Original Article

Yupingfeng formula inhibits lung cancer metastasis via modulating NK cells in a mouse model

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Abstract: Objective: Yupingfeng (YPF) is a classic Chinese herbal formula, consisting of Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu), and Saposhnikoviae Radix (Fangfeng). It has been used to effectively cure and prevent respiratory tract diseases. The present study aimed to elucidate the anti-metastatic effects of YPF on lung cancer in tumor-bearing mouse, elucidating the underlying antineoplastic mechanisms of action. Methods: A mouse model of metastatic lung cancer was established by intraosseous inoculation. Model mice were orally administered with YPF (2:2:1 or 3:1:1) or water for 30 days. Body weight was measured once a week. The survival curve was observed. Animal living image system was used to monitor tumor development. Lung metastatic nodules were detected via India ink perfusion under microscope. Numbers of NK cells and T-cells were explored by flow cytometric analysis. CFSE-based cytotoxic activity and CD107a assays were applied to test cytotoxicity and degranulation of NK cells and T-cells. Anti-NK1.1 antibody (PK136) was used to deplete NK cells through intraperitoneal injections (ip). ELISA was employed to test liver and kidney function after YPF treatment. Results: YPF (3:1:1) remarkably prolonged survival time of tumor-bearing mice and inhibited lung metastatic nodules by enhancing NK cell population and NK cell-dependent killing activity. Moreover, YPF is a safe herbal formula, showing minimum toxicity to the liver and kidneys. Conclusion: YPF may be used as a potential complementary therapy and alternative for treatment of lung metastasis. However, its possible antineoplastic mechanisms need to be revealed.

Keywords: Chinese herbal formula, Yupingfeng, metastatic lung cancer, NK cells

Introduction

Metastasis, the spread of cancer cells from a primary tumor site to distant secondary tumor sites, is one of the greatest challenges in cancer treatment. Over 90% of cancer-associated mortality can be attributed to this metastatic process [1]. Moreover, metastatic lung cancer is the leading cause of cancer-related deaths, worldwide, with more than 50% of cancer patients diagnosed with metastatic lung cancer [2].

A classic Chinese herbal formula, Yupingfeng (YPF) has been widely used in Asian countries. It consists of three types of herbs, Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu), and Saposhnikoviae Radix (Fangfeng). According to Traditional Chinese Medicine theory, YPF is good at notifying “Qi” to consolidate superficies and arrest perspiration. Therefore, it has been widely used to treat “Qi”-deficient diseases, which mainly include disorders in the upper respiratory tract, such as colds, flu, allergic rhinitis, asthma, and bronchitis. Recent accumulating evidence has shown that YPF’s therapeutic effects are associated with immune regulation. For instance, YPF markedly reduces bleomycin (BLM)-induced pulmonary fibrosis (PF) and alveolitis in rats by suppressing activation of high mobility group box 1 (HMGB1), one of the inflammatory mediators. Moreover, YPF can reverse epithelial-mesenchymal transition (EMT), a key origin of myofibroblasts [3]. In addition, YPF can also significantly decrease levels of other fibrotic factors, such as hydroxyproline (HYP), type I collagen (Col-I), transforming growth factor-beta1 (TGF-
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β1), and α-smooth muscle actin (α-SMA) [4]. Furthermore, combination of YPF with chemotherapy and radiotherapy can dramatically reduce levels of Foxp3+ Tregs and hyaluronic acid (HA) in patients with nasopharyngeal carcinoma [5].

Most cancer patients die because of metastases. Lungs are the most frequent site for development of metastasis due to their unique anatomic characteristics and physiological function. In scientific research, the first step is to establish an appropriate metastatic animal model. The current study established an experimental mouse model of metastatic lung cancer via intraosseous inoculation of tumor cells to mimic development of human tumors. YPF has been used for treatment of lung cancer patients [6]. A previous study [7] has shown that YPF can inhibit Lewis lung carcinoma (LLC) growth in subcutaneous mouse models via NK-dependent regulation. Therefore, the present study investigated the therapeutic effects of YPF on lung metastasis through immune regulation. Moreover, this study explored the effects of YPF on survival of tumor-bearing mice (metastatic LLC mouse model), evaluating its role in cytotoxicity and degranulation of NK cells and T-cells. Results indicate that lung metastases were obviously inhibited by YPF (3:1:1) treatment through NK cell-dependent modulation. This finding suggests that YPF could be used as a potential therapeutic alternative for treatment of lung metastasis.

