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Research article

Network pharmacology and experimental verification reveal the mechanism of Qingfei Tongluo mixture in treating pulmonary fibrosis ☆

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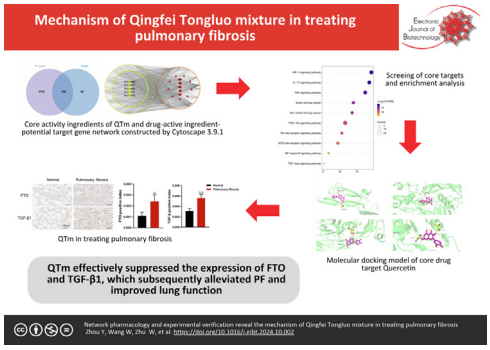
GRAPHICAL ABSTRACT

Network pharmacology and experimental verification reveal the mechanism of Qingfei Tongluo mixture in treating pulmonary fibrosis.



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ABSTRACT

Background: Pulmonary fibrosis (PF) is a chronic interstitial lung disease posing significant health risks. This study aimed to investigate the therapeutic mechanism of Qingfei Tongluo mixture (QTm) in treating PF by combining network pharmacology and experimental verification.
Results: A total of 246 active ingredients in QTm were identified, with 159 potential targets for PF treatment. Quercetin, a key active ingredient, was associated with the TGF-β1 signaling pathway. Gene Ontology and KEGG enrichment analyses identified 42 core genes, with a notable implication of the TGF-beta signaling pathway in PF. Immunohistochemistry showed elevated FTO and TGF-β1 levels in PF tissues. Animal experiments demonstrated that QTm improved alveolar structure, reduced interstitial lesions, and enhanced lung function while decreasing hydroxyproline content and the expression of FTO and TGF-β1 proteins.

☆ Audio abstract available in Supplementary material.

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Conclusions: QTm may inhibit PF progression by suppressing FTO/TGF-β1 expression, thereby improving lung function. These findings suggest that QTm holds potential as a treatment for PF.

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1. Introduction

Pulmonary fibrosis (PF) is a chronic and irreversible interstitial lung disease that primarily affects middle-aged and older individuals [1]. This condition is marked by extensive proliferation of interstitial cells, accumulation of extracellular matrix, and pathological changes in alveolar epithelial cells [2]. Idiopathic pulmonary fibrosis (IPF), the most prevalent form of PF, presents a grim prognosis with a median survival rate of merely 2–4 years following diagnosis and a five-year survival rate of less than 30% [3,4]. Epidemiological data reveal that the global incidence of IPF ranges from 0.09 to 1.30 cases per 10,000 individuals and continues annually [5]. Currently, the therapeutic options for PF are notably inadequate, lacking highly effective therapeutic drugs or approaches. Pirfenidone and Nintedanib, the two drugs recommended by contemporary guidelines, provide only limited benefits. They modestly slow the decline in lung function but do little to stop the progression of the disease or improve quality of life. Additionally, these medications are costly and associated with notable adverse reactions [6]. Consequently, there is a pressing need to investigate and develop safe and efficacious drugs for treating PF.

In traditional Chinese medicine, PF is classified under the categories of "lung Bi" and "lung potency". This condition is believed to stem from damage to lung qi, which not only disrupts normal breathing but also heightens the risk of blood stasis. The repeated invasion of pathogenic factors into the lungs and inefficient transformation of vital energy are thought to impair lung body fluids, consequently increasing susceptibility to the formation of blood stasis pathological products [7,8]. Thus, the pathogenesis of PF in Chinese medicine is often described as a combination of "Qi deficiency, Yin deficiency, and blood stasis" [9]. The Qingfei Tongluo mixture (QTm), used in this study, is a fusion of the well-known Qianjin Weijing Decoction and Liujunzi decoction. This formulation is designed to enhance qi, nourish Yin, purge the lungs and eliminate phlegm, as well as promote blood circulation and clear collaterals. Previous clinical investigations conducted by our research group have demonstrated that QTm significantly reduces the annual decline rate of FVC value in patients with PF over 52 weeks. Additionally, it has been shown to decrease the frequency of acute occurrences. Despite these promising results, the precise mechanism by which QTm exerts its beneficial effects on PF remains to be elucidated.

Network pharmacology is a burgeoning interdisciplinary field that leverages computer simulation, data analysis, and extensive database searches to elucidate the active components of drugs and their mechanisms of action in treating diseases. This approach aligns with the holistic principles of Traditional Chinese Medicine (TCM) by examining the complex interactions between drugs and diseases from a comprehensive perspective [10]. By utilizing both network pharmacology and animal experimentation, the research seeks to identify the key bioactive compounds in QTm that interact with known therapeutic targets of PF. Furthermore, this study endeavors to elucidate how these interactions modulate molecular pathways associated with PF, with a special focus on the FTO/TGF-β1 axis. In doing so, the research aims to provide scientific validation of traditional Chinese medicine (TCM) formulations for modern therapeutic applications, thereby addressing the urgent need for effective and accessible treatments for PF.

2. Materials and methods

2.1. Sample collection

The study included a cohort of 30 patients diagnosed with PF who were admitted to our hospital from January 2022 to December 2022. The inclusion criteria were as follows: 1) Participants aged 18 and above; 2) Pulmonary fibrosis diagnosis confirmed through imaging and histological examination; 3) Presence of a well-documented medical history and clinical manifestations, such as dyspnea, cough, chest pain, etc.; 4) Willingness to voluntarily participate in the study and provide informed consent. Exclusion criteria: 1) Pregnant or lactating women; 2) Individuals with severe cardiovascular, hepatic, or renal organ dysfunction; 3) Those presenting an active pulmonary infection or other systemic infection; 4) Allergic to drugs or other contraindications; 5) Individuals suffering from severe mental illness or cognitive dysfunction.

Surgical procedures were performed to collect both normal lung tissue samples and PF lung tissue samples. This study has been received and approved by the Medical Ethics Committee in Tongde Hospital of Zhejiang Province (Ethics number: Zhejiang Tongde fast audit No. [2021] 020), and informed consent was obtained from either the patients themselves or their guardians, demonstrating voluntary participation.

2.2. Network pharmacological analysis

2.2.1. Network construction of drug - ingredient - potential target

The QTm is composed of mulberry bark, reed root, winter melon seed, coix seed, peach kernel, Salvia miltiorrhiza, princelestine ginseng, Nansha ginseng, Poria, white art, pinellia, sand kernel, platycodon grandiflorum and licorice. In this study, we utilized the TCM System Pharmacological Analysis Platform (TCMSP) [11] to screen all active ingredients from 14 TCMs, selecting candidate active ingredients based on their oral bioavailability (OB) of more than 30% and drug-likeness (DL) over 0.18. The TCMS database was employed to search for targets of these candidate active ingredients. PF-related disease genes were obtained from GeneCards [12] and OMIM [13] databases. By intersecting with the targets of active ingredients identified earlier in our study, the potential target genes of QTm for treating PF were determined. The network diagram of TCM-active ingredients-potential target was constructed using Cytoscape 3.9.1 software, and the top five active ingredients were selected as the core components by analyzing the Degree values of each component node.

