Original Paper

Bushenshugan Formula Attenuates the Development of Lung Cancer by Inhibiting Epithelial-Mesenchymal Transition

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Key Words

Bushenshugan Formula • Epithelial-mesenchymal transition • Lung cancer

Abstract

Background/Aims: BushenShugan Formula (BSF) is a traditional Chinese medicine that has therapeutic effects on middle- and late-stage lung adenocarcinoma in clinical application. It was reported that Bushen Chinese medicine suppressed the onset of pre-metastatic niches in a murine model of spontaneous lung metastasis. However, the mechanisms of BSF on human lung adenocarcinoma remain unknown. **Methods:** Cell proliferation was determined by CCK8 and colony formation. Cell apoptosis and cell cycle were detected by flow cytometry. Cancer stem cells properties were examined by spheroid body formation. The migration and invasion abilities were analyzed by wound healing assay and transwell invasion assay. The mRNA expressions were determined by qRT-PCR. Western blotting analysis showed the protein levels. **Results:** BSF was shown to inhibit the proliferation of A549 cells in time- and concentration-dependent manners. Colony formation assays also indicated the antiproliferative effect of BSF against A549 cells. Cellular mechanistic studies demonstrated that BSF arrested the cell cycle in G2/M phase and induced apoptosis. Importantly, BSF could inhibit the epithelial-mesenchymal transition(EMT) of A549 cells through PI3K/AKT/NF-κB pathway. **Conclusions:** BSF effectively inhibited tumour growth, suggesting that it is a promising anticancer treatment for further clinical development.

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Introduction

Lung cancer is a malignant tumour that is seriously harmful to human health. The incidence and mortality of lung cancer are the highest among malignant tumours worldwide. Lung cancer is a highly metastasizing cancer and the number one cause of death in humans; it is classified into two broad histologic classes in terms of growth and spread: small-cell lung carcinoma (SCLC) and non-small cell lung carcinomas (NSCLC). Although some early-stage lung cancer patients can be successfully treated, 70% of patients present with advanced stage when diagnosed, the cancer has already spread locally or to distant organs, and have lost the chance of operation [1]. Metastasis is the leading cause of death by cancer. Chemotherapy has advanced considerably over the last 30 years, but the 5-year relative survival rate for lung cancer remains only 17.4% [2]. Elucidation of the mechanisms of lung cancer and development of various treatments (surgery, chemotherapy, and radiotherapy) has made advances in recent years, especially for NSCLC. Cisplatin (DDP) is a well-known chemotherapeutic drug for the treatment of NSCLC, but poor compliance and drug resistance remain concerns. Thus, there is an urgent need to develop novel anticancer agents, which may include highly effective Chinese medicines.

EMT is an important regulator in lung cancer cells. In addition, it is also a key process that occasionally occurs during lung cancer metastasis and invasion in which lung cancer cells acquire a more aggressive phenotype [3, 4]. Based on these significant findings, EMT is present in a variable proportion of lung cancer cells and results in up-regulation of mesenchymal markers, such as N-cadherin, snail and vimentin, and down-regulation of epithelial markers, such as E-cadherin. EMT is an important mechanism of tumour invasion and metastasis, suggesting that EMT may be a key factor in metastasis of lung cancer and providing a new direction for the treatment of lung cancer.

Modern clinical pharmacology research and preliminary results have shown that Chinese medicine has a multi-target effect and can inhibit the recurrence and metastasis of tumour and significantly improve patient quality of life [5-8]. There are many varieties and sources of Chinese medicine in China, and further analysis of the traditional theory of TCM (Traditional Chinese Medicine) and identification of effective anti-tumour monotherapies or compounds based on modern scientific results is one direction for anti-tumour research in the future. Bushen Chinese medicine has been shown to suppress the onset of the pre-metastatic niche in a murine model of spontaneous lung metastasis [9]. BSF is derived from Wuzi Yanzong pills and composed of Schisandra, Bupleurum, Chinese angelica, Medlar, Dodder seed, Ligustrum lucidum, Plantago, Fructus trichosanthis and 10 other Chinese herbal medicines. BSF has good anti-lung cancer metastasis effects, and it could prevent tumour recurrence, as the clinical empirical formula exhibits potent therapeutic effects on human lung carcinoma in our previous studies [10-13]. However, the mechanisms of BSF on human lung adenocarcinoma and the effect on EMT remain unknown. In this study, we aimed to investigate whether BSF inhibited EMT and explored the possible anticancer mechanisms on human lung cancer.

