Modulation of radiation-induced tumour necrosis factor-α and transforming growth factor β1 expression in the lung tissue by *Shengqi Fuzheng* injection

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Abstract. Radiation-induced lung injury (RILI) is one of the most common and severe side effects of thoracic radiotherapy. Therefore, novel therapeutic approaches to improve the effectiveness of RILI treatment are required. The present study was designed to determine the effectiveness of a traditional Chinese medicine regimen, *Shenqi Fuzheng* injection (SFI), in the treatment of RILI. SFI is composed of extracts from *codonopsis pilosula* and *radix astragali*. Here, we determined the protective effects of SFI on RILI with a single-dose irradiation (RT) of 12 Gy in C57BL/6 8-week-old mice. The mice were divided into four groups treated with i) phosphate-buffered saline (PBS; pH 7.4, 20 ml/kg/day) alone as normal a control; ii) SFI only (20 ml/kg/day); iii) RT + PBS (20 ml/kg/day); and iv) RT + SFI (20 ml/kg/day). SFI and PBS were administered via intraperitoneal injection 1 week before and 2 weeks after RT. The pathology of RILI and any clinical signs of toxicity were monitored. The expression of tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 in the lungs was analyzed by RT-PCR and immunohistochemistry. TNF-α and TGF-β1 expression was increased by RT, but was reversed by SFI treatment during the radiation pneumonic and fibrotic phases ($P<0.05$). Lung histology at 24 weeks revealed a significant decrease in structural damage and collagen deposition in the RT + SFI group compared to the RT + PBS group. In conclusion, TNF-α and TGF-β1 are key mediators for the pathogenesis of RILI, and SFI reduces TNF-α and TGF-β1 expression after RT. This may be a key mechanism behind the preventive effects of SFI on lung injury after radiation.

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Introduction

Pulmonary radiation is an important curative and palliative modality in the treatment of thoracic cancer, but is often associated with toxicity to neighbouring normal tissues, which limits the deliverable dose intensity (1). The radiosensitivity of lungs represents a critical late toxicity issue after radiation therapy; clinical manifestations of lung toxicity, termed radiation-induced lung injury (RILI), mainly include radiation pneumonitis and lung fibrosis. RILI is the most common and most severe side effect of thoracic radiotherapy (2-4). It is usually unavoidable, seriously affects the quality of life of patients, and suggests a poor prognosis (5). The incidence of RILI is 10-20% (6); 50-100% of patients with RILI develop radiological evidence of regional lung injury, while 50-90% experience a decline in pulmonary function (7). Early RILI manifests as radiation pneumonitis, occurs between 1 and 3 months of radiation, and is characterized by the loss of type I pneumocytes, increases in capillary permeability, interstitial edema, alveolar capillary congestion and the accumulation of inflammatory and immune cells from peripheral blood in the alveolar space. Radiation pneumonitis may trigger multiple repair mechanisms to restore lung function and remodel lung fibrosis (8-10). The duration (hours to days) before the obvious appearance of radiation pneumonitis is referred to as the 'latent' period, since no clear histopathologic signs are observed. If radiation pneumonitis develops, it leads to pulmonary fibrosis characterized by the loss of capillaries, thickened alveolar septa and obliteration of the alveolar space. The clinical symptoms of RILI are characterized by cough, dyspnea and fever.

Several studies have shown that pro-inflammatory cytokines play direct and indirect roles in the development of RILI (11-13). Tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 are among the most crucial pro-fibrosis cytokines. TNF-α and TGF-β1 expression is elevated in lung tissue after irradiation (12,14,15). Therefore, in this study, TNF-α and TGF-β1 were used as biomarkers to evaluate the effects of RILI treatment.