2.2.2. Core target screening and enrichment analysis

Pass the names of potential target genes through the STRING database [14] to construct Protein-Protein Interaction (PPI) networks, which were further topologically analyzed using Cytoscape plug-ins. Genes with degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC) values not less than the median are considered core genes [15,16]. GO biofunctional

enrichment analysis and KEGG pathway enrichment analysis were performed using the R-package clusterProfiler (version 4.8.3) [17].

2.2.3. The binding ability of active ingredients to key target genes was analyzed by molecular docking

To prepare for molecular docking, the active component corresponding to TGF-β1 was obtained as a small ligand molecule from the TCMSP database. Subsequently, the PDB file of TGF-β1 was acquired from the PDB database [18]. The docking between TGF-β1 and its corresponding active ingredient was identified by Auto-Dock (version 4.2.6), followed by visualization using Pymol software (version 2.5).

2.3. Immunohistochemistry

The collected lung tissues were fixed with 4% neutral formaldehyde, sampled, dehydrated, and embedded in paraffin. Following sectionalization, the expression levels of FTO and TGF-β1 in the lung tissues were detected through immunohistochemistry. Firstly, the paraffin sections were baked at 60°C for 1 h before being dewaxed and hydrated. Subsequently, the treated sections were placed in a boiling antigen repair solution under high pressure for hot repair. The serum working solution was then added and incubated at a temperature of 37°C for 30 min within a closed environment. Excess serum was absorbed using filter paper before adding the mono-antibody working solution which was left to incubate overnight at 4°C. The slices were then rewarmed at 37°C for 1 h followed by the addition of diluted secondary antibody which was incubated at 37°C for 20 min. DAB-H_2O_2 was utilized for color development throughout 10 min, and the staining was observed by microscope. Finally, hematoxylin was added for re-dyeing, and the slices were transparent and sealed with neutral gum. The parts to be analyzed in each section need to be photographed under a microscope for image analysis. IOD (Total Optical Density) = $\sum$ (Area (positive expression site) × Density (average optical density of the site)). Positive index refers to IOD divided by the total pixels in the photo (IOD area is calculated in pixel units). The immunohistochemical section with DAB color development exhibits brownish yellow as its positive area while the positive index indicates even density within the photo. The antibodies used include TGF-β1 (#AF1027, Affinity, USA), FTO antibody (#DF8421, Affinity, USA), Goat Anti-Rabbit IgG (H+L) HRP (#S0001, Affinity, USA).

2.4. Animal modeling

Forty SPF C57BL/6 male mice (weighing 18 ~ 20 g) were purchased from Hangzhou Medical College, with production license No. SCXK (Zhejiang) 2019–0002, and were raised by Laboratory Animal Center of Zhejiang Academy of Traditional Chinese Medicine with license No. SYXK (Zhejiang) 2019–0010. The mice were maintained in a controlled environment with room temperature set at (20 ± 2)°C, relative humidity maintained at (60 ± 10)%, and subjected to a 12-hour light–dark cycle. They were provided standard feed ad libitum. This study has been approved by the Experimental Animal Welfare Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (Ethics number: Zhejiang Research Animal Ethics Review No. [2021] 013).

The mice were randomly divided into four groups: the normal group, the model control group, the prednisone treatment group (8 mg/kg, prepared with normal saline), and the QTm treatment group (15 g/kg), with ten mice in each group. After one week of adaptive feeding, except for the normal group, mice in the other groups were anesthetized by intraperitoneal injection of 0.5% pentobarbital sodium (50 mg/kg). They were then immobilized and intubated through their mouths with a flat-head microsyringe combined with a laryngoscope. Subsequently, intratracheal injection bleomycin (3 mg/kg) was administered to establish animal models of PF. After ten days of modeling, mice in each treatment group received intragastric administration of drugs at the same dose. The normal and model control groups received intragastric administration of equal volumes of normal saline once daily for 28 consecutive days at an intragastric volume ratio of 1 mL/100 g. The drugs used included prednisone acetate tablets (batch number: H33021207; 5 mg/tablet; 100 tablets/bottle; Zhejiang Xiju Pharmaceutical), QTm (batch number: Z20200045000; 500 mL/bottle; each mL is equivalent to 0.724 g decoction pieces; provided by Zhejiang Tongde Hospital Preparation Room), Bleomycin hydrochloride for injection (batch number: H20090885; 15 mg/bottle; Nippon Chemical Co., LTD.).

2.5. Detection of lung function

After the final administration, mice in each group were anesthetized via intraperitoneal injection of 0.5% pentobarbital sodium (50 mg/kg). The trachea was exposed anteriorly to the neck, and tracheal intubation was performed. Pulmonary function indexes of mice were assessed using a pulmonary function test system, which included forced vital capacity (FVC), respiratory volume (FEV), expiratory volume at 25 ms (FEV25), expiratory volume at 50 ms (FEV50), FEV at peak expiratory volume (FEVPEF), and mean mid-expiratory flow (MMEF).

2.6. Observation of lung histopathology

After conducting the lung function test, the mice were euthanized for cervical vertebrae dissection and their lung tissues were extracted, fixed with 4% neutral formaldehyde, sampled, dehydrated, embedded in paraffin, sectioned and subjected to routine procedures including HE staining. The resulting histopathological changes in the lungs were observed under a microscope.

2.7. Determination of hydroxyproline content

The content of hydroxyproline (HYP) in lung tissue was determined using the alkaline hydrolysate method. According to the instructions of the kit (batch number: A030-2-1, Nanjing Jiancheng Bioengineering Institute, China), the quantitative lung tissue sample was weighed and mixed with 1 mL hydrolysate, then hydrolyzed in a boiling water bath for 20 min. After the water was cooled, the pH was adjusted to approximately 6.0 ~ 6.8, followed by the addition of distilled water up to a final volume of 10 mL and thorough mixing. Decolorization was achieved by adding an appropriate amount of activated carbon, followed by centrifugation at 3500 r/min for 10 min. Finally, 1 mL supernatant was taken from each tube and its absorbance was measured at a wavelength of 550 nm.

2.8. Western blot

The protein extraction solution was prepared following the instructions provided by the protein extraction kit (KGP2100, KGPBIO, China). The lung tissue was dissected and added to the working fluid for protein extraction. The tissue homogenization was performed using magnetic beads, and the tissue protein samples were obtained through centrifugation. The protein concentration in the sample was determined using a BCA protein quantification kit (P0010S, Beyotime, China) and stored at −80°C for future use.