Materials and Methods

Animals

Sixty male SD rats, weighing 250-300g, were supplied by the Center of Experiment Animal of Zhengzhou University [SCXK (Yu) 2010-0002, Henan, China]. The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Zhengzhou University. All rats were maintained under a regulated temperature (18-22°C) and relative constant humidity (50%-60%). Animals were treated according to protocols established by the ethics committee of Zhengzhou University, and the *in vivo* experiments were carried out in accordance with the approved guidelines and approved by the ethics committee of Zhengzhou University.

Serum Preparations

BSF from the First Affiliated Hospital of Zhengzhou University in this study was a Chinese recipe including the following Chinese herbal medicines: Bupleurum 6g, Schisandra 6g, Angelica 6g, Red peony root 6g, Medlar 10g, Dodder 9g, Plantain 15g, Raspberry 9g, Astragalus 18g, Epimedium 9g, Houttuynia 15g, Trichosanthes 12g, Allium white 9g, Prunella 9g, *Ligustrum lucidum* 9g, *Houttuynia cordata* 15g, Polyporus 9g, Gentian 2g, 1 centipede. The concentration of the BSF solutions were 1.25g/mL (low dose) and 3.75g/mL (high dose), and the solutions were stored at 4°C for use. DDP was from Shandong Qilu Pharmaceutical Co., Ltd. The 60 male rats were divided into 6 groups using a random digits table method (n=10 per group); the groups included the BSF Low-dose group, BSF High-dose group, cisplatin(DDP) group, Low-dose combined with DDP group, high-dose combined with DDP group and saline group. According to a human and murine body surface area conversion method, the BSF low-dose group and high-dose group were respectively given 15g/kg and 30g/kg BSF by gavage once during 7days. The DDP group was given BSF solution intraperitoneally at a dose of 8g/kg on the first, third and fifth day. The low-dose combined with DDP group and high-dose combined with DDP group were respectively given 15g/kg and 30g/kg BSF by gavage once a day and 8g/kg DDP on the first, third and fifth day. The saline group received an equal volume of 0.9% sodium chloride solution orally once during 7days. Chloral hydrate anaesthesia was performed 2 hours after the last gavage, and then, we collected the blood from the abdominal aorta under sterile conditions and separated the serum. The sera were inactivated at 56°C for 30 minutes, then filtered by a filter (0.22μm) to remove bacteria, and finally stored at -20°C. Drug-containing medium contained 10% serum from the BSF Low-dose, BSF High-dose, DDP, low-dose combined with DDP, high-dose combined with DDP and saline groups.

Cell lines and cell culture

The human lung cancer A549 cells were provided by the Cancer Experimental Center of Zhengzhou University. All cells were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% foetal bovine serum(FBS) (HyClone, USA), 100U/mL penicillin, and 100μg/mL streptomycin at 37°C in a humidified 5% CO₂ incubator (Thermo, USA).

Cell Proliferation Assay

A549 cells were seeded in a 96-well culture plate at a density of approximately 4×10⁴ cells/well, 6 wells per group. Then, 100μL suspension per well was cultured in 1640 medium containing 10% FBS, and 12h later, the medium was discarded, and the cells were treated with 100μL media containing serial concentrations of BSF and cultured for another 72 hours. Cell Counting Kit-8 (CCK-8) solution at 10μL was added to each well and incubated for another 1h before it was discarded. The absorbance(A) was measured by a microplate reader at a wavelength of 450nm. The inhibitory ratio was calculated by the following formula: Inhibition rate (%) = (1-experimental group A/normal group A)×100%. The experiment was repeated three times under the same conditions.