Although several agents have been used for the treatment of RILI (including glucocorticoid, angiotensin-1-converting

enzyme inhibitors, non-steroidal anti-inflammatory drugs, adrenocorticotropic hormone and cortisone) (16), they show limited effectiveness and their adverse effects are significant, which limits their clinical application (17). A novel therapy for RILI with high efficacy and low toxicity is required. We have been interested in developing a Chinese traditional herbal medicine as a novel RILI therapy. The present study was designed to evaluate the effectiveness of a Chinese traditional medicine regimen, called *Shenqi Fuzheng* injection (SFI), in the treatment of RILI. SFI is composed of extracts from the traditional Chinese medicinal herbs *codonopsis pilosula* and *radix astragali*. In patients with cancer, SFI reduces the side effects of chemotherapy (18) and improves immune function (19) and quality of life (18). In a previous study, we demonstrated the effects of SFI in the treatment of radiation pneumonitis and its influence on the levels of TNF-α, and TGF-β at various stages (pre-, mid- and post-radiation) of irradiation (RT) (20). The objective of this study was to determine whether the administration of SFI has a protective effect on RILI, and to investigate the possible mechanisms behind these effects.

Materials and methods

Animals. Female C57BL/6 (C57BL/6NCrl) 8-week-old mice were obtained from Tongji Medical College (Wuhan, P.R. China) and housed 4-6 per cage in laminar flow hoods in a pathogen-free environment (22±2˚C, 55±10% humidity and 12-12 h/light-dark cycle) with free access to a standard laboratory diet and water. Mice were allowed to acclimatize for 1 week prior to the experiments. The animal use and care protocol was reviewed and approved by the Medical Sciences Animal Care and Use Committee of Huazhong Science and Technology University.

Shenqi Fuzheng injection preparation. Livzon Pharmaceutics Ltd. (Zhuhai, P.R. China) provided the medicinal herbs used to prepare the *Shengqi Fuzheng* injection (Z19990065). Two raw herbs, *codonopsis pilosula* (Campanulaceae) and *radix astragali* (Leguminosae) were employed at a ratio of 1:1. The two herbs (10 g of each) were autoclaved and subsequently mixed with deionised water for a final volume of 10 ml, then maintained overnight at room temperature. The extract was centrifuged for 10 min at 1,000 x g (2,500 rpm) and the supernatant removed and filtered using a 0.2-μm filter, providing a stock solution extract of 0.08 g/ml. The injection was identified and authenticated by the National Institute of Science Communication and Information Resources (21). The SFI was then used for high performance liqid chromatography (HPLC) analysis.

Irradiation. The mice were randomly divided into four groups: i) phosphate-buffered saline (PBS; pH 7.4, 20 ml/kg/day) alone as normal controls; ii) SFI only (20 ml/kg/day); iii) RT + PBS (20 ml/kg/day); and iv) RT + SFI (20 ml/kg/day). SFI and PBS were administered via intraperitoneal (i.p.) injection 1 week before irradiation. The mice were anesthetized with i.p. ketamine (67.5 mg/kg) and xylazine (4.5 mg/kg) and then irradiated. A single dose of 12 Gy was delivered to the lungs in a single fraction via a posterior field with a linear accelerator. The dose distribution of the thoracic irradiation was evaluated by the ADAC Pinnacle three-dimensional treatment planning system. A plastic jig was used to restrain the mice and lead strips were placed to shield the head and abdomen (14). The irradiation protocol was: beam energy, 15 MV photons; dose rate, 2.0 Gy/min; source-surface distance (SSD), 100 cm; size of radiation field, 18x10 cm. The sham-irradiation areas (other than lung area) were shielded using lead blocks. Post-irradiation, 20 ml/kg/day of SFI or PBS were administered for 14 days. Sham-irradiated control animals were maintained under identical conditions for the course of the experiment.

Tissue isolation. After anaesthesia by i.p. injection, the lung tissues of 3 mice were harvested at 1, 24 and 72 h, and at 2, 4, 8, 16 and 24 weeks post-irradiation. The right lung lobes were snap frozen in liquid nitrogen for RNA isolation and subsequent reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The left lobes were stored in 4% buffered formaldehyde for immunohistochemical (IHC) analysis.