The Loading Buffer was prepared by the kit instructions. Protein samples were mixed with Loading Buffer and 0.1 × Sample Buffer, resulting in a final sample concentration of 0.5 mg/mL. Each hole should be filled with 1 μL of Loading Buffer and can be supple-

mented with sample diluent. After mixing, the samples were denatured at 95°C for 5 min, followed by a cooling period of 5 min and subsequent vortex centrifugation before being placed on ice for further use. Ladder, denatured samples, primary antibody, secondary antibody, luminescent solution, and Wash Buffer were added to the Simple Wes protein test plate (SM-W001, protein simple, USA), and the protein was isolated by capillary electrophoresis. Protein expression levels were analyzed using Compass for SW 5.0 software to determine the relative ratio between the target protein and internal reference protein as an indicator of relative protein expression levels. The antibodies used included FTO (ab126605, 1:10,000, Abcam, UK), TGF-β1 (ab215715, 1:1000, Abcam, UK), GAPDH (ab9485, 1:2500, Abcam, UK), Goat Anti-Rabbit IgG H&L HRP (ab6721, 1:2000, Abcam, UK).

2.9. Statistical analysis

All data were presented as mean ± standard deviation (SD). The statistical analysis was performed using GraphPad 8.0 software. Independent sample T-test was used for comparison between two groups, and one-way ANOVA was used for comparison among multiple groups to ensure homogeneity of variance. Data with irregular or inconsistent variance were statistically analyzed using the rank sum test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of core active ingredients of QTm

The search for relevant targets was conducted using the TCMSP database, resulting in the identification of 264 candidate active ingredients following verification and recirculation. Utilizing the GeneCards and OMIM databases, we identified 3892 genes associated with PF. By intersecting these genes with the targets of TCM components, we identified 159 potential target genes of the QTm for treating PF (Fig. 1A). A network diagram illustrating the interactions between the drug, active ingredients, and potential target genes was constructed using Cytoscape 3.9.1, as depicted in Fig. 1B. Core ingredients were selected for further analysis and research based on the top 5 Degree values of each component node (refer to Table 1). It was noteworthy that quercetin, the primary bioactive compound, included TGF-β1.

3.2. Screening core targets and enrichment analysis

We imported the 159 intersecting targets into STRING to construct a PPI network (Fig. 2A). Through rigorous screening where DC, BC, and CC were all higher than the median, we identified 42 core nodes (Fig. 2B). Further examination involved GO enrichment and KEGG pathway analysis for these core genes. The results of the GO enrichment analysis revealed significant associations with biological processes related to cellular response to biotic stimulus, response to oxygen levels, and cellular response to chemical stress. Cell component annotations included caveola, membrane microdomain, and membrane raft, among others. Molecular function annotations primarily involved DNA binding, transcription factor binding, phosphatase binding, and cytokine activity (Fig. 2C). KEGG pathway analysis identified several enriched pathways such as the HIF-1 signaling pathway, IL-17 signaling pathway, TNF signaling pathway etc (Fig. 2D). Importantly, the pivotal cytokine in the TGF-beta signaling pathway demonstrated a strong correlation with PF.

3.3. Molecular docking analysis of the binding ability of active ingredients to key target genes

It is a well-established principle in molecular docking that a binding energy below 0 kcal/mol indicates a spontaneous interaction between two molecules, with lower values reflecting stronger affinity. Quercetin, identified as the most active compound, was chosen for molecular docking studies with TGF-β1, as depicted in Fig. 3 and Table 2. The binding energies between Quercetin and various structural forms of TGF-β1 were all below 0 kcal/mol, confirming a favorable interaction between them. The results of molecular docking are detailed in Fig. 3 and Table 2. Based on these findings, TGF-β1 was selected as a crucial research target to further explore the mechanisms by which the QTm aids in the improvement of PF.

3.4. Expression of FTO and TGF-β1 in PF patients

The m^{6}A demethylase FTO has been demonstrated to play a regulatory role in the expression of TGF-β1, influencing the progression of renal interstitial fibrosis [19]. To investigate whether FTO/TGF-β1 was abnormally expressed in PF, we performed immuno-

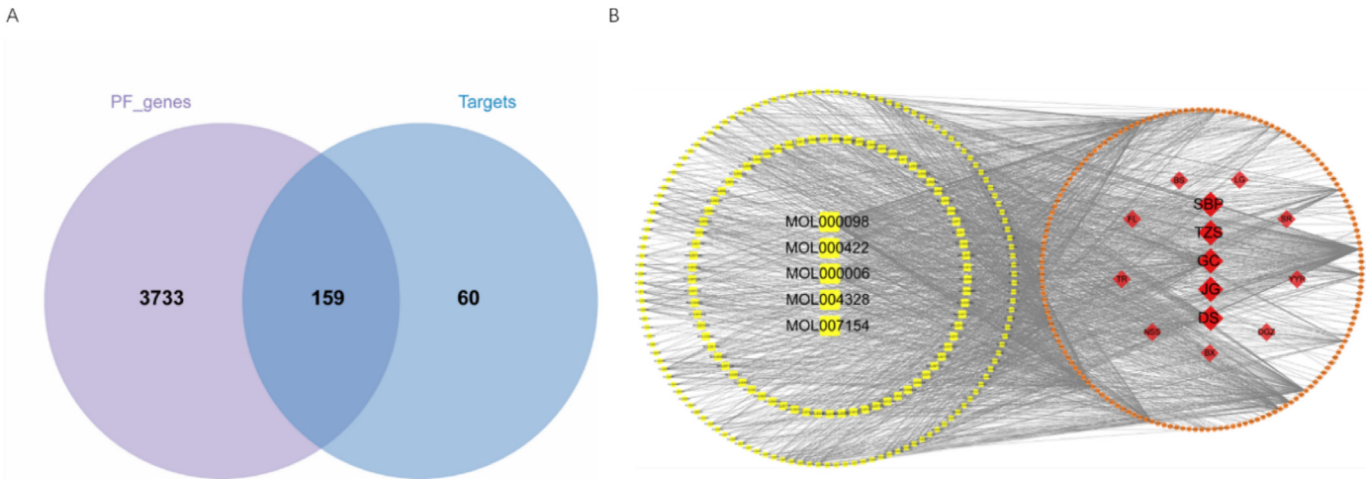

Fig. 1. Determination of core active ingredients of QTm. (A) Gene Venn Diagram of intersection between the selected component targets and the PF-related genes; (B) Drug-active ingredient-potential target gene network constructed by Cytoscape 3.9.1.

Table 1
QTm treatment of PF network of the central five active ingredients.