Colony formation assay

A549 cells were trypsinised and seeded at 1000 cells per well in 6-well dishes. Cells were allowed to attach overnight and then exposed to different concentrations of BSF for 14 days. After they were fixed with 4% paraformaldehyde, the cells were stained with 0.1% crystal violet. The colony formation rate was calculated and analysed as the following equation: colony formation rate = (number of colonies/number of seeded cells) ×100%.

Migration assay

To determine cell motility, A549 cells (1×10⁵ cells/well) were seeded in 24-well plates and incubated at 37°C/5% CO₂ overnight. Wounds were made using 200μL pipette tips. Cells were then washed twice with Hank's balanced salt solution to remove floating cells, and fresh medium was added. Cells migrating from the edge of the wound were photographed from five random fields of view during different time periods.

Transwell Invasion Assay

The invasion assays with A549 cells were performed as described previously. The invasion of the cells was measured with Matrigel-coated transwell inserts. Matrigel matrix (25μL/well) was placed in

the upper chamber of the transwell filter (pore size of 8μm) and was incubated for 30 minutes at 37°C for gelling. A549 cells were trypsinised and seeded at 2×10^4 cells (300μL) per well in basic 1640 medium with Matrigel matrix (25μL/well) in the upper chambers. Drug-containing medium was placed in the lower well. After 24 h incubation at 37°C, cells in the upper side were scraped with a cotton swab, and invaded cells were fixed with 4% paraformaldehyde and stained with Giemsa. The cells were rinsed with PBS, air-dried and observed under a microscope equipped with a camera.

Spheroid body formation

A549 cells (1000 cells/mL) were seeded on ultra-low attachment plates (Corning, Lowell, MA, USA) and incubated at 37°C/5% CO₂ to form spheroid bodies. Cells were cultured in DMEM/F12 (Gibco, Carlsbad, CA, USA) with B-27 supplements (Gibco), 10% FBS, basic fibroblast growth factor (Sigma-Aldrich, Poole, UK) and epidermal growth factor (Calbiochem, San Diego, CA, USA) for 7 days at 37°C/5% CO₂.

Cell Cycle Assay

Cells were seeded in 24-well plates at a density of approximately 1×10^5 cells/well and incubated for 24h, and then, the medium was discarded, and cells were cultured with the drug-containing medium for 72h. A549 cells were digested with trypsin without EDTA and collected in a 1.5mL centrifuge tube by centrifugation at 1000rpm/min. After washing twice with cold saline and discarding the supernatant, 1mL precooled 70% ethanol was added at 4°C overnight. The next day, the cells were resuspended in 500μL precooled phosphate buffered saline (PBS) containing 25g/L RNase and 2.5μL PI (10 g/L) for staining at 37°C for 30 min. Cell cycle analysis was performed by flow cytometry, and the cells in the G0/G1, S and G2/M phases were measured using FlowJo software.

Cell Apoptosis Assay

The cells were digested with trypsin without EDTA. The density of cells was adjusted to 1.0×10^5 /ml, and 1 mL of cells was seeded in each well of 24-well plates. BSF-induced apoptosis of A549 cells was quantified by an Annexin V-FITC/PI dual staining assay [14]. Briefly, cells were treated with different concentrations of BSF for 72 h. Then, the cells were collected, centrifuged and resuspended with PBS. After centrifugation, the cells were resuspended with 500μL binding buffer and incubated with 5μL Annexin V-FITC and 4μL PI at 4°C for 0.5 h in the dark. Then, the cells were analysed immediately by flow cytometry.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

After collecting A549 cells treated for 72 h, total RNA was extracted using TRIzol (Invitrogen, USA). The first-strand cDNA was synthesised from 1μg of total RNA using a Super Quick RT cDNA Kit (CWbio, China) according to the manufacturer's instructions. Then, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using SYBR Green PCR Master Mix (Qiagen, Germany) and assessed by Agilent Mx3005P. GAPDH was used as an internal control. Primer sequences for qRT-PCR are shown in Table 1.