Histology and immunohistochemistry. For histological analysis, the lung tissues were fixed in 4.5% neutral buffered formalin, paraffin-embedded and sectioned at an average thickness of 4 μm. Midsagittal sections of the lung lobes encompassing the largest surface area were collected for analysis. The mounted sections were stained by H&E or immunohistochemistry. For H&E staining, the sections of left lung tissues were fixed in 10% neutral formaldehyde for 24 h, dewaxed and rehydrated with xylene and alcohol. H&E staining was performed and semi-quantitative analysis methods were used to analyze the degree of alveolitis according to the method of Szapiel *et al* (22).

For TGF-β1 and TNF-α IHC staining, the formalin-fixed and paraffin-embedded tissue sections were dewaxed in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by heating the sections for 60 min with citrate buffer at 96˚C, then endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After blocking the sections with normal rabbit serum, rabbit-anti-mouse primary anti-TGF-β1 (R&D system, Minneapolis, MN, USA) and TNF-α antibody (Abcam Inc, Cambridge, MA, USA) diluted 1:100 in PBS were applied to each section. The sections were incubated at 4˚C in the humidified chamber overnight with biotinylated anti-rabbit antibody (Invitrogen, USA) diluted 1:200 in PBS for 1 h, and then with avidin-biotin-peroxidase complex (ABC complex; Dako, Glostrup, Denmark) for 30 min at room temperature. After being washed with PBS, the sections were incubated with diaminobenzidine (DAB; Sigma) until a brown colour was visible, then placed in PBS to stop the reaction. Finally, the sections were counterstained with hematoxylin for 45 sec, dehydrated with ascending alcohol concentrations and mounted in Entellan (Merck, Darmstadt, Germany).

The results of immunohistochemistry were evaluated using a semi-quantitative scoring method (23). The slides were prepared for transmitted light microscopy (Olympus Provis microscope with a DP10 digital camera). TNF-α or TGF-β1 staining was scored and subsequently calculated in 50 representative high power fields for each tissue sample. Cells with yellow/brown granules were regarded as positive. The

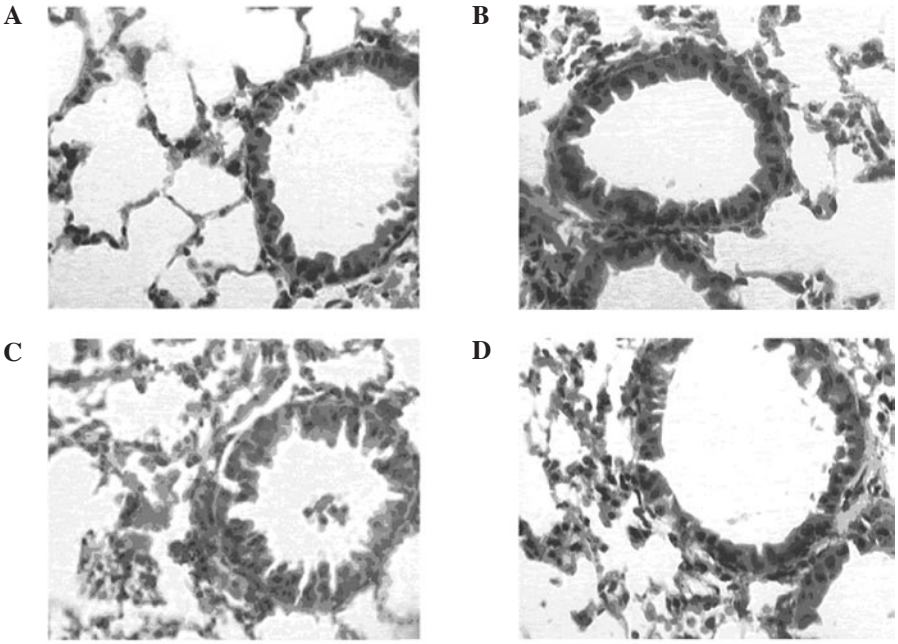

Figure 1. Qualitative histopathological analysis of radiation-induced lung injury in C57BL/6 mice. H&E staining of the PBS and SFI groups showed no clear alveolitis (A and B). H&E staining of the RT + PBS and RT + SFI groups showed overt alveolitis characterized by edema of the alveolar wall, hyperemia, widened alveolar septa and inflammatory cell infiltration at 4 weeks (C and D). Original magnification x400.

score according to the degree of positive staining in the lung tissue was defined as follows: 0, no immunostaining; 1, weak (light yellow); 2, moderate (yellow-brown); 3, strong (brown). The score according to the proportion of positive cells in the tissue was defined as follows: 0, negative; 1, <10%; 2, 11-50%; 3, 51-75%; 4, >75%. After calculating the product of the two immunohistochemical scores, a score of >3 was defined as a positive immune response.