MOLID	Active ingredients	Degree	TCM
MOL000098	quercetin	106	GC; SBP
MOL000006	luteolin	46	DS; TZS; JG
MOL000422	kaempferol	39	GC; SBP
MOL007154	tanshinone IIA	27	DS
MOL004328	naringenin	24	GC

histochemistry to assess the expression levels of FTO and TGF-β1 in tissue samples from patients with PF. The results, as illustrated in Fig. 4A–B, revealed that the expression levels of both FTO and TGF-β1 were significantly higher in PF tissues compared to normal lung tissues. This increase in the positive index for FTO and TGF-β1 suggests a potential involvement of FTO/TGF-β1 in the pathophysiology of PF.

3.5. Lung histopathological observation and determination of hydroxyproline content

To further elucidate the mechanism of QTm, we established a mouse model of PF for experimental verification. As depicted in Fig. 5A–D, HE staining revealed the pathological changes in lung tissue following different treatments. It was evident that the lung tissue structure in the normal group appeared clear and devoid of any observed pathological changes such as inflammatory cell infiltration (Fig. 5A). In contrast, the model control group exhibited disorganized pulmonary alveolar structures and obvious pulmonary interstitial hyperplasia, accompanied by a significant influx of inflammatory cells (Fig. 5B), confirming the successful establishment of the PF model. Treatment with either prednisone (8 mg/kg) or QTm (15 g/kg) markedly improved the condition of lung tissues in the mice. Notably, these treatments preserved the alveolar structure, resulted in only slight pulmonary interstitial hyperplasia, and reduced inflammatory cell infiltration (Fig. 5C–D). These observations suggest that QTm effectively ameliorates PF in mice, supporting its potential therapeutic benefits.

HYP is an amino acid uniquely present in collagen fibers, and measuring its content in tissues has become the most commonly used method for evaluating the severity of fibrosis. In this study, we quantified the HYP content in lung tissues of mice across different groups. Results as depicted in Fig. 5E, compared to the normal group, the content of HYP in the lung tissue of mice in the model control group was significantly increased ($P < 0.01$), which further indicated the successful establishment of the PF model. However, when comparing to the model control group, the content of HYP in lung tissue of mice treated with prednisone (8 mg/kg) and QTm (15 g/kg) exhibited a significant reduction ($P < 0.01$), although it remained higher than that in the normal group. These findings suggested that QTm could effectively reduce the severity of PF in mice.

3.6. Comparison of lung function indexes of mice in different treatment groups

Functional tests were performed in each group before final administration to assess the therapeutic effect of QTm on lung

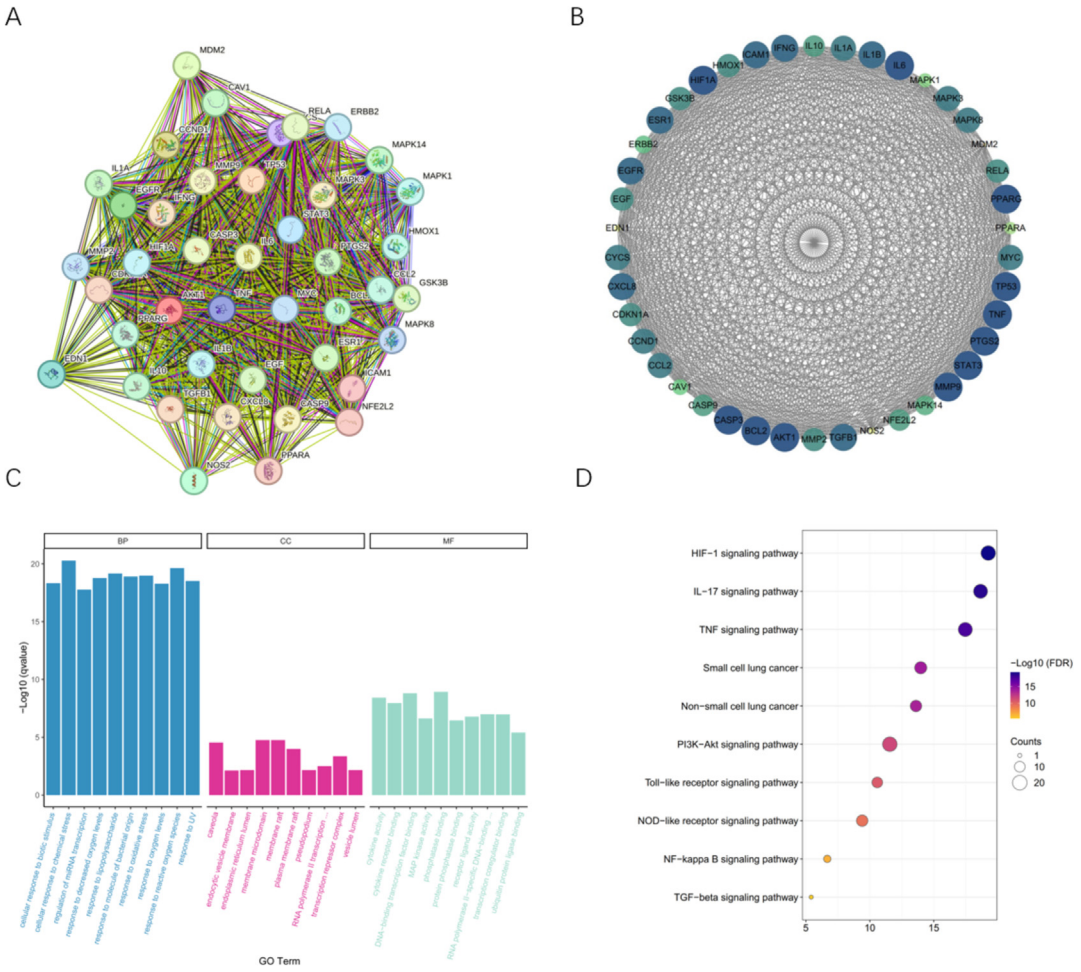

Fig. 2. Screening of core targets and enrichment analysis. (A) PPI network of the target protein of core drug therapy for PF; (B) The visibility graph of the core node; (C) GO Biofunctional enrichment analysis of core target; (D) KEGG pathway enrichment analysis.