Western blot

First, 1×10^6 cells were lysed with mixed RIPA buffer containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1% PMSF (100×) (Cwbio, Beijing, China) on ice for approximately 30mins and then centrifuged at 1500 g for five min at 4°C. Next, 30μg proteins were loaded onto a 12% SDS-PAGE gel and transferred to a PVDF (Millipore, Bedford, MA) membrane. The membrane was blocked in TBST buffer containing 5% non-fat milk at room temperature (22°C) for 1 h and then probed with Bcl2 (1:1000, Genetex, GTX34425), P53 (1:500, Abcam, ab131442), Bax2 (1:1000, Genetex, GTX109683), CyclinD1 (1:3000, Proteintech, 60186-1-Ig), E-cadherin (1:2000, Abcam, ab40772), N-cadherin (1:800, Abcam, ab18203), Vimentin (1:1000, Abcam, ab92547), α-SMA (1:1000, Genetex, GTX100034), snail (1:1000, Abcam, ab53519), P-PI3K (1:1000, CST, 13857), PI3K (1:1000, CST, 4249), P-AKT (1:1000, CST, 4691), AKT (1:2000, CST, 4064), P-NF-κB (1:1000, CST, 3033) and NF-κB (1:1000, CST, 6956) antibodies at 4°C

Table 1. RT-PCR Primer Design

Gene	Primer sequence
GAPDH	QF: GGTATCGTGGAAGGACTCATGAC QR: ATGCCAGTGAGCTTCCCGTCAGC
N-cadherin	QF: CCACAGTACCCAGTCCGATCC QR: ACTAAGAGGGAGTCATACGGTGG
E-cadherin	QF: GGATTGCAAAATCTGCCATTTC QR: AACGTTGTCCCGGTGTCA
Vimentin	QF: AAAGTGTGGCTGCCAAGAAC QR: CTGCACCTGTCTCCGGTACT
α-SMA	QF: CCACCGCAAATGCTTCTAAGT QR: GGCAGGAATGATT GGAAAGG
Snail	QF: AATGCTCATCTGGACTCTGTC QR: TCTTGACATCTGAGTGGGTCTG

overnight, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then, the membrane was exposed to chemiluminescent reagent (ECL) for approximately 5–10 min. The membranes were exposed to X-ray photographic films in a darkroom, and the band densities were later quantified with Image J software (NIH, USA).

Statistical Analysis

All data are expressed as the mean $\pm$ standard deviation (SD) of three independent biological experiments. Statistical significance was assessed using the two tailed Student's t-test (for comparisons of two treatment groups) and one-way ANOVA (for comparisons of three or more groups). * $P < 0.05$ was considered statistically significant.

Results

Effects of the BSF serum on proliferation and tumorigenicity of A549 cells

All groups showed significantly inhibited proliferation of A549 lung cancer cells compared with the saline group (Fig. 1A). The inhibition rate of the BSF High-dose group was second only after the DDP group. The inhibition rate of the high-dose combined with DDP group was the strongest, and after treatment for 24 h, 48 h, and 72 h, the inhibitory rates were 45.39%, 54.76% and 59.94%, respectively.

Abnormal proliferation of cancer cells results in malignant tumours. Colony formation assays directly assess the proliferative ability of a single cancer cell to form a cell colony *in vitro*. The tumorigenicity of A549 cells and the inhibitory effect of BSF on colony formation of A549 cells were measured by colony formation assays. BSF effectively inhibited the colony-forming ability of DDP-treated A549 cells compared with those treated with cisplatin alone. The colony formation ability decreased drastically in the high-dose BSF combined with DDP group. Representative images and quantitative analysis of the colony formation rate are presented in Fig. 1B and C.

Effects of the BSF serum on apoptosis and the cell cycle of A549 cells

To investigate the cytotoxic mechanism of BSF, we determined the effects on A549 cell apoptosis by using flow cytometry. The results of apoptosis assays are shown in Fig. 2A and B. BSF was found to be a potent inducer of apoptosis in a dose-dependent manner. The apoptosis rate of the high-dose combined with DDP group was the highest among all the groups.