Materials and methods

Preparation of YPF

All of the Chinese herbal granules used in the study were purchased from Tianjiang Pharmaceutical Co. Ltd. (Jiangyin, Jiangsu, China), one of six approved manufacturers for Chinese herbal granules in China. YPF formula was composed of Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu), and Saposhnikoviae Radix (Fangfeng), at a ratio of 2:2:1 (18 g, 18 g, 9 g) or 3:1:1 (30 g, 10 g, 10 g). They were mixed well in still water and stored in a refrigerator at 4°C for further analysis. The granules were well qualified by HPLC [7].

Reagents

Mice were euthanized through CO2 suffocation when any mouse became moribund. India ink or 0.5 × OCT/0.5 × PBS suspension was applied to pulmonary perfusion using a 25-gauge½-inch needle on a 3-mL syringe (BD Bioscience, USA). Next, the metastatic lung nodules were counted under a microscope or embedded in OCT and frozen. Frozen tissue sections were stained with H&E.

Animal model and inoculation

Six-week-old C57BL/6 mice (Shanghai SLAC Laboratory Animal Co. Ltd.) were maintained in accordance with NIH guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. Cancer cell lines LLC (ATCC) and LLC-Luc cells were maintained in DMEM supplemented with 10% of fetal calf serum (FCS) and 1% of penicillin-streptomycin. Confluent LLC and LLC-Luc cells were digested with Trypsin-EDTA (0.25%) (Gibco, USA) for 5 minutes, then re-suspended in PBS for intraosseous inoculation. Various amounts of cells (1 × 10^4, 2 × 10^4, 5 × 10^4, 1 × 10^5, and 1.5 × 10^5) were re-suspended in a volume of 30 μL. Intraosseus inoculations were performed by directly inserting an insulin syringe (27 gauge½-inch needle, BD Bioscience, USA) into the right tibia, then pushing the plunger on the syringe (Figure 1A). On the same day of inoculation, the mice were intragastrically administered with YPF at a daily dose of 116 mg or 129 mg per mouse (equal to 45 g or 50 g of clinical dose). The same volume of water was used as the control. For NK cell depletion, 100 μg of PK136 was intraperitoneally administered on day 1 after inoculation and every 3 days thereafter. Body weights of the mice were monitored once a week. Each experiment was performed in triplicate.

Metastatic analysis

Mice were euthanized through CO2 suffocation when any mouse became moribund. India ink or 0.5 × OCT/0.5 × PBS suspension was applied to pulmonary perfusion using a 25-gauge½-inch needle on a 3-mL syringe (BD Bioscience, USA). Next, the metastatic lung nodules were counted under a microscope or embedded in OCT and frozen. Frozen tissue sections were stained with H&E.

Mononuclear cell preparation

Mononuclear cells were isolated from the spleen by smearing and pushing the tissue
through a 40-μL cell strainer (Corning, USA) twice. Isolated cells were then treated with erythrocytolysin. Subsequently, the cells were purified through centrifugation using Easy Sep™ Mouse NK Cell and T-cell Enrichment Kit (STEMCELL Technologies, Canada). Mononuclear cells were collected and subjected to further analyses.

**Whole-body bioluminescence imaging**

Whole-body bioluminescence imaging was performed once a week for 5 consecutive weeks to monitor metastatic lung tumor growth from the 7th day after inoculation. D-luciferin potassium salt reconstituted in DPBS was intraperitoneally injected to the mice at a dose of 150 mg/kg. Mice were anesthetized with 2% isoflurane and imaged by Caliper IVIS Lumina II (Caliper Life Sciences, USA) 10 minutes after the luciferin injection. Region of interest (ROI) was defined as a circle with radius of 3.2 cm over the lung area. Total flux (photos/s) and average radiance (p/s/cm²/sr) within the ROI were quantified using Living Image [8].