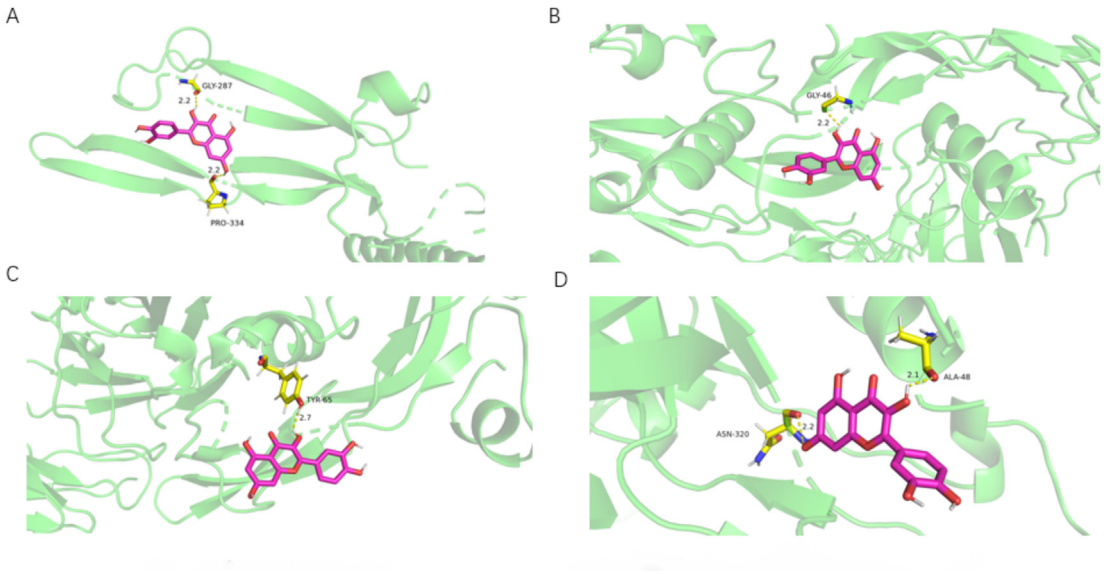

Fig. 3. Molecular docking model of core drug target Quercetin and different structural TGF-β1. (A-D corresponds to 5VQP, 3KFD, 4KV5 and 8C7H structures in turn.

Table 2
The docking results of the core drug target and the corresponding active ingredient.

Compound	Structures	Resolution (Å)	Binding energy (kcal/mol)
Quercetin	5VQP	2.90	−4.35
	8C7H	2.70	−2.73
	3KFD	3.00	−1.88
	4KV5	3.00	−1.32

function. As depicted in Fig. 6, the induction of PF by bleomycin resulted in a significant reduction in FVC, FEV, PEF, and MMEF in mice compared to the normal group ($P < 0.01$). Conversely, the levels of FEV25/FVC% and FEV50/FVC% were significantly increased ($P < 0.01$). As expected, following treatment with QTm (15 g/kg), there was a notable restoration of these parameters, particularly FEV25/FVC% and FEV50/FVC%, suggesting a significant improvement in lung function. These results indicated the potential of QTm in effectively enhancing pulmonary function in the context of PF.

3.7. Expression of FTO and TGF-β1 in mouse lung tissue

To assess the impact of different treatments on the protein expression levels of FTO and TGF-β1 in lung tissues, we employed Simple Western (wes) and immunohistochemistry techniques across various treatment groups. The results obtained from WB analysis revealed a significant increase in the relative expression levels of FTO and TGF-β1 in the lung tissue of the model control group compared to the normal group ($P < 0.05$). However, both the prednisone (8 mg/kg) treatment group and QTm (15 g/kg) treatment group exhibited a remarkable ability to reduce the relative expression of these proteins when compared to the model control group ($P < 0.05$) (Fig. 7A–B). Further confirming these findings, the results from the immunohistochemistry, displayed in Fig. 8, showed a marked increase in the positive index for FTO and TGF-β1 proteins in the lung tissues of the model control group relative to the normal group ($P < 0.01$). Remarkably, treatment with either prednisone (8 mg/kg) or QTm (15 g/kg) significantly decreased the positive index for these proteins when compared to the model control group ($P < 0.01$).

In summary, these results suggested that QTm effectively mitigated PF and improved lung function by downregulating the expression of FTO/TGF-β1 in lung tissues.

4. Discussion

PF is a prevalent form of interstitial lung disease that poses a serious threat to human health. Recent research has underscored the potential of TCM in both preventing and treating PF. Polyphenol compounds, such as curcumin, gallic acid, and resveratrol, have

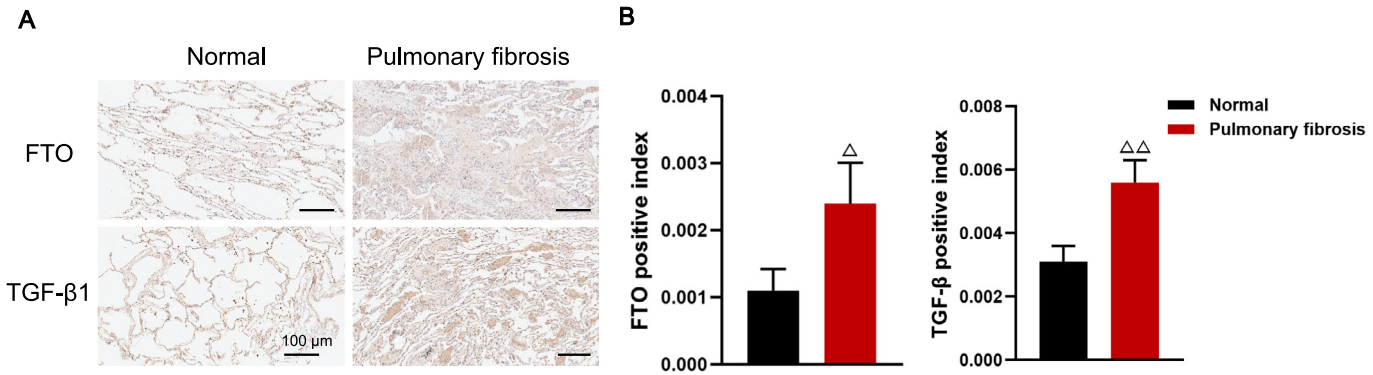

Fig. 4. Expressions of FTO and TGF-β1 in normal and fibrotic lung tissues were detected by immunohistochemistry. (A) Immunohistochemical results (100 ×); (B) Quantitative statistics of expression levels of FTO and TGF-β1 in different tissues. Compared with the normal group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