The cell cycle involves a series of events that lead to cell division and replication. The cell cycle consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis). As a hallmark of A549 cells, cell cycle dysregulation contributes to the aberrant cell proliferation and development of cancer. Therefore, targeting cell cycle progression is an effective method for novel anti-tumour drug discovery. To determine whether the

Fig. 1. Effects of the BSF serum on the proliferation and tumorigenicity of A549 cells. (A) Cell viability was evaluated at the indicated time points using CCK8 assays. (B) The colonies of A549 cells with >100 cells were quantified. Original magnification: $200\times$. (C) The bar graphs represent the average number of cloned cells under each group of processing conditions: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value $\pm$ SD. * $P < 0.05$ compared with group a.

Fig. 2. Effects of the BSF serum on the apoptosis and cell cycle of A549 cells. (A) Cell apoptosis was measured by flow cytometric analysis. (B) The bar graphs represent the percentage of apoptotic cells. (C) The cell cycle was measured by flow cytometric analysis. (D) The bar graphs represent the percentage of G2/M, S and G0/G1 phase A549 cells in each group. (E) The expression levels of the cell apoptosis and cell cycle-related proteins Bcl-2, P53, Bax and CyclinD1 were detected by western blot. (F) The bar graphs represent the average quantitative value of normalized expression to β -Actin in each group: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value $\pm$ SD. * $P < 0.05$ compared with group a.

inhibitory effects of BSF on A549 cell growth were correlated with cell cycle arrest, we further investigated the effects of BSF on cell cycle progression. As shown in Fig. 2C, high-dose BSF combined with DDP could induce cell cycle arrest in the G2/M phase in A549 cells.

Some apoptotic and cell cycle regulatory proteins were also detected by western blot analyses (Fig. 2E and 2F). The expression levels of Bcl-2 and CyclinD1 were inhibited by BSF, and the high-dose BSF combined with DDP treatment was the most effective. However, BSF could increase the expression of Bax and P53, and DDP had a synergistic effect.

Effects of the BSF serum on the cancer stem cells (CSCs) of A549 cells

Cancer stem cells (CSCs) are a specific subpopulation of tumour cells that selectively possess tumour initiation and self-renewal capacities, as well as the ability to give rise to bulk populations of non-tumorigenic cancer cell progeny through differentiation [15]. We examined the CSC properties of A549 cells by BSF. We generated floating spheroid bodies to assess the self-renewal and differentiation capacities of these cells. As shown in Fig. 3A and B, BSF altered the morphology of spheroid cells, and the sizes of the spheroid bodies were reduced. These results suggest that BSF strongly inhibited CSC properties of A549 cells.

Effects of the BSF serum on the migration and invasion of A549 cells

The migratory effects of BSF against A549 cells were analysed by wound-healing assays according to the previous method. Wounds were made using pipette tips, and cell motility was observed after 24 h, as shown in Fig. 4A and B. Based on the results, high-dose BSF combined with DDP significantly reduced cell motility of A549 cells. For wound-healing assays, phase contrast microscopy images of control A549 cells treated with BSF cultures were acquired at 0 and 24 h after wounding. The high-dose BSF combined with DDP group showed significantly reduced cell motility of A549 cells.

BSF inhibited the invasion of A549 cells. After cells were cultured with drug-containing medium for 24 h, the invasion of A549 cells showed different degrees of decline in Fig. 5A and B. While the BSF high-dose group showed significantly inhibited invasion of A549 cells,

Fig. 3. Effects of the BSF serum on the cancer stem cells of A549 cells. (A) Bright-field microscopy images of spheres generated from A549 cells after the sphere-formation assay. Original magnification: 200×. (B) The bar graphs represent the number of spheres in each group: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value ± SD. *P<0.05 compared with group a.