RT-PCR analysis. Frozen samples of pulmonary tissue (75 mg) were homogenized in 1 ml of TRIzol reagent (Gibco BRL, Life Technologies, USA). Total RNA preparation was performed according to the manufacturer's instructions. Tissue lysates were stored at -85˚C before processing or were used directly in the procedure according to the manufacturer's protocol. Ribonucleic acid concentrations were determined by spectrophotometric absorption at 260 nm. One-step RT-PCR was performed according to the manufacturer's instructions (Promega). Total RNA (4 μl) was used as a template to synthesize cDNA in a 25-ml reaction mixture containing 2.5 mM oligo d(T)16 primers and M-MLV Reverse Transcriptase (Promega) at 37˚C for 1 h, followed by 95˚C for 4 min. The PCR was performed with a 20-ml reaction mixture (5 μl 10X buffer, 2 μl template cDNA, 4 μl of each dNTPs, 5 μl $MgCl_2$, 1 μl of each primer and 0.5 μl Taq polymerase), to which ddH_2O was added for a final volume of 50 μl. The conditions were as follows: 94˚C for 4 min, followed by 30 cycles at 94˚C for 30 sec, 57˚C for 40 sec, 72˚C for 40 sec and, finally, 72˚C for 5 min. The primer sequences for PCR amplification were as follows: TGF-β1 sense (5'-GGA GCA ACA TGT GGA ACT CTA CC-3') and anti-sense (5'-GAC GTC AAA AGA CAG CCA CTC AG-3'); TNF-α sense (5'-AGG CGG TGC CTA TGT CTC A-3') and anti-sense (5'-GAG GCC ATT TGG GAA CTT CT-3'). As an internal control, a GAPDH cDNA fragment was simultaneously amplified using sense (5'-TCA CCA CCA TGG AGA AGG C-3') and anti-sense (5'-GCT AAG CAG TTG GTG GTG CA-3') primers. The above primers were synthesized by Dalian Baosheng Bioengineering Co (Dalian, P.R. China). PCR product sizes were 109 bp (TGF-β1), 123 bp (TNF-α) and 168 bp (GAPDH), respectively. The PCR products were visualized by electrophoresis of 7 μl of reaction mixture on a 1% agarose gel containing 0.5 mg/ml ethidium bromide (EB; Sigma) at 70 V for 40 min, and quantitated by densitometry using a dual-intensity transilluminator equipped with Gel-Pro Analyzer version 3.1. β-actin was used as an internal control. The R-value of mRNA expression was evaluated by the band-intensity ratio of TNF-α or TGF-β1 to GDPDH.

Statistical analysis. Data analysis was performed using SPSS13.0 statistical software with one-way analysis of variance (ANOVA) followed by Bonferroni correction. A P-value of ≤0.05 was considered to be significant.

Results

Histological changes. Qualitative and quantitative analyses of the histopathologic alterations of RILI in C57BL/6 mice were performed, and the results were in agreement with those described previously (24). H&E staining showed no obvious alveolitis in the PBS and SFI groups, but overt alveolitis was found in the RT + PBS and RT + SFI groups, characterized by edema of the alveolar wall, hyperemia, widened alveolar septa and by inflammatory cell infiltration at various time points, from 1 h to 4 weeks (Fig. 1). The mean grade of alveolitis was 0-1 in the RT + SFI group, which is significantly lower (better) than that in the RT + PBS group (grade 2-3; $P<0.05$), indicating the effectiveness of SFI in preventing RILI.