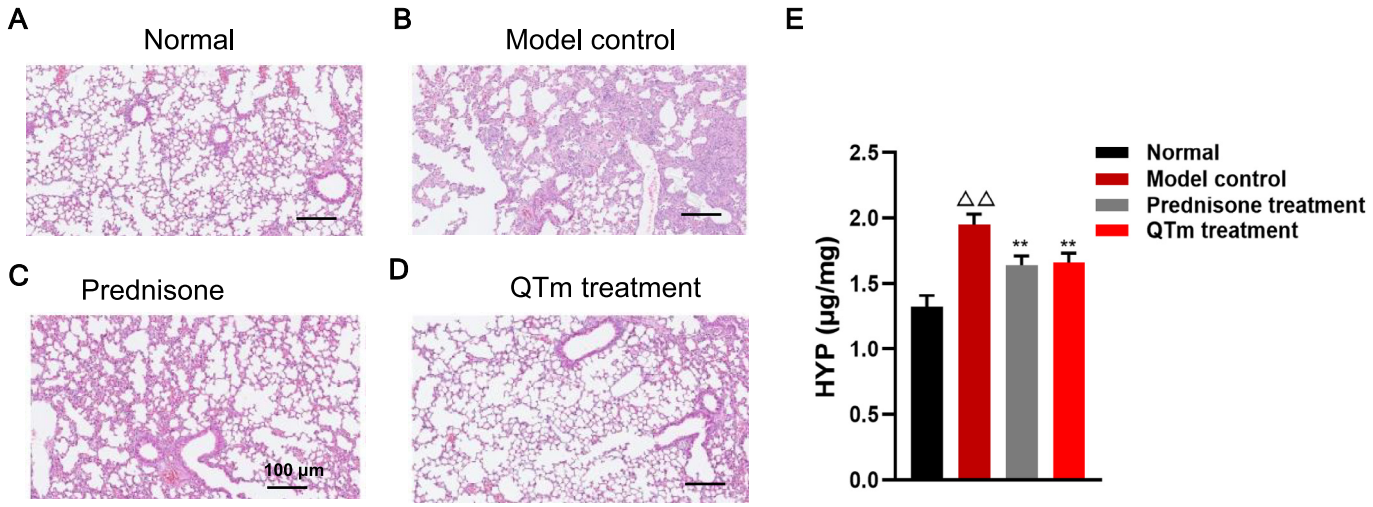

Fig. 5. HE staining and hydroxyproline content detection in mouse lung tissue. (A-D) Lung histological results of mice in normal group (A), model control group (B), prednisone treatment (C) and QTm treatment group (D) (100 ×); (E). Comparison of hydroxyproline content in lung tissue of mice in each group. Compared with normal group, $^{\triangle\triangle}P < 0.01$; Compared with model control group, $^{**}P < 0.01$.

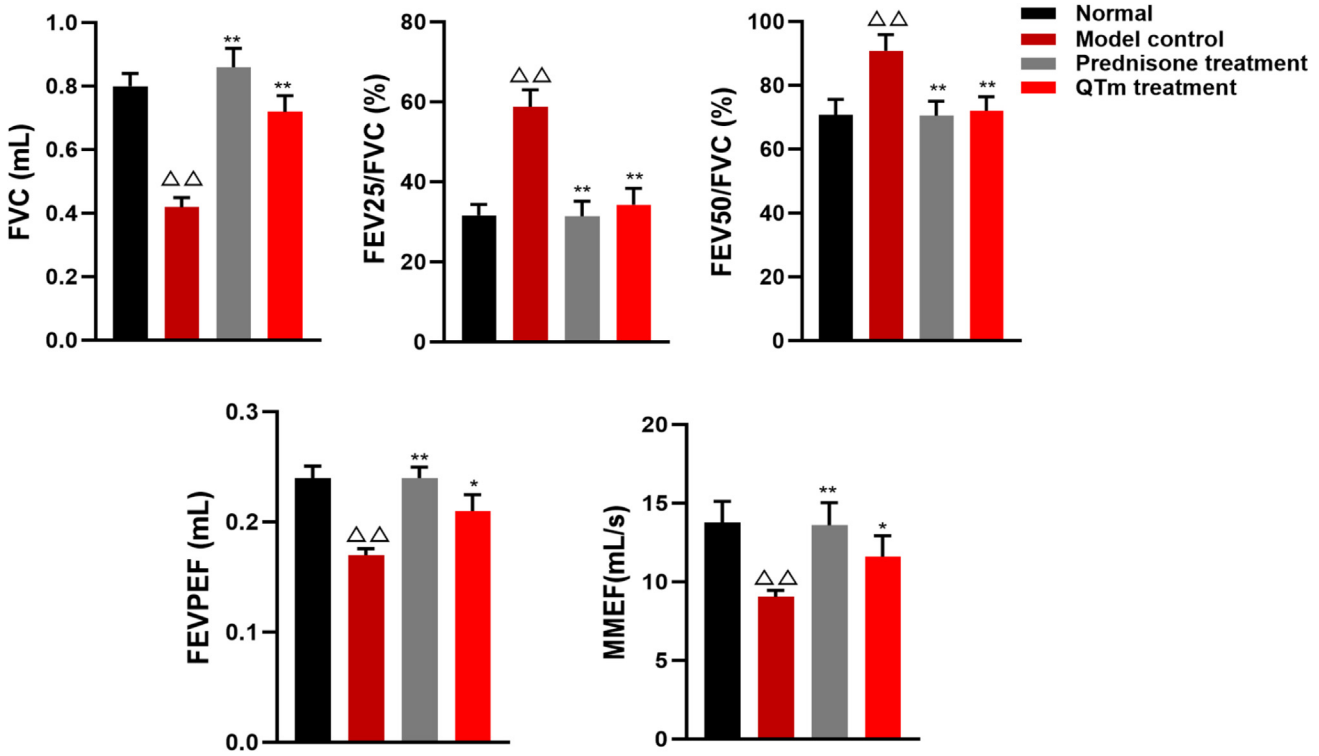

Fig. 6. Comparison of lung function indexes of mice in different groups. (A-E) Comparison of lung function indicators FVC (A), FEV25/FVC% (B), FEV50/ FVC% (C), FEVPEF (D) and MMEF (E) in mice. Compared with normal group, $^{\triangle\triangle}P < 0.01$; Compared with model control group, $^{**}P < 0.01$, $^{*}P < 0.05$.

demonstrated their anti-PF effects through a variety of mechanisms [20]. Monomeric compounds, including flavonoids, alkaloids and terpenoids, have been found to inhibit the activation and differentiation of fibroblasts, playing a crucial role in PF treatment [21]. In this study, we discovered that QTm effectively reduced the expression of FTO/TGF-β1 in lung tissues, mitigated the severity of PF, and ultimately achieved the effect of improving lung function.