**CFSE-based cytotoxic activity**

LLC cells were labeled with 0.25 μM CFSE in PBS at 37°C for 8 minutes in the dark. They were then washed three times with RPMI-1640 (Thermo Fisher Scientific, USA) containing 20% FCS. Freshly isolated splenocytes and CFSE-labeled LLC cells were used as effector cells and target cells, respectively. They were co-incubated at an E:T ratio of 10:1, 25:1, 50:1, or 100:1. Cell tracker violet was then added (5 μM) in the cell suspension to label viable cells for 5 hours at 37°C. Subsequently, PI (1 μL/mL) was added and incubated at room temperature for another 15 minutes in the dark. Ratio of cell death was analyzed with Attune tm Acoustic Focusing Cytometer (Thermo Fisher Scientific, USA) and FlowJo software.
CD107a assay

Degranulation of cytotoxic contents from NK cells and T-cells was determined through analysis of the degranulation marker CD107a with flow cytometry [9]. Purified NK cells or T-cells were co-incubated with LLC target cells (E:T ratio: 10:1, 25:1, 50:1, or 100:1) at 37°C for 5 hours in 5% CO₂. Additionally, PE-labeled anti-CD107a antibody or its corresponding isotype control was added to label the cells. After 5 hours of co-culturing, cells were washed and stained with anti-CD3, anti-NK1.1, or anti-CD8 antibody to confirm CD107a expression in NK cells and cytotoxic T-cells. Stained cells were fixed with 1% paraformaldehyde. CD107a expression in NK cells and CD8⁺ T-cells was assessed by Attune™ Acoustic Focusing Cytometer and FlowJo software.

ELISA assay

Serum alanine aminotransferase (ALT, Bioo Scientific, USA), aspartate aminotransferase (AST, Bioo Scientific, USA), albumin (ALB, Genway Biotech, USA), and creatinine (Cr, Bioo Scientific, USA) levels were measured by the standard colorimetric method at 510 nm, UV spectrum 340 nm, 450 nm, and 510 nm, respectively.

Statistical analysis

Data are expressed as mean± standard deviation (M ± SD) of at least three independent experiments. Statistical comparisons of bioluminescence measurements from LLC-Luc metastasis were analyzed via one-way analysis of variance (ANOVA), with Tukey’s multiple comparison post-hoc test (Figure 3). Data was first transformed to the natural logarithm due to significantly unequal variances. Student’s t-test was performed for two-group comparisons and Log-rank test was performed for survival comparisons. All analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). P < 0.05 indicates statistical significance.

Results

Mouse model of metastatic lung cancer successfully established by intraosseous inoculation of LLC cells

The present study established the mouse model of metastatic lung cancer through intraosseous inoculation. For intraosseous inoculation, the tibia was punctured through the top of the bone. Cell suspension was directly injected into the bone marrow (Figure 1Ab) and a primary tumor was successfully developed in the tibia (Figure 1Ac). After 70 days of inoculation of 1 × 10⁴ LLC cells, there were almost no obvious metastatic lung nodules. When 2 × 10⁴ or 5 × 10⁴ LLC cells were inoculated (Figure 1B), it took 70 days or 60 days to generate lung metastases, respectively. However, too many tumor cells were inoculated, such as 1.5 × 10⁵ LLC cells per mouse. The lung metastases became too heavy. Therefore, 1 × 10⁵ tumor cells per mouse seemed to be the most appropriate dose to establish the mouse model of metastatic lung cancer (Figure 1C). After 35 days of inoculation, 28 metastatic lung nodules were detected under a microscope. To further confirm the establishment of this metastatic mouse model, luminescence live imaging was used to detect metastatic foci in lung tissues (Figure 1D). Results showed that the luminescent signal was mainly localized in right legs of the mice during the first 3 weeks after intraosseous inoculation. Luminescent signal in lungs was first detected at the 4th week and markedly increased at the 5th week, indicating the generation and development of metastatic foci. At the end of the 5th week, after intraosseous inoculation, the mice were euthanized. Heavy metastatic lung nodules were observed. Bones and lung tissues were collected and stained with H&E to determine histological structure changes. Evidence confirmed that this mouse model of metastatic lung cancer was successfully established (Figure 1D, 1E).