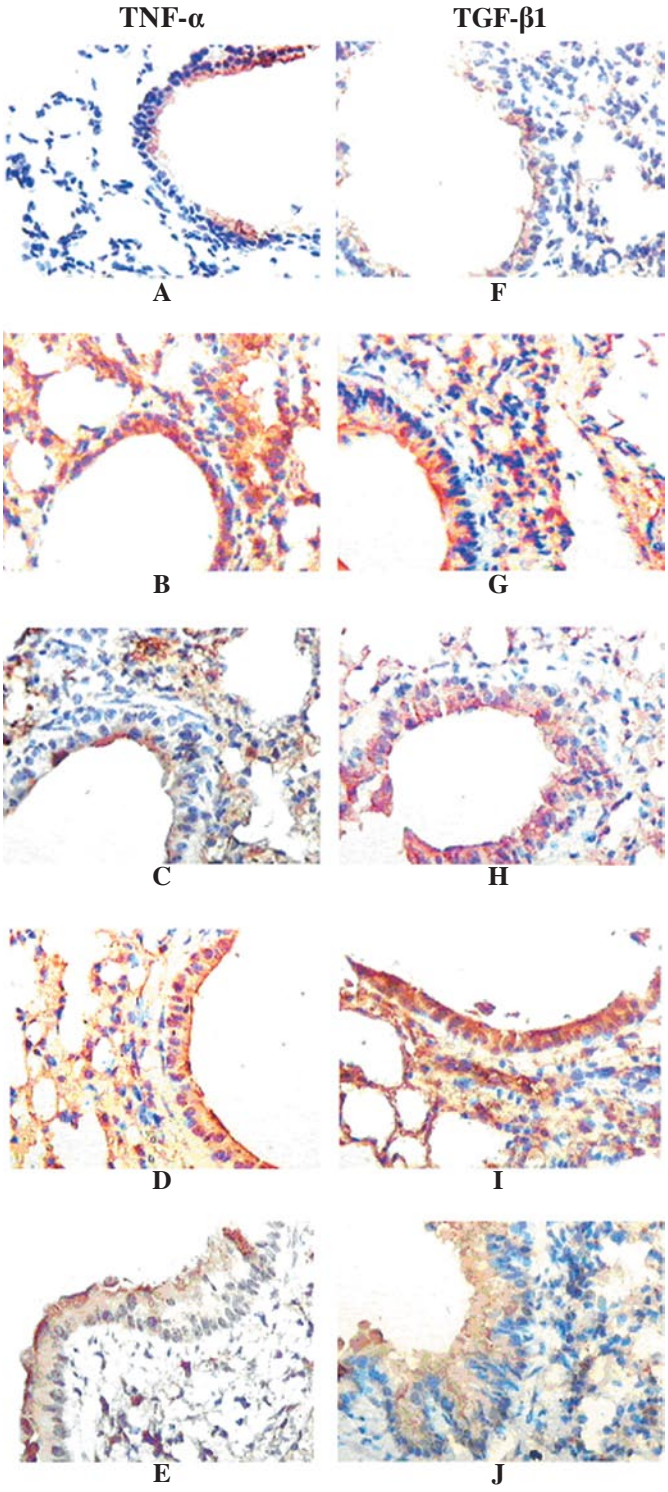

Figure 2. Immunohistochemical analysis of TNF-α and TGF-β1 expression. In the PBS and SFI only groups, TNF-α (A) and TGF-β1 (D) positive staining was restricted to the single cells of the bronchiolar epithelium. Immunohistochemical staining of TNF-α (B) and TGF-β1 (G) in lung tissue obtained 4 weeks after 12 Gy thoracic irradiation in the RT + PBS group, which showed strong immunostaining of the bronchional epithelium and of interstitial inflammatory cells. Immunohistochemical staining of TNF-α (C) and TGF-β1 (H) in lung tissue obtained 8 weeks after 12 Gy thoracic irradiation in the RT + PBS group, which showed strong immunostaining of the bronchional epithelium and of interstitial inflammatory cells. Samples collected at 4 and 8 weeks after 12 Gy RT with SFI showed decreased expression of TNF-α (D) and TGF-β1 (I) in the bronchional epithelium and fewer interstitial inflammatory cells. Samples collected at 4 and 8 weeks after 12 Gy RT with SFI showed decreased expression of TNF-α (F) and TGF-β1 (J) in the bronchional epithelium and fewer interstitial inflammatory cells. Original magnification, x400.