We utilized network pharmacology to analyze the main active components of the QTm, identifying 159 potential target genes for the treatment of PF through a cross-analysis with known targets. Additionally, we also evaluated the connectivity degree of each active ingredient's node within the network and observed that quercetin, as the top-ranked compound, contained TGF-β1. To refine our analysis, we further screened the 159 potential target genes, selecting those where DC, BC, and CC all exceeded the median values. This process identified 42 core genes. Subsequent KEGG enrichment analysis revealed that these core genes were primarily involved in pathways such as the IL-17 signaling pathway and TGF-beta signaling pathway, indicating the potential mechanisms through which QTm may exert its effects. These findings suggested that QTm may be effective in the treatment of PF by modulating

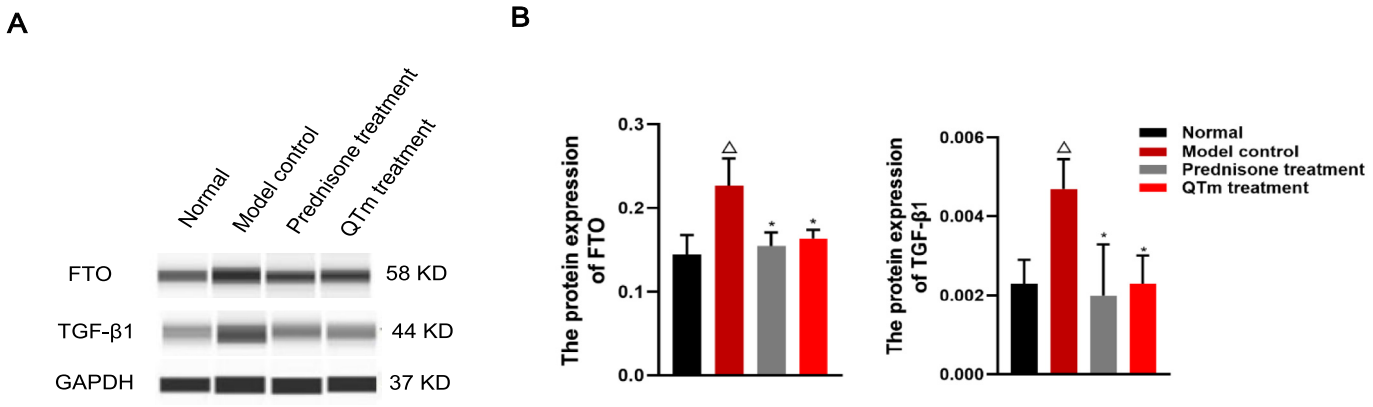

Fig. 7. Western blot analysis of FTO and TGF-β1 protein expression in lung tissues of mice in each group. (A) WB was used to detect the expression of FTO and TGF-β1 protein in lung tissues of mice in different treatment groups; (B) Quantitative results of expression of FTO and TGF-β1 protein. Compared with normal group, $^{\triangle}P < 0.05$; Compared with model control group, $^{*}P < 0.05$.

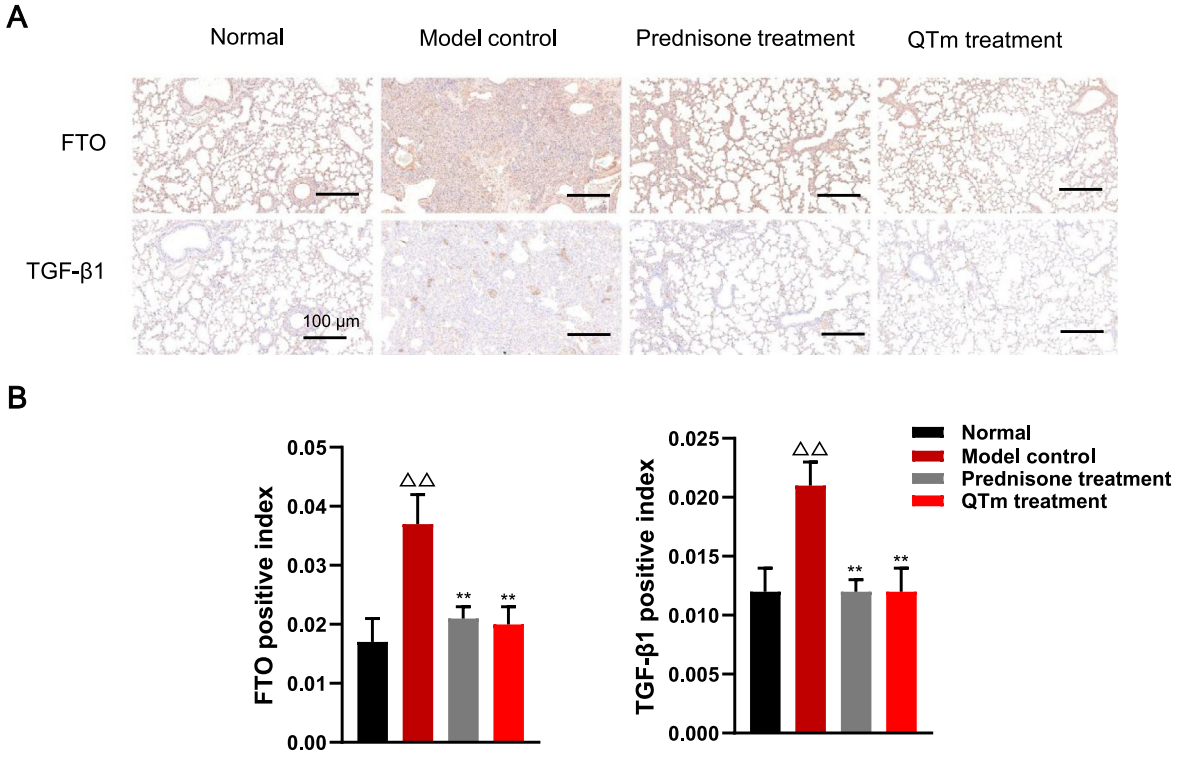

Fig. 8. The expression of FTO and TGF-β1 in lung tissues of mice in different treatment groups were detected by immunohistochemistry. (A) Immunohistochemical results (×100); (B) Quantitative statistics of expression levels of FTO and TGF-β1 in different tissues. Compared with normal group, $^{\triangle\triangle}P < 0.01$; Compared with model control group, $^{**}P < 0.01$.

the inflammatory response and regulating immune processes. Additionally, based on the molecular docking results of quercetin with TGF-β1, we selected TGF-β1 as our primary research target for further validation through animal experiments. This approach aimed to deepen our understanding of QTm's therapeutic potential in PF treatment.

FTO, the first identified RNA demethylase, is involved in the regulation of multiple signaling pathways. In HER2-positive breast cancer, FTO is highly expressed and has been shown to promote tumor cell invasion and migration through the FTO/miR-181b-3p/ARL5B signaling pathway [22]. Animal studies involving myocardial infarction have demonstrated that overexpression of FTO can reduce fibrosis and enhance angiogenesis, thereby playing a crucial role in heart contraction during heart failure [23]. Furthermore, it has been reported that TGF-β1 treatment increases FTO expression in kidney tissue of UUO mice, leading to reduced m^6A modification of lncRNA GAS5, which in turn promotes the EMT process and inflammatory response [19]. In this study, we examined the expression levels of FTO and TGF-β1 in tissue samples from patients with PF and observed a significantly higher positive index for both proteins compared to normal lung tissues. These findings suggested that FTO/TGF-β1 may play a pivotal role in the pathogenesis and progression of PF, highlighting potential targets for therapeutic intervention.

