL-10 in peripheral blood of Lewis lung cancer bearing mice

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Abstract. Objective: To observe the effect of Shanxian Granule (SXG) on the proliferation of Lewis lung cancer cells and IFN-γ, TNF-β and IL-10 in peripheral blood of Lewis lung cancer bearing mice, and explore the molecular mechanism of SXG 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 SXG group, calculating the inhibition rate by weighing the tumor of Lewis lung cancer bearing mice, the changes of IFN-γ, TNF-β and IL-10 in peripheral blood were detected by ELISA. Results: 1. The inhibition rate of SXG group on Lewis lung cancer was 45.99%, significantly higher than that of chemotherapy group (P < 0.05); 2. IFN-γ and TNF-β in peripheral blood of mice in model group and chemotherapy group were significantly lower than those in control group (P < 0.05), while IL-10 was significantly higher (P < 0.05), and IFN-γ and TNF-β in peripheral blood of mice in SXG group were significantly higher than those in model group and chemotherapy group (P < 0.05), while IL-10 was significantly lower (P < 0.05). There was no significant difference in TNF-β and IL-10 between the two groups (P > 0.05). Conclusion: SXG can obviously inhibit the growth of tumor tissue in Lewis lung cancer bearing mice, increase IFN-γ and TNF-β and decrease IL-10 in peripheral blood, suggesting that the anti-tumor effect of SXG may be related to restoring the homeostasis of immune function of cancer patients, improving the immunity of the body and strengthening the 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: SXG (SXG), preparation center of Affiliated Hospital of Shaanxi University of Chinese Medicine (batch number 150328), cisplatin injection; main reagents: Hyclone DMEM DMEM high glucose culture fluid, ELISA kit (Wuhan New Enlightenment Biotechnology Co., Ltd.).
1.3. main instruments
Carbon dioxide incubator (Germany BindCD-150), ultra-clean workbench (Suzhou purification), inverted microscope (Auter BDS-200), automatic Enzyme Marker (Bio-Tek ELX808IU, USA), etc.

2. Experimental Methods and Index Detecting

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 1×10^8/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 2×10^7 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 SXG 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, SXG Suspension (0.05g/ml) was prepared by SXG (g): Normal Saline (mL) = 1:20 and stored in refrigerator at 4℃ for reserve.

2.4. medication: The medication was given the next day after grouping. The control group, model group and chemotherapy group were given 0.4mL normal saline respectively, and SXG group was given 0.4ml (1g/kg/d) SXG 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 SXG 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, SXG group was given 0.4ml SXG 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 blood was collected immediately by puncturing abdominal aorta then centrifugation and Cryopreservation at -80℃.

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. The changes of IFN-γ, TNF-β and IL-10 in peripheral blood of mice were detected by ELISA: The frozen serum was dissolved rapidly in 37℃ water bath and the strips needed for the experiment were removed from the sealed bag. Each group has three compound holes (n = 3) with blank holes reserved. Added prepared serum and standard, reacting 90min at 37℃- washed plate twice- added the IFN-γ biotinylated antibody working fluid, reacting 60min at 37℃-washed plate three times - added enzyme conjugate working fluid (except blank hole), reacting 30min at 37℃- washed plate five times with TMB coloring- terminated coloring-measured OD value (450 nm). The detection methods of TNF-β and IL-10 were same as the above.
2.7. Statistical Analysis of Data: In this experiment, the data was analyzed by SPSS19.0 statistical analysis software and expressed by $\bar{x} \pm s$, 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).

| Group            | Pre-treatment weight (g) | Weight after experiment (g) | Tumor weight (g) | Tumor inhibition rate (%) |
|------------------|--------------------------|-----------------------------|------------------|--------------------------|
| Model group      | 19.53±0.66               | 25.13±1.62                  | 3.25±0.36        | —                        |
| Chemotherapy group | 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: Compare with model group, ☆P<0.05, □P>0.05; Compared with chemotherapy group,▽P<0.05