Fig. 4. Effects of the BSF serum on the migration of A549 cells. The migratory capabilities of A549 cells were evaluated by scratch assays. (A) Representative microscopic images of the bottom chamber are shown. (B) The bar graphs represent the fold change of cell migration distance in each group: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value ± SD. *P<0.05 compared with group a.

Fig. 5. Effects of the BSF serum on the invasion of A549 cells. The invasive capabilities of A549 cells were evaluated by using transwell invasion assays. (A) Representative microscopic images of the bottom chamber are shown. (B) The bar graphs represent the average number of invaded cells on the underside of the membrane in each group: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value ± SD. *P<0.05 compared with group a.

this treatment showed synergy combined with DDP. These results also suggested that DDP might have synergistic effects on the inhibition of invasion. The differences between the experimental groups and control group were significant (P<0.05).

Effects of the BSF serum on EMT of A549 cells and related molecular mechanisms

As shown in Fig. 6A, the DDP group showed no significant inhibitory effects on the EMT-related genes, but BSF in this assay decreased the expression levels of N-cadherin and

Fig. 6. (A) Effects of the BSF serum on genes related to EMT. E-cadherin, N-cadherin, Vimentin, α -SMA and Snail were decreased at the mRNA level. Relative gene expression was determined by normalising the expression of each target gene to GAPDH. (B) The expression levels of the EMT-related proteins E-cadherin, N-cadherin, Vimentin, α -SMA and Snail were examined through western blot. (C) The bar graphs represent the average quantitative value of normalized expression to β -Actin in each group. (D) The expression levels of PI3K-AKT-NF/ κ B-related proteins. PI3K, P-PI3K, AKT, P-AKT, NF/ κ B and P-NF/ κ B were examined through western blot. (E) The bar graphs represent the average quantitative value of normalized expression to β -Actin in each group: group a. Saline group, b. BSF Low-dose group, c. BSF High-dose group, d. Low-dose combined with DDP, e. High-dose combined with DDP, f. DDP. The data are expressed as the mean value $\pm$ SD. * $P < 0.05$ compared with group a.

Vimentin and increased the expression of E-cadherin in a concentration-dependent manner. Importantly, BSF combined with DDP exhibited the most potent regulatory effect on target genes. The protein expression levels are shown in Fig. 6B and Fig. 6C. BSF could increase the expression of E-cadherin and decrease the expression levels of N-cadherin, Vimentin, α -SMA and Snail. All these results demonstrated that DDP displayed synergy with BSF in the EMT progression. To explore the molecular mechanisms related to EMT of A549 cells, we examined the expression levels of proteins related to the PI3K-AKT signalling pathway. As shown in Fig. 6D and Fig. 6E, BSF combined with DDP could decrease the expression levels of P-PI3K, P-AKT and P-NF- κ B.

Discussion

NSCLC is one of the most common malignant tumours and a leading cause of cancer-related death worldwide [16]. Although the National Comprehensive Cancer Network (NCCN) panel recommended platinum-based chemotherapy regimens as a preferred therapy for patients with NSCLCs, these treatments have many side effects, including fever, anaemia, and alopecia, which have significantly limited their use and decreased patient quality of life [17]. Since metastasis and recurrence are considered to be responsible for the failure of treatments, the mechanism of lung cancer metastasis has been a hot spot in cancer research [18, 19]. Therefore, there is an urgent and increasing need to develop effective novel methods or pharmacotherapies to solve this problem.

EMT is an evolutionarily conserved process that plays an important role in the embryonic development and the progression of tumours. Meanwhile, EMT is believed to be involved in metastasis and drug resistance [3]. A recent study also suggested that EMT could increase

the motility and invasiveness of cancer cells [20-22]. E-cadherin and N-cadherin are major biological markers in EMT progression, and they could be regulated during the therapeutic process [20, 22, 23]. In addition, Vimentin is widely used as a marker of the epithelial to mesenchymal transitions (EMTs) that occur during embryogenesis and metastasis [24, 25]. As a member of the zinc finger inhibitory transcription factor family, Snail could bind directly to the promoter region of E-cadherin, thus inhibiting the expression levels of E-cadherin and then promoting EMT [26, 27]. The PI3K/AKT signalling pathway mediates the process of EMT and has attracted widespread attention as a potential target for the prevention and treatment of metastatic tumours [28, 29]. NF- κ B is a ubiquitously expressed transcription factor that has been reported to act downstream of the PI3K/Akt pathway. This protein is a major target of activated AKT that regulates genes involved in cancer cell migration and invasion [30].