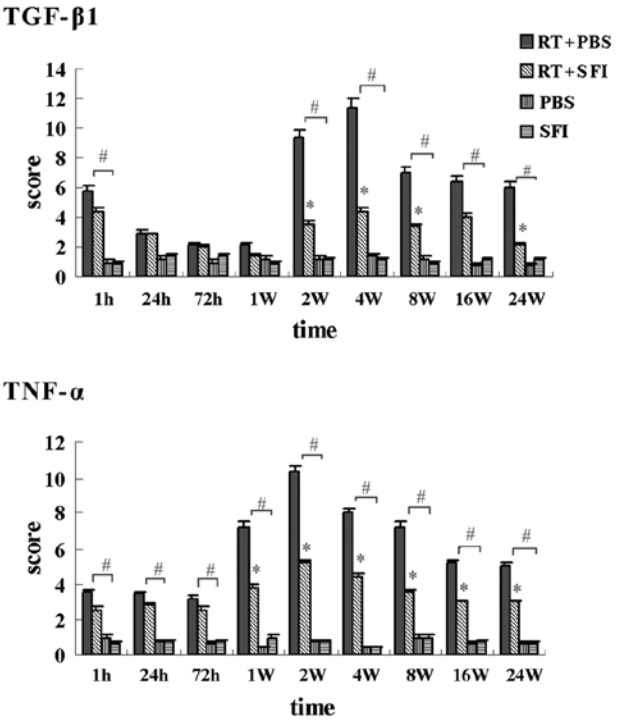

Figure 3. Immunohistochemistry score of TNF-α and TGF-β1 in the groups. Data are the mean ± SD of triplicate determinations using three mice. *Statistically significant differences between the RT + PBS and RT + SFI groups. #Statistically significant differences between the RT and sham-RT groups.

Immunohistochemical analysis of TNF-α and TGF-β1. There were no significant changes in the expression of TNF-α and TGF-β1 at any time point in the PBS and SFI groups (P>0.05). As shown in Fig. 2, significant increases in TGF-β1 and TNF-α expression were observed at every time point post-irradiation for all the RT groups compared to the PBS and SFI groups by IHC analysis. IHC staining of TNF-α and TGF-β1 in the lung tissue of the PBS and SFI groups showed that positive staining was restricted to single cells of the bronchiolar epithelium, while TNF-α and TGF-β1 staining obtained 4 weeks after irradiation showed strong immunostaining of the bronchial epithelium and of interstitial inflammatory cells. Treatment with SFI attenuated the expression of TNF-α and TGF-β1 in lung tissue post-irradiation. The immunohistochemical scores of TGF-β1 and TNF-α varied with time (Fig. 3). The TNF-α immunohistochemical score peaked at 2 weeks post-irradiation, while the TGF-β1 score peaked at 4 weeks post-irradiation. Although the changes in TGF-β1 and TNF-α expression followed the same trend in both the RT + SFI and RT + PBS groups, the former had significantly lower levels of expression than the latter for all time points 1 week (TNF-α) or 2 weeks (TGF-β1) after irradiation (Fig. 3, P<0.05). This indicates that SFI had a significant impact on the production of pro-inflammatory cytokines after radiation therapy.