YPF (2:2:1) had no inhibitory effects on lung metastases

YPF formula contained three types of herbs, including Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu), and Saposhnikoviae Radix (Fangfeng). The 2:2:1 ratio is a very classic ratio of these herbs, according to ancient Chinese medical books. Based on a primary study, the ratio of 2:2:1 plays a preventive role in subcutaneous mouse models of non-small cell lung cancer. It reveals that pretreatment of YPF (2:2:1) for 2 weeks not only shrinks the tumor, but also significantly prolongs survival time of mice via NK cell-dependent cytotoxicity [7]. Therefore, this study further investigated the immunomodulatory effects of YPF on
metastatic lung tumors. Mice were orally administered with 116 mg YPF (2:2:1) for 30 days after inoculation. It was found that such administration neither suppressed lung metastases (Figure 2A, 2B) nor initiated bone tumors. However, there was a trend of prolonged survival time in the YPF (2:2:1) treatment group (Figure 2C).

**YPF (3:1:1) inhibited lung metastases and prolonged survival of tumor-bearing mice**

Previous studies have shown that the ratio of 3:1:1 has been widely used in both clinic and scientific research fields [10-12]. This such ratio has been documented by Pharmacopoeia of the People’s Republic of China (2005). To determine the effects of YPF (3:1:1) on metastatic lung cancer, the mouse model of LLC-metastatic lung cancer was established. The mice were orally administered with YPF (3:1:1), at a daily dose of 129 mg, following inoculation.

Live imaging showed that luminescence intensity in primary tumors was similar between the two groups in the first 2 weeks after intraosseous inoculation (Figure 3A). However, a luminescent signal was detected in lung tissues of the control group, while it was not detected in the YPF (3:1:1)-treated group. Moreover, the luminescent signal was continuously increased in the control group during the 4th and 5th weeks after inoculation. Markedly, administration of YPF (3:1:1) delayed lung metastases for at least 1 week, compared with the control group. Furthermore, the luminescence intensity in lung metastatic foci of the YPF (3:1:1)-treated group was significantly lower than that of the control group at the end of 5th week (Figure 3B). In addition, the survival time of LLC-bearing mice could be significantly prolonged after YPF (3:1:1) treatment (P < 0.01) (Figure 3C). Taken together, although YPF (3:1:1) had almost no suppressive effects on initial tumors in the leg, it dramatically inhibited lung metastases, com-
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pared with the control group (P < 0.01) (Figure 3D, 3E).

YPF (3:1:1) enhanced cytotoxicity in the mouse model of metastatic lung cancer via increasing NK cell population and its degranulation

