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# Pirfenidone prevents radiation-induced intestinal fibrosis in rats by inhibiting fibroblast proliferation and differentiation and suppressing the TGF-β1/Smad/CTGF signaling pathway

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ABSTRACT

Radiation-induced intestinal fibrosis (RIF) is a chronic toxicity following radiation, and can be very difficult to treat. Pirfenidone is a promising anti-fibrotic agent that inhibits fibrosis progression in various clinical and experimental studies. This study was aimed to explore whether pirfenidone could protect against RIF, and to evaluate the underlying mechanism. An animal model of RIF was induced by exposure of a single dose of 20 Gy to the pelvis. Rats were orally administered with pirfenidone (200, 400 md/kg/d) for 12 weeks. Primary rat intestinal fibroblasts were cultured to determine the effects of pirfenidone on TGF-β1-induced (5 ng/ml) proliferation and transdifferentiation of fibroblasts. The expression of collagen I, α-SMA, and TGF-β1/Smad/CTGF pathway proteins were analyzed by qRT-PCR and/or western blot analysis. The cell proliferation rate was determined by CCK-8 assay. The results indicated that pirfenidone significantly attenuated fibrotic lesion in irradiated intestines and reduced collagen deposition by inhibiting TGF-β1/Smad/CTGF pathway in rat models. Moreover, in primary rat intestinal fibroblasts, pirfenidone decreased the up-regulation of TGF-β1-induced collagen I and α-SMA by suppressing TGF-β1/Smad/CTGF signaling pathway. Altogether, our findings suggested that pirfenidone attenuated RIF by inhibiting the proliferation and differentiation of intestinal fibroblasts and suppressing the TGF-β1/Smad/CTGF signaling pathway.

## 1. Introduction

Radiation-induced intestinal fibrosis (RIF) is a chronic toxicity of pelvic radiation. It is characterized by transmural accumulation of extracellular matrix within intestinal layers, which induces loss of intestinal compliance, and might leads to strictures formation requiring surgical resection (Larsen et al., 2007). Currently, there is no reliable clinical or laboratory marker available for RIF; progressive fibrosis and consequent intestinal strictures are not often diagnosed until irreversible. Moreover, current drug therapies have limited effect on fibrotic development, making surgery the only practical option for severe symptomatic intestinal fibrosis (Hamama et al., 2012; Henson et al., 2012). Bear in mind that excessive synthesis and deposition of collagens are the main pathological process of fibrotic diseases and the pathogenesis of fibrosis often shares common signaling pathways, it is of great value to learn from other fibrotic diseases in terms of pathogenesis and treatment (Urban et al., 2015).

Pirfenidone (5-methyl-N-phenyl-2- (1H)-pyridone), a promising anti-fibrotic agent, has exhibited remarkable antifibrotic properties in various clinical and animal-based experimental studies (Azuma et al., 2005; Di Sario et al., 2002; Duan et al., 2015; Hewitson et al., 2001; Rodríguez-Castellanos et al., 2015; Shi et al., 2011). In vitro studies have shown that pirfenidone inhibits proliferation and/or activation of a variety of fibroblasts responsible for the pathogenesis of fibrosis (Hewitson et al., 2001; Lin et al., 2009; Shi et al., 2011). Due to its great efficacy and safety, pirfenidone has been approved for the treatment of idiopathic pulmonary fibrosis in Europe and Japan (Cottin, 2013; Takeda et al., 2014). Recently, pirfenidone has been reported to inhibit the proliferation and secretory activities of fibroblasts isolated from patients with Crohn's disease (Kadir et al., 2016). In addition, this agent also showed promising antifibrotic effects in a newly developed mouse model of intestinal fibrosis, suggesting that pirfenidone might be used as an antifibrotic agent for intestinal fibrosis (Meier et al., 2016).

In the present study, we explored whether pirfenidone could protect against RIF in a rat model. Additionally, primary rat intestinal fibroblasts were incubated with transforming growth factor-β1 (TGF-β1) to

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possibly mimic in vivo situation, and we investigated the inhibitory effects of pirfenidone on critical fibrotic process, such as fibroblast proliferation as well as TGF-β1-induced myofibroblasts differentiation and fibrogenic activities. We also evaluated pirfenidone effects on the TGF-β1/Smad/CTGF signaling pathways underlying these antifibrotic activities both in vivo and in vitro.

## 2. Materials and methods

### 2.1. Animal experiments

Six-week-old male Sprague–Dawley (SD) rats weighing 180–200 g were purchased from the Laboratory Animal Center, Fujian Medical University (Fuzhou, China). Rats were maintained in a controlled environment (23 ± 3 °C and 12 h light/dark illumination cycle) and fed with a standard chow diet and water. Experimental protocols were approved by Institutional Animal Care and Use Committee of Fujian Medical University.

After acclimatization for one week, rats were randomly divided into four groups (n = 10): (i) control group, (ii) radiation group, (iii) radiation plus low-dose pirfenidone group (200 mg/kg/d via oral gavage), and (iv) radiation plus high-dose pirfenidone group (400 mg/kg/d via oral gavage). The RIF model was developed as described previously (Haydont et al., 2007; Okoshi et al., 2008). Briefly, rats were anesthetized with chloral hydrate (0.3 ml/100 g; Sigma, USA) intraperitoneally, and then restrained and taped by the legs on an acryl plate at a supine position. A single dose of 2000 cGy (Varian Clinac, USA, 6MV-X, and source to surface distance 100 cm) was delivered to a 3 × 4 cm area of the lower pelvis containing 2 cm lengths of the rectum in the middle of the field. Pirfenidone was dissolved in 0.5% Carboxymethyl cellulose solution (vehicle). Rats were administered orally with pirfenidone (200, 400 mg/kg/d) or vehicle once a day after irradiation. The well-being of rats was inspected daily, and body weight was measured weekly. Twelve weeks after irradiation, all rats were killed. Three 5 mm segments of the rectum which was 1 cm proximal to the anus were excised, fixed in 10% formalin for histologic and immunohistochemical staining, or immediately snap-frozen in liquid nitrogen for RNA extraction.