TNF-α and TGF-β1 expression based on RT-PCR analysis. As observed with IHC analysis, there were no significant changes in the mRNA levels of TNF-α or TGF-β1 in the PBS and SFI groups at any time point (Fig. 4, P>0.05). The mRNA expression of TNF-α and TGF-β1 was increased at 1 h post-

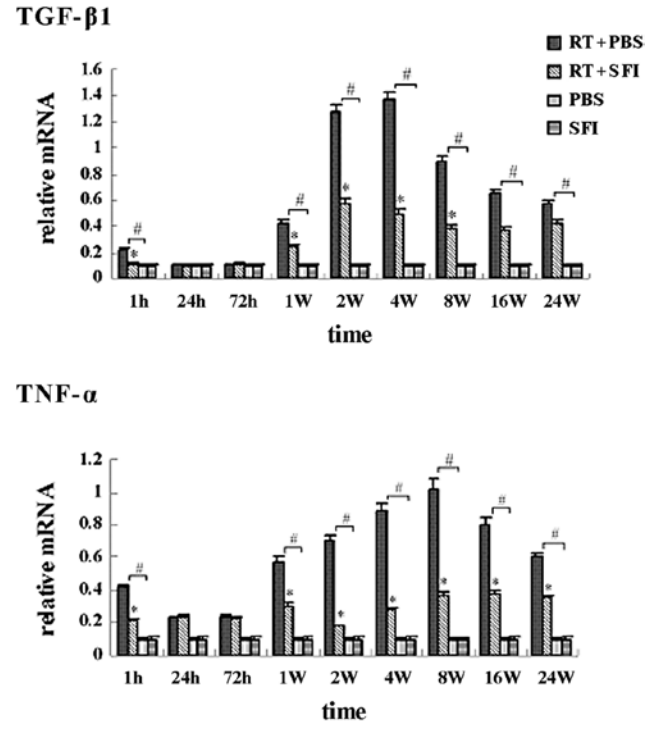

Figure 4. TNF-α and TGF-β1 mRNA expression in the groups. Data are the mean ± SD of triplicate determinations using three mice. *Statistically significant differences between the RT + PBS and RT + SFI groups. #Statistically significant differences between the RT and sham-RT groups.

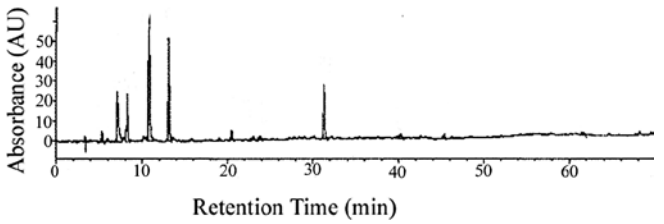

Figure 5. High performance liqid chromatography fingerprinting of *Shengqi Fuzheng* injection.

irradiation, but was decreased during the latent period of RILI, achieving the same level as the control groups (Fig. 4, P>0.05). The expression of both genes increased significantly during the period of radiation pneumonitis, reaching a peak during the pulmonary fibrosis phase. As illustrated in Fig. 4, significant increases in TGF-β1 and TNF-α mRNA expression were found in all the RT groups compared to the control groups at all time points post-irradiation, except for 24 and 72 h (P<0.05). The mRNA levels of both genes in the RT + SFI group were significantly lower than those in the RT + PBS mice (Fig. 4, all P<0.05) except for two time points, 24 and 72 h (P>0.05). TNF-α mRNA expression peaked at 8 weeks after irradiation, while TGF-β1 mRNA expression reached its peak at 4 weeks after irradiation.

HPLC fingerprinting of Shengqi Fuzheng injection. Using HPLC fingerprint analysis, *codonopsis pilosula* and *radix astragali* were determined to be the only two ingredients in the injection (Fig. 5).

Discussion

Lung cancer is a major health problem worldwide and has the highest morbidity and mortality rate among all types of cancer in China (25). The efficacy of current therapeutic approaches such as surgery is often limited. Since many lung cancer patients are frequently diagnosed at an advanced stage, approximately 30% of patients are not candidates for surgery. Thus, chemo- or radiotherapy is the main treatment option (26). Radiotherapy plays a major role in lung cancer treatment, with 53.6% of small cell lung cancer and 64.3% of non-small cell lung cancer patients in different stages of the disease receiving radiotherapy. Approximately 46% of patients undergo radiotherapy as the first treatment (27). However, side effects, including RILI, limit the success of the treatment. At present, there is a trend emphasizing the quality of life in cancer patients receiving radiotherapy in addition to its cancer-killing effects. RILI arises from the injury of normal lung tissues, limiting the effectiveness, dose and schedule of radiotherapy and reducing the quality of life of patients. Adrenocorticotropic hormone and cortisone are the main drugs for RILI (16), but their effectiveness is poor and their adverse effects are significant. Thus, the prevention of RILI with novel drugs represents a new avenue for dealing with these side effects of radiation therapy.