3.2. Effect of SXG on IFN-γ, TNF-β and IL-10 in Peripheral Blood of Lewis Lung Cancer were Detected by ELISA on Bearing Mice (Table 6)

| Group            | n  | TNF-β    | IFN-γ | IL-10    |
|------------------|----|----------|-------|----------|
| Blank group      | 3  | 611.67±30.22 | 97.86±13.90 | 35.27±4.08 |
| Model group      | 3  | 323.76±23.43□ | 31.19±7.62□ | 59.10±16.30○ |
| Chemotherapy group | 3  | 291.00±20.45□☆ | 20.21±3.35□☆ | 69.10±7.88□☆ |
| SXG group        | 3  | 546.94±39.88△☆▽ | 89.42±8.62△☆▽ | 42.10±10.88△☆▽ |

Note: Compare with blank groups, □P<0.05, △P>0.05; Compare with model group, ☆P<0.05; Compared with chemotherapy group,▽P<0.05

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, 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-γ, 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.\textsuperscript{[4]} Differentiated Th1 and Th2 cell subsets, which secrete different cytokines, can stimulate their own differentiation and development and inhibit each other's differentiation and development, and interact with each other. Th1 cytokines such as IL-2, IFN-γ can stimulate the proliferation and differentiation of CTL. IFN-γ can enhance the killing ability of NK cells.\textsuperscript{[5]} TNF can directly kill tumor cells.\textsuperscript{[6, 7]} Thus inhibiting the development of tumors. Th2 cytokines, such as IL-10, are inhibitory cytokines. They can inhibit the antigen presenting ability of macrophages and the activity of NK cells, thus promoting the immune escape and promoting the growth of tumors. Studies have shown that IL-10 mediates immune mechanism by acting on different immune cell subsets and exerting immune suppression in various ways, resulting in anti-tumor immune escape. In normal physiological state, the body maintains the dynamic balance of cellular immunity and humoral immunity by regulating Th1/Th2. Th1/Th2 drift means the Th1/Th2 is out of balance and the transform, Th1/Th2 drift has been found in lung cancer, breast cancer, bladder cancer, gastric cancer, colorectal cancer, leukemia and other types of cancer patients. Th2 cytokines are strongly expressed in the tumor-bearing body, while Th1 cytokines are weakened. The study of digestive tract tumors showed that the serum levels of IL-2, IFN-γ and other Th1 cytokines in patients with gastric cancer and colorectal cancer were significantly lower than those in the normal group, while the levels of IL-4, IL-6, IL-10 and other Th2 cytokines were significantly higher. Therefore, the drift of the bearing cancer from Th2 to Th1 provided a new idea for the immunotherapy of tumors.

By applying basic theory of traditional Chinese medicine and combining with many years of clinical experience and modern pharmacological research, SXG is developed by Oncology Department of the First Affiliated Hospital of Shaanxi University of Chinese Medicine as an anti-cancer compound preparation based on the 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.\textsuperscript{[6, 12]} In addition, clinical studies have shown that SXG can improve the patients' anti-tumor immunity and clinical symptoms, inhibit metastasis and recurrence of the tumor.\textsuperscript{[13, 14]} Experimental studies have found that SXG can inhibit the metastasis of Lewis lung cancer, and also have an effect on the expression of apoptosis-related genes bcl-2/bax, fasl/fas, caspase-3, Hsp70 and VEGF.\textsuperscript{[15]}

The results showed that SXG could significantly inhibit the growth of LLC bearing mice, Th1 cytokines IFN-γ and TNF-β in peripheral blood in model group were lower than those of control group; Th2 cytokine IL-10 in model group was higher than those of control group; reminding that imbalance of Th1/Th2 and Th1/Th2 drift occurred in Lewis lung cancer bearing mice. Besides, Th1 cytokines IFN-γ and TNF-β in the chemotherapy group were lower those of model group, and SXG group were higher than model group; Th2 cytokine IL-10 in the chemotherapy group was higher than those of model group, and SXG group were lower than model group; but there was no significant difference between the SXG group and the control group. 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
SXG can obviously inhibit the growth of tumor tissue in Lewis lung cancer bearing mice, increase IFN-γ and TNF-β and decrease IL-10 in peripheral blood, suggesting that the anti-tumor effect of SXG may be related to restoring the homeostasis of immune function of cancer patients, improving the immunity of the body and strengthening the immune surveillance function.

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