### 2.2. Histological analysis

Tissue sections (5 μm in thickness) were stained with hematoxylin and eosin (HE) and Van Gieson (VG) to enable histological evaluation of intestinal fibrosis. The severity of intestinal fibrosis was scored as 0 (absent), 1 (mild), 2 (moderate) or 3 (severe) depending on the density and extent of VG positive connective tissue staining and disruption of tissue architecture, as described previously (Lawrance et al., 2003). The thickness of submucosa was measured randomly at five points in each sample. The depth of submucosa was measured from muscularis mucosae to muscularis propria at the corresponding position. Morphometric analysis was done by 5 measurements randomly taken in 5 different fields (400×) by two pathologists blinded to treatment assignment.

### 2.3. Immunohistochemical analysis

Immunohistochemical staining with anti-collagen I (ab34710) and anti-α-smooth muscle actin (α-SMA, ab5694) antibodies (Abcam, USA) in the specimen was performed using an avidin–biotin complex immunoperoxidase method. Five different fields were selected randomly from each slice using a bright field light microscope (400×) by two pathologists in a blind fashion. The degree of immunostaining was scored depending on the intensity of staining: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown) (Salmanidis et al., 2013). The percentage of positively-stained cells was graded as: 0 (< 5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (> 75%). The two scores were multiplied together and a weighted score of each sample was defined as follows: 0 score (−), 1–4 scores (+), 5–8 scores (++), 9–12 scores (+++). Quantitative comparison of immunohistochemical staining was performed by using the built-in functions of the Image-Pro-Plus image analysis software (Media Cybernetics, Silver Spring, MD, USA).

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines, interleukin-1β (IL-1β), IL-6, IL-12, IL-17, and tumor necrosis factor-α (TNF-α) levels were measured in tissue extracts using specific ELISA kits [IL-1β (bsk00027), IL-6 (bsk00042), IL-12 (bsk000374), IL-17 (bsk00054), and TNF-α (bsk00163); Bioss Antibodies, China] according to the manufacturer's instructions.

### 2.5. Isolation and culture of primary rat intestinal fibroblasts

Primary rat Intestinal fibroblasts were isolated from irradiated intestinal tissues of 18-week-old healthy male SD rats using trypsin digestion method, as described previously (Phan et al., 1985). The cells were cultured at 37 °C in 5% CO2 atmosphere using Dulbecco's modified minimal essential medium (DMEM)/F-12 solution containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin. Experiments were performed on passages 3 to 5 cells.

### 2.6. Cytotoxicity and cell proliferation assay

Before addingTGF-β1 and/or pirfenidone, we synchronized the cell cycle by incubating all the cells in DMEM/F-12 containing 2% FBS for 24 h. After treatments with various concentrations of pirfenidone (0, 0.01, 0.05, 0.1, 0.5, 1 mg/ml) for 24 h, the cytotoxic effects of pirfenidone were evaluated by the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Beyotime, China) according to the manufacturer's instructions. We further explored the effect of pirfenidone on TGF-β1-induced cell proliferation. The primary rat pulmonary fibroblasts were assigned to ten groups: (i)control, cells were incubated in phosphate-buffered saline (PBS) for 24 h; (ii–v) pirfenidone, cells were incubated with pirfenidone (0.01, 0.05, 0.1, 0.5, 1 mg/ml) for 24 h; (vi–x) TGF-β1 + pirfenidone (0,0.01, 0.05, 0.1, 0.5, 1 mg/ml): cells were incubated with different concentration of pirfenidone for 1 h and then exposed to TGF-β1 (5 ng/ml) for 24 h; Cell proliferation was determined by performing Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). The number of cells was assessed by measuring absorbance at 450 nm using Multiskan Go microplate reader (Thermo Scientific, USA).

### 2.7. Cell experiments

The experiments were bifurcated into two parts. In the first part of the experiment, we incubated cells with a potent and non-specific alkaline phosphatase inhibitor, L-p-Bromotetramisole oxalate (Santa Cruz, USA), to evaluate the role of phosphorylation of TGF-β1/Smad pathway and the subsequence effect of TGF-β1-induced secretion of collagen I and myofibroblast differentiation. The cells were divided into three groups as follows: (i) control, cell were incubated with phosphate buffer saline (PBS) for 24 h; (ii) TGF-β1, cells were incubated with TGF-β1 (5 ng/ml) for 24 h; (iii) TGF-β1 + L-p-Bromotetramisole oxalate, cells were pre-treated with L-p-Bromotetramisole oxalate for 1 h and then treated with TGF-β1 (5 ng/ml) for 24 h.

In the second part of the experiment, the cells were divided into five groups as follows: (i) control, cell were incubated with PBS for 24 h; (ii)TGF-β1, cells were incubated with TGF-β1 (5 ng/ml) for 24 h; (iii–v) TGF-β1 + pirfenidone (0.1, 0.5, 1 mg/ml), cells were pre-treated with TGF-β1 (5 ng/ml) for 1 h and then treated with different concentration of pirfenidone for 24 h; Firstly, we explored the inhibitory effects of