Accumulating evidence has indicated that YPF (3:1:1) can modulate immune system [3-5]. According to the current in vitro experiment, YPF (3:1:1) showed no effects on migration and invasion of tumor cells (Figure S1). This study showed that YPF (3:1:1) could inhibit lung metastases in a metastatic mouse model. However, its underlying mechanisms remain largely unexplored. Based on current data, YPF (3:1:1) could dramatically enhance the cytotoxicity of splenocytes, compared with the control group (Figure 4A). NK cells and T-cells play a critical role in tumor immune surveillance. This function is mainly associated with their killing ability, in which degranulation is a prerequisite. When degranulation occurs, secretory lysosomes are released and the lysosome-associated membrane protein-1 (CD107a) is translocated onto the cell surface, rendering its accessibility for antibody binding. Therefore, CD107a is a marker for degranulation of NK cells and CD8+ T-cells [13, 14]. The current study first analyzed the population of cytotoxic NK cells and T-cells in spleens of control and YPF (3:1:1) treatment groups by flow cytometry. Results showed that YPF (3:1:1) induced an obvious change in NK cells in spleens but not in T-cells (Figure 4B), suggesting that YPF positively regulated NK cells. However, regarding NK cells or T-cells, which one plays the key role in increasing cytotoxicity of YPF remains unknown. Therefore, this study further investigated the killing ability of NK cells and T-cells upon YPF (3:1:1) administration. This study isolated and purified NK cells and T-cells from splenocytes, then respectively co-cultured them with LLC cells for 5 hours (at an E:T ratio of 100:1). After 5 hours of co-culturing, anti-CD3, anti-NK1.1, and anti-CD8 antibodies were added to samples. Results showed that YPF (3:1:1) could remarkably elevate the degranulation of NK cells (Figure 4C, 4D), but not CD8+ T-cells (Figure 4E, 4F). In conclusion, enhanced cytotoxicity of splenocytes could be attributed to the increased cytotoxicity of NK cells, but not T-cells, suggesting that YPF could enhance NK cell-mediated killing.

NK cell depletion reversed YPF (3:1:1)-mediated tumor suppression

Since YPF (3:1:1) significantly increased the cytotoxicity of lymphocytes via enhancing NK cell population and its degranulation, this study
Figure 4. YPF (3:1:1) enhances NK cell-mediated killing activity. A. Cytotoxic activity. Mononuclear lymphocyte cells were isolated from spleens of tumor-bearing C57BL/6 mice treated with water or YPF (3:1:1) on day 30 after inoculation. Target cells (LLC) co-cultured with effect cells (splenocytes) at the E-T ratios of 10:1, 25:1, 50:1 and 100:1. The values represent the mean ± SD of three independent experiments, *P < 0.05, ***P < 0.001 versus the control group. B. Mononuclear lymphocyte cells were stained with CD3, NK1.1, CD8 antibodies, then collected and analyzed by flow cytometry, ***P < 0.001. C, D. NK cells were purified from splenocytes and then co-cultured with LLC cells (E:T 100:1) for 5 hours. They were stained with CD107a and NK1.1, followed by analysis with flow cytometry. Expression of CD107a was observed and analyzed, **P < 0.01. E, F. T cells were purified from splenocytes and then co-cultured with LLC for 5 hours. They were stained with CD107a, CD3, and CD8 antibodies, followed by analysis with flow cytometry. Expression of CD107a was observed and analyzed.
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Further explored whether YPF-mediated inhibition on tumor metastasis was dependent on NK cells. Depletion of NK cells was carried out by intraperitoneal injections of purified anti-NK1.1 antibody PK136 (Figure 5A, 5B). After oral administration of YPF (3:1:1), YPF (3:1:1)-triggered inhibitory effects on lung metastases were significantly reversed by PK136 antibodies, including reduced splenocyte cytotoxicity (Figure 5C), shortened survival times (Figure 5D) and increased lung nodules (Figure 5E, 5F). Results suggest that suppression of tumor metastasis by YPF was dependent on NK cells.

Figure 5. NK cell depletion reverses YPF-induced tumor suppression. The metastatic lung cancer mouse model was established and treated by YPF (3:1:1) as described in Section 2. Briefly, 100 mg of purified mouse anti-NK1.1 antibody (PK136) per mouse was applied by IP injection, N = 12. A, B. Mononuclear lymphocyte cells were isolated from mouse spleen and then stained with CD3 and NK 1.1 antibodies. This was followed by analysis with flow cytometry. The values represent the mean ± SD of three independent experiments, ***P < 0.001 versus the control group. C. The cytotoxic ability of splenocytes was detected after NK cell depletion. D. Survival curve, N = 12. E, F. After euthanasia, their lung tissues were collected to apply India ink perfusion and lung metastatic tumors (white nodules) were counted under a microscope, N = 12.
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Toxicity study after oral administration of YPF (3:1:1)

Body weights and general appearances of each mouse were regularly monitored during the experiment. There was no reduction in body weight after YPF treatment with two different ratios (2:2:1 and 3:1:1) (Figure 6A). Biochemical functions of the liver and kidneys were also assessed. There were no obvious differences between the control and YPF treatment groups (Figure 6B, 6C). Furthermore, livers and kidneys from the control and YPF (3:1:1) treatment groups were stained with H&E. Histological structures of livers and kidneys were examined and compared microscopically. Data showed that there were almost no histological changes after YPF (3:1:1) treatment (Figure 6D), suggesting that YPF (3:1:1) inhibited lung metastases with minor adverse effects. Therefore, YPF (3:1:1) could be a potential therapeutic candidate for lung metastases.