Several clinical factors are associated with lung toxicity, including tumor location (28-30), patient performance status (31,32), pulmonary function before radiotherapy (32) and smoking status (31,33). In addition, many studies have demonstrated that dosimetric factors are crucial for the estimation of lung tolerance, including mean lung dose, the percentage of the total lung volume exceeding 20 Gy (V20), the percentage of the total lung volume exceeding 30 Gy (V30), effective volume and normal tissue complication probability from the lung dose-volume histogram (34-37). However, the accuracy of these parameters is limited (38).

RILI frequently involves several cell types within lung tissue, including type II pneumocytes, endothelial cells, macrophages and phoroblasts (39). It has been demonstrated that type II pneumocytes and endothelial cells release pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, which could activate macrophages, leading to the production of pro-fibrosis cytokines such as PDGF and TGF-β and inducing the hyperplasia of phoroblasts and the synthesis of cell matrix proteins by a series of autocrine or paracrine secretions. Cytokines such as TGF-β1 and TNF-α initiate and sustain the inflammatory and fibrogenic processes associated with RILI (12,14,15,24,40). Several studies suggest that TGF-β has an important role at the beginning and end of the pathologic changes during chronic fibrosing processes (41,42). TGF-β1 also plays an important role in repairing tissue injury and is one of the most critical cytokines in the formation of fibrosis (43). The rise of TGF-β1 levels in plasma and bronchoalveolar lavage fluid directly reflects the severity of RILI (44-48), and TGF-β1 has been suggested as a sensitive plasma marker of RILI post-irradiation (47). The importance of TNF-α in the development and progression of RILI has also been suggested. TNF-α as a cytokine network regulator plays a key role in initiating local inflammatory responses, and then further attracts and activates macrophages, lymphocytes and stromal

cells, indirectly enhancing the generation of pro-inflammatory cytokines. TNF-α also modulates fibroblast proliferation and differentiation and ECM protein synthesis in the subsequent fibrotic phase. Therefore, regulating TNF-α and TGF-β1 expression may provide a novel approach to the prevention and treatment of RILI.

In the present study, we demonstrated the therapeutic effects of the *Shenqi Fuzheng* injection, a traditional Chinese medicine preparation, on RILI in experimental mice. Previous studies have shown that the effectiveness of the combination of SFI and glucocorticoid is significantly higher than that of glucocorticoid alone, with the time to alleviate clinical symptoms being remarkably shorter (48). In our previous study (20,49), SFI significantly decreased the fibrogenic expression of TNF-α and TGF-β1 in patients after chest irradiation and maintained anti-fibrogenic cytokine IL-10 levels, decreasing the incidence of radiation pneumonitis. These results indicate that SFI can be used as an effective adjuvant drug in the treatment of radiation pneumonitis, based on its effects on the regulation of the cytokine network.

In the present study, we demonstrated that SFI treatment significantly attenuated lung injury assessed by histological, IHC and PCR analyses. Mice in the RT alone group exhibited radiation pneumonitis characterized by acute inflammation and edema, followed by increased collagen and fibrosis during the subsequent fibrotic phase post-irradiation. SFI treatment may attenuate RILI, as determined by H&E staining after radiotherapy of the severity of alveolitis, IHC staining of mouse lung tissue TNF-α and TGF-β1 protein expression, and RT-PCR detection of TNF-α and TGF-β1 mRNA expression. The efficacy of treatment with SFI in RILI was found to be related to the enhanced suppression of radiation-induced TNF-α and TGF-β1 expression. These results provide a basis for future clinical studies of SFI as a novel agent in the prevention and treatment of RILI in patients with lung cancer.

Acknowledgements

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