To further elucidate the therapeutic mechanism of QTm, we established a mouse model of PF using bleomycin injections for *in vivo* validation. Results from HE staining revealed significant improvements in lung tissue integrity following QTm treatment. Notably, the alveolar structures remained relatively intact with only slight pulmonary interstitial hyperplasia and minimal

inflammatory cell infiltration. Moreover, the HYP content assay indicated that QTm effectively reduced the degree of PF in mice by significantly decreasing the hydroxyproline content in lung tissues. This suggested a mitigation of collagen deposition, a hallmark of defibrotic processes. Additionally, pulmonary function tests further supported these findings, showing partial restoration of FVC along with improvements in PEF, MMEF, and the ratios of FEV25/FVC% and FEV50/FVC%. These results collectively suggested that QTm had the potential to enhance pulmonary function. Furthermore, the expression levels of FTO and TGF-β1 in mouse lung tissues were analyzed using WB and immunohistochemistry. These studies confirmed that QTm could improve lung function and ameliorate PF by suppressing the expression of the FTO and TGF-β1 proteins and reducing the excessive deposition of extracellular matrix. This comprehensive set of results validated our hypothesis regarding the beneficial effects of QTm in the treatment of PF.

While the bleomycin-induced PF model is widely used due to its reproducibility and the ability to induce fibrotic responses, it does not fully capture the complexities of human pulmonary fibrosis. Specifically, this model tends to mimic acute injury followed by rapid fibrosis resolution, which differs significantly from the chronic, progressive nature of PF seen in humans. In human cases, PF is characterized by gradual accumulation of extracellular matrix and irreversible damage over months to years, a process that is less accurately reflected in the rapid onset seen in bleomycin models. Moreover, bleomycin-induced fibrosis often involves prominent inflammation, which is not as dominant in the pathology of human PF, where chronic and low-grade inflammation is more typical.

These limitations suggest that while the bleomycin model offers useful insights, it falls short in modeling chronic disease progression, extracellular matrix deposition, and fibrotic remodeling over longer durations. Future studies should consider employing models that better emulate these aspects. Potential alternatives include the use of humanized mouse models or the emerging use of organoids derived from human lung tissue, which offer greater relevance to human disease. Human lung organoid models, in particular, may provide a more accurate platform to study the cellular and molecular mechanisms involved in chronic PF, as they better reflect the human lung environment and allow for long-term studies of fibrotic progression. Integrating these models into future studies could help overcome the limitations of the bleomycin-induced model and provide deeper insights into therapeutic interventions that may be effective in chronic human PF.

In this paper, we focused on screening 159 potential targets and identifying the key roles of TGF-β1 and FTO signaling pathways in PF by network pharmacology methods. However, the pathological process of pulmonary fibrosis is extremely complex and involves multiple signaling pathways and biological processes, such as IL-17 signaling pathway and TNF signaling pathway. Future studies can utilize more advanced bioinformatics methods, such as multi-omics integration analysis, to further explore the regulatory role of QTm in a wider range of molecular networks, especially the effects on immune regulation and cellular metabolic pathways. Secondly, the optimization of QTm dosage and composition is an important direction for future research. In this paper, the efficacy of QTm in the PF model was verified by animal experiments, but a comprehensive evaluation of the specific efficacy of different dosage and composition combinations is still lacking. In the future, the formulation and dosage of QTm can be further optimized to maximize its therapeutic effect through quantitative analysis of drug components and dose–response experiments. In addition, the synergistic effects between different herbal components can be explored to determine their optimal combinations. Third, clinical translational studies on QTm are also particularly critical. Although the effectiveness of QTm was verified by animal models in this paper, its practical application in human clinic needs to be further confirmed. In the future, large-scale, multicenter clinical randomized controlled trials can be designed to systematically evaluate the efficacy, safety, and long-term effects of QTm in patients with different types of pulmonary fibrosis. Meanwhile, combined with modern molecular diagnostic techniques, personalized treatment strategies for QTm can be explored in the future to develop precise treatment plans for patients with different pathological types or molecular features of pulmonary fibrosis. In addition, the potential of combination therapy of QTm with western drugs is also an important direction for future research. This article mentions the limited efficacy and significant side effects of western drugs currently in clinical use (e.g., pirfenidone and nidanib). Future studies could further explore the possibility of combining QTm with existing western drugs and evaluate its role in reducing the adverse effects of western drugs and enhancing the therapeutic efficacy. This combined Chinese and Western medicine treatment program is expected to provide new ideas for the comprehensive treatment of PF. Finally, the potential application of QTm in other respiratory diseases is also worth further exploration. QTm demonstrated its therapeutic effect on pulmonary fibrosis in this paper, and future studies could be extended to other chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma. The mechanism of QTm's broad-spectrum anti-inflammatory and antifibrotic effects will be evaluated through studies in different disease models, further expanding its clinical applications.

5. Concluding remarks

In summary, this study utilized network pharmacology to identify quercetin as the active component of the QTm and TGF-β1 as a potential key target for the treatment of PF. These findings were preliminarily validated through animal experiments. The results demonstrated that QTm effectively suppressed the expression of FTO and TGF-β1, which subsequently alleviated PF and improved lung function.

This research not only underscored the critical role of network pharmacology in leveraging TCM for the prevention and treatment of PF but also provided empirical support and a theoretical framework for using QTm to alleviate symptoms and slow the progression of the disease in patients. This study contributed valuable insights into the therapeutic mechanisms of QTm and highlighted its potential as a beneficial treatment option in the management of PF.

CRediT authorship contribution statement

Ying Zhou: Write articles, Formal analysis, Data curation, Conceptualization. **Wenlong Wang:** Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Wanping Zhu:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tingting Cai:** Visualization, Validation, Supervision, Software, Resources, Project administration. **Nannan Wang:** Visualization, Validation, Methodology, Investigation. **Xia Liu:** Methodology, Investigation, Funding acquisition, Formal analysis. **Wenmin Wang:** Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kequn Chai:** Supervision, Software, Resources, Project administration, Data curation.

Ethical approval (humans)

The experiment was conducted with the human subjects' understanding and consent, and had received and approved by the Medical Ethics Committee in Tongde Hospital of Zhejiang Province (Ethics number: Zhejiang Tongde fast audit No. [2021] 020).

Ethical approval (animals)

The animal experiments were approved by the Experimental Animal Welfare Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (Ethics number: Zhejiang Research Animal Ethics Review No. [2021] 013).

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Supplementary material

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Data availability

Data will be made available on request.

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