Discussion

Metastases are the main cause of high mortality in cancer patients. Therefore, it is crucial to mimic the metastatic tumor microenvironment in scientific research. Multiple recent studies have shown that bone marrow offers a superior and more appropriate environment for improving vascularization, increasing tumor growth, and promoting distant metastases in tumor development [15, 16]. Cutrera et al. [17] proved that intraosseous inoculation is a unique type of inoculation, in which tumor cells are directly injected into the bone marrow cavity instead of leaking into the bloodstream due to the inoculation. Generally speaking, metastatic cascade includes primary neoplasms, vascularization, invasion, embolisms, circulation, adherence, extravasation, and proliferation [18]. Intravenous injection is the most common method to establish a metastatic mouse model. However, the first four steps of metastatic cascade (primary neoplasm, vascularization, invasion, embolism) are omitted when a large single dose of tumor cells is directly released into the bloodstream. Therefore, tumor development via intraosseous inoculation is more likely to mimic the initiation and development of human tumors, by which all above-mentioned processes are involved in tumor development. The present study established a mouse model of metastatic lung cancer by intraosseous inoculation. Obvious lung metastasis could be ob-

Figure 6. Toxicity study of YPF (3:1:1) treatment. A. Mouse body weight measurement, N = 10. B-D. Detection of serum levels of AST/ALT and creatinine (Cr) reflected liver or kidney function post control and YPF (3:1:1) treatment, N = 10. The values represent the mean ± SD of three independent experiments. E, F. The histological observation (H&E staining) of kidneys and livers in control and YPF (3:1:1) treatment groups, N = 8.
served by just injecting $2 \times 10^4$ tumor cells. Moreover, initial tumor development and distant metastases could be induced by as few as $2 \times 10^4$ tumor cells into the bone marrow environment. However, if the number of injected tumor cells was too low, it took a long time to generate the primary bone tumor. Alternatively, if the number of injected tumor cells was too high, mice would have a quite short life span due to the excessive primary tumor burden and heavy metastases.

The compatibility of Chinese herbal drugs plays a vital role in herbal formulas, according to Traditional Chinese Medicine theory, because every herb has its own “four properties and five tastes” and “channel tropism”. Only when they are mixed together, at appropriate ratios, can the mixture take effect. YPF formula is composed of Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu), and Saposhnikoviae Radix (Fangfeng). Previous studies have proven that the pharmaceutical effects of YPF formula (combination of these three herbs) are superior to individual herbs or two-herb combinations [10, 19]. The reason may be attributed to the idea that more components become active in the final formula due to herb compatibility. YPF has two common formulas according to component weight ratios. The first one is 2:2:1, while the other one is 3:1:1. Both are well-documented in ancient Chinese books and scientific literature. They have been extensively used in clinical practice for thousands of years, based on “syndrome differentiation and treatment”. The current study tested the formula with a ratio of 2:2:1, finding that this formula had inhibitory effects neither on lung metastases nor shrinkage of primary bone tumor size in the mouse model of metastatic lung cancer (Figure 2). However, lung metastases were significantly inhibited by the YPF formula with a ratio of 3:1:1 (Figure 3). Based on compatibility of Chinese herbal drugs, Astragali Radix (Huangqi) is the principal component in the whole formula, meaning that it plays a pivotal role in YPF. Astragali Radix (Huangqi) consists of more than 100 compounds, including polysaccharides (AMP), flavonoids, amino acids, saponins, and other trace elements. Many studies have revealed the pharmacological functions of Astragali Radix (Huangqi), including antitumor [20], antioxidant [21], anti-inflammatory [22], antihyperglycemic [23], and antiviral activities [24]. Moreover, most of these therapeutic effects are related to its modulatory role in the immune system. For instance, AMP can increase body weight, spleen index, and thymus index, as well as the phagocytic function of macrophages in H22 hepatocellular carcinoma-bearing mice. It can also promote secretion of serum IL-2, IL-12, and TNF-α, but decrease IL-10 levels in serum. These may be the underlying mechanisms of its inhibitory effects on tumor development [25]. Flavonoids are another component from Astragali Radix. They suppress the production of pro-inflammatory cytokines, such as IL-6, IL-12 p40, and TNF-α, in bone marrow-derived dendritic cells (BMDCs), suggesting that flavonoids are a natural source of inflammation inhibitors [26]. In the current study, YPF (3:1:1), in which the usage of Astragali Radix (Huangqi) is the largest, was shown to strengthen the immune regulatory effects of YPF on lung metastases.