Cell apoptosis and the cell cycle play an important role in the pathogenesis of cancers. The development of cancer mainly depends on the balance of pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2 [31]. The p53 protein is a regulatory factor of many processes necessary for the proper functioning of cells. It plays a critical role as a tumour suppressor, inducing cell cycle arrest and apoptosis [32]. CyclinD1, as a major member of the cyclins, plays an important role in cell cycle progression [33].

Lung cancer according to TCM is a disease that involves excess in the body but deficiency in the holistic concept of TCM, and the deficiency is the root cause, while the excess is the manifestation. Lung cancer lesions are located in the lungs but are often related to the liver, spleen, stomach and kidney. Liver governs conveyance and dispersion and affects emotions. Lung governs the qi, whose diffusion and decrease depend on the conveyance and dispersion functions of the liver. Lung is the mother of the kidney and governs regulation of water passages, and kidney governs water metabolism. In early research, we found that the kidney regulates the lung via androgens and their receptor, which may be the material basis of the mutual relationship of the lung and kidney [34]. In the late stage of lung cancer, symptoms include kidney essence deficiency and emotional depression. Studies demonstrated that Bushen prescription can improve the patient's immune system [35, 36] and inhibit lung cancer growth and metastasis through a variety of mechanisms [9, 37]. Dispersing stagnated liver qi to regulate qi for the treatment of lung cancer is still rare, but the incidence of depression in the patients who were diagnosed with lung cancer is approximately 15%-44% according to statistics [38].

With broad applications in the treatment of lung cancer [39, 40], breast cancer [41], colorectal cancer [42] and other tumours, traditional Chinese medicines have been explored and have provided new therapeutic targets. BSF had beneficial effects on the kidney and the liver in clinical practice. BSF was also shown to benefit the kidney and nourish semen, dispersing stagnated liver qi to regulate qi, dissipating phlegm and resolving masses. In our earlier research, BSF contributed to the improvement of human immunity and inhibited proliferation of tumour cells by apoptosis. However, the effect of BSF on EMT remains unknown.

In this work, BSF could effectively inhibit the proliferation of A549 cells in time- and dose-dependent manners and had synergistic effects combined with DDP. The inhibition rates at 24 h, 48 h and 72 h were 45.39%, 54.76% and 59.94%, respectively. The colony formation ability decreased drastically with high-dose BSF combined with DDP. Annexin V/PI staining with flow cytometry suggested that the herb serum could induce cell apoptosis in the concentration-dependent manner and arrest the cell cycle at G2/M phase. Western blot analysis showed that BSF combined with DDP could decrease the expression levels of cyclinD1 and Bcl2 but increased the expression levels of P53 and Bax. Floating spheroid bodies demonstrated that BSF strongly inhibited the CSC properties of A549 cells. In further research, BSF decreased the gene and protein expression levels of N-cadherin, Vimentin, Snail and α -SMA, while it increased the expression levels of E-cadherin. Further, BSF combined with DDP could decrease the protein expression levels of P-PI3K, P-AKT and NF- κ B.

In summary, BSF could inhibit the proliferation, decrease the colony formation, induce cell apoptosis, inhibit the invasion and block the CSC properties of A549 cells. Cellular mechanisms suggested that BSF inhibited the process of EMT and affected the expression of N-cadherin, Vimentin, Snail, α -SMA and E-cadherin through PI3K/AKT/NF- κ B pathway. Thus, BSF as a traditional Chinese medicine might provide new insight into the treatment of NSCLC. In addition, elucidation of the pharmacological mechanisms and the specific therapeutic targets of BSF is underway.

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Disclosure Statement

The authors declare that they have no competing interests.

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