Chinese medicine takes effect relatively slowly. Its therapeutic effects are not overly strong, due to the characteristics of its natural compounds. Therefore, although YPF (3:1:1) could not inhibit the development of primary bone tumors, the general situation of mice in the YPF treatment group was superior to that of the control group. However, this study fed mice with YPF at the same day of inoculation without any pre-treatment. However, if YPF (3:1:1) administration was combined with some chemotherapies, immune cell therapies, or even amputation, it is believed that the survival time of mice could be prolonged. After oral administration, YPF (3:1:1) dramatically enhanced cytotoxicity in the mouse model of metastatic lung cancer. Moreover, it could increase NK cell population in the spleen (Figure 4B) and also enhance the degranulation of NK cells (Figure 4C, 4D), but not CD8+ T-cells (Figure 4E, 4F). However, such inhibitory effects of YPF (3:1:1) on lung metastases could be markedly reversed once NK cells were depleted by anti-mouse NK1.1 antibody (PK136) (Figure 5). NK cells play an important role in host immunity against cancer by preventing neoplastic development in a process called “cancer immunosurveillance” [27, 28]. However, their activity can be eluded in the tumor microenvironment, interfering with NK cell activation pathways or the receptors that modulate NK-mediated killing activity [29]. Besides, the population of NK cells is always
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pretty low in cancer patients [30, 31]. Therefore, increasing NK cell population and enhancing NK-mediated killing activity have been proposed as a potential strategy for cancer immunotherapy [32, 33]. In the present study, YPF (3:1:1) significantly increased the cytotoxicity of lymphocytes in the mouse model of metastatic lung cancer via increasing NK cell population and its degranulation in the spleen. Strikingly, the depletion of NK cells significantly decreased the inhibitory effects of YPF on tumor metastasis, providing convincing evidence that YPF (3:1:1) enhances the cytotoxic ability of NK cell-dependent lymphocytes.

Metastases is the leading cause of cancer-related deaths, worldwide. Except for surgeries, chemotherapy is the most commonly used treatment for cancer patients. However, anticancer chemotherapy has been largely restricted due to its serious adverse effects, such as liver or kidney injuries. Current data demonstrated that YPF (3:1:1) treatment could inhibit lung metastasis, in vivo, with minimum side effects, suggesting that the levels of biochemical makers of livers and kidneys, such as ALT, AST and Cr, were still within the normal range post YPF (3:1:1) treatment. Therefore, YPF (3:1:1) might be used as a safe and potent immune regulatory drug for cancer immunotherapy.

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Disclosure of conflict of interest

None.

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References

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[30] Abel AM, Yang C, Thakar MS and Malarkannan S. Natural killer cells: development, matura-


Figure S1. YPF (3:1:1) has no direct inhibitive effect on non-small-lung cancer cells. In the migration assays, the non-small-lung cancer cell lines LLC and H460 were pre-treated with either control or YPF (3:1:1) for 2 days and then seeded in Transwell plates for